Broth Dilution Testing of *Candida albicans* Susceptibility to Ketoconazole

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We performed a detailed investigation of the kinetics of ketoconazole activity in the setting of broth dilution testing of *Candida albicans* susceptibility. Turbidimetric readings reflected parallel quantitative colony counts. The method of endpoint determination markedly affected the results. Determinations of 50% inhibitory concentrations clearly separated the ketoconazole-resistant strains from the susceptible strains.

Broth dilution testing of *Candida albicans* susceptibility to the imidazole antifungal agents has been difficult to use because of a marked inoculum effect (3, 4, 13) and the lack of a clear visual endpoint (10), the latter caused by partial growth inhibition over a wide range of drug concentrations. Additional factors which influence MICs include the medium composition (2), pH (9), and temperature and duration of incubation (13). Of possible significance are growth phase of the yeast cells (1), stationary versus shaking incubation, and oxygen tension.

Galgiani and Stevens (3, 4) described a susceptibility test based on reading broth dilution tubes turbidimetrically and determining the drug concentration producing 50% inhibition compared with the drug-free control (IC$_{50}$). This test is reported to be inoculum independent, and it also eliminates the subjectivity of visual MIC readings. Modifications of this method, described by Ryley et al. (IC$_{50}$; 11) and Johnson et al. (IC$_{50}$; 7), separated ketoconazole-resistant *C. albicans* strains from routine clinical isolates. Although similar, the latter tests require additional dilution of the broth dilution tubes with graphic determination of the endpoint (IC$_{50}$) or special equipment (IC$_{50}$) to perform. The IC$_{50}$ assay may have better potential for use as a susceptibility method in clinical laboratories because it is easily done as an adjunct to the routine broth dilution procedure by reading the undiluted tubes with a spectrophotometer and by making a simple mathematical calculation. The ability to separate isolates showing clinical resistance from routine clinical isolates is an important aspect of the assay and requires further investigation.

In the present study, we compared ketoconazole IC$_{50}$ determinations with visual MIC readings for several strains of *C. albicans* demonstrating in vivo susceptibility or resistance to ketoconazole. In addition, experiments involving detailed time-kill studies and parallel turbidimetric readings were designed to investigate the kinetics of ketoconazole activity against *C. albicans* and the correlation between turbidity with quantitative yeast counts in the setting of the broth dilution test as it is commonly performed. These studies attempt to provide a further understanding of the problems inherent in such testing, with particular reference to endpoint determination.

Three strains of *C. albicans* were used (ATCC 11651, Moon, and B980) which have demonstrated susceptibility to ketoconazole in an animal model (6). Two ketoconazole-resistant strains of *C. albicans*, designated AD (or NCPF 3302) and KB (or NCPF 3303), were cultured from patients with chronic mucocutaneous candidiasis (CMC) who relapsed during prolonged ketoconazole therapy (5, 11). Both strains demonstrated resistance to ketoconazole in vitro and in animal models (11). The organisms were maintained by serial transfer on yeast morphology agar slants.

The method of broth dilution susceptibility testing used was a modification of that previously described (3). Ketoconazole was dissolved in Me$_2$SO, diluted in supplemented yeast nitrogen base at pH 7.0 (1), and serially diluted twofold in broth from 200 to 0.2 µg/ml. The drug-free broth contained Me$_2$SO at a final concentration of 0.5% (vol/vol). Samples (1 ml) of the dilutions were placed in disposable plastic tubes (12 by 75 mm). Yeast inocula were prepared from 18-h broth cultures incubated at 37°C with shaking at 150 rpm, which were diluted in broth medium to a concentration of 1 x 10$^5$ to 2 x 10$^5$ CFU/ml as determined by plate counts. Samples (1 ml) of inoculum were added to the tubes and mixed with a vortex mixer. For each yeast strain, dilutions were set up in triplicate; in addition, the tests were repeated on a different date. After stationary incubation for 24 and 48 h at 37°C, visual MICs, defined as the complete lack of visible growth, were read. At 0, 6, 24, and 48 h, the tubes were also read turbidimetrically with a Coleman 295 spectrophotometer at 540 nm, by using drug-free medium as a blank. The 24- and 48-h readings were used to calculate IC$_{50}$s, defined as the lowest concentrations with %T ≤ %T$_{control}$ + 0.15.

**Table 1.** Ketoconazole MICs and IC$_{50}$s for five strains of *C. albicans* and a reference strain of *C. pseudotropicalis*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Visual MIC at:</th>
<th>IC$_{50}$ at:</th>
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<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
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<td></td>
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<tr>
<td>ATCC 11651</td>
<td>&gt;100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B980</td>
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<td>≥100&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Moon</td>
<td>≥100</td>
<td>≥100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AD</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>KB</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>C. pseudotropicalis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 28838</td>
<td>100</td>
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* All values in micrograms per milliliter.

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FIG. 1. Kinetics of yeast viability (CFU per milliliter) at selected ketoconazole concentrations (micrograms per milliliter) with simultaneous optical density measurements (percent transmittance). C. albicans strains: A, ATCC 11651; B, B980; C, Moon. Each curve represents the mean and range of two experiments.
%T<sub>control</sub>, where %T is percent transmittance and control is a drug-free tube (3). A commonly used control yeast, *Candida pseudotropicalis* ATCC 28838, with a MIC of 0.05 μg/ml (12), was included in testing, for reference.

Tubes from the broth dilution susceptibility tests were sampled (0.05 ml) at 0, 6, 24, and 48 h for quantitative counts. Tenfold serial dilutions were prepared in saline containing 0.02% Tween 80, and 1-ml samples were then combined with 9 ml of molten Sabouraud agar in petri plates (15 by 100 mm). The plates were incubated for 48 h at 30°C and counted; after an additional 3 days, they were reexamined for additional colonies.

The susceptibility test results are shown in Table 1. Each of the five strains of *C. albicans* had an MIC of ≥100 μg/ml. For the three susceptible strains, the visible growth was absent or very slight at 100 μg/ml, gradually increasing as the concentration of ketoconazole fell to 0.1 μg/ml, with no abrupt differences between tubes that would allow an endpoint determination. At the lowest concentration tested (0.1 μg/ml), growth remained markedly less than in the control tube. For the CMC relapse isolates, an abrupt increase in visible turbidity was seen at the concentration corresponding to the IC<sub>1/2</sub>, but slight growth persisted at higher concentrations, including 100 μg/ml. Replicates agreed within one tube for all determinations.

Viability curves of the ketoconazole-susceptible yeast strains during exposure to ketoconazole are depicted in Fig. 1. Slow fungicidal activity was observed against the B980 and Moon strains at a concentration of 100 μg/ml. After 5 days of incubation, additional colonies were occasionally counted which were not visible after 2 days on viability plates prepared from the highest concentrations. Although diluted 1,000-fold or greater, enough ketoconazole may have been present in the agar to delay yeast growth. A substantial dose-related fungistatic effect of ketoconazole was observed over the range of concentrations from 50 to 1 μg/ml. There was good correlation between the turbidimetric readings and the quantitative counts, although there were minor variations in optical density for a given number of viable units between strains.

In contrast to the three ketoconazole-susceptible strains, the CMC relapse isolates (Fig. 2) required at least 8- to 60-fold-higher concentrations of ketoconazole for inhibition, above which a fungistatic effect was observed similar to that shown in Fig. 1. A moderately strong (but incomplete) level of fungistatic activity was seen at the concentration corre-
responding to the IC_{1/2}. Turbidimetric readings again reflected quantitative colony counts.

In the standardization of broth dilution susceptibility testing with ketoconazole versus *C. albicans*, perhaps the greatest confusion has arisen from differences in definition of the endpoint. We observed differences greater than 1,000-fold in concentrations required for inhibition of susceptible organisms depending on the method of reading the tubes. When MIC was defined as the absolute lack of visible growth, our determinations failed to separate the ketoconazole-susceptible strains from the CMC relapse isolates. Hence, such a definition is unlikely to have clinical utility. Other kinds of visual readings have been used, such as the definition of MIC as the concentration which inhibits clearly visible growth, with a faint haze or slight turbidity being ignored (12). The turbidity-versus-time curves in Fig. 1 illustrate that there are such slight differences in turbidity over the entire range of ketoconazole concentrations tested that a valid visual endpoint determination is not possible (other than at $\leq 0.1 \mu g/ml$). We believe that a visual reading without a precise definition is likely to lead to unacceptable error caused by subjectivity in readings. Evidence that this is true is found in a recent study by Leifer and Stevens (8), who reported visual MIC readings ranging from 0.39 to 100 $\mu g/ml$ for eleven clinical isolates of *C. albicans* that all had IC_{1/2}s of $\leq 0.097 \mu g/ml$ (which presumably would have time-versus-turbidity curves similar to those shown in Fig. 1). If visual readings could be carefully standardized between laboratories so that a defined level of turbidity was used for endpoint determination (such as a certain McFarland standard), such a system might also have potential usefulness.

Although ketoconazole does demonstrate fungicidal activity under some in vitro conditions (8), our time-killing studies suggest that there is little fungicidal activity at clinically useful concentrations. Further, it has been shown that there is a substantial problem with drug carry-over in methods of determination of minimal fungicidal concentration which involve direct plating of undiluted samples (8). Hence, performance of determinations of minimal fungicidal concentration by such methods is unlikely to provide useful information and may actually be misleading.

We found calculation of IC_{1/2} from turbidimetric readings of broth dilution tubes to be easy to perform and reproducible. There was clear separation of ketoconazole-susceptible strains of *C. albicans*, with IC_{1/2}s of $\leq 0.1 \mu g/ml$, from resistant strains, which had higher IC_{1/2}s. The turbidimetric readings were generally a reliable reflection of actual colony counts, although minor variations in optical density occurred between strains for a given number of viable units.

As originally described (3), the IC_{1/2} assay is inoculum size independent because it is a comparison of growth rates, rather than of absolute levels of growth attained. In this regard, readings at 24 h or earlier are more valid than those at 48 h, when growth has reached a plateau phase. The 48-h IC_{1/2} determinations in this study appeared to have little to add to the 24-h values.

Other questions remain regarding the use of the IC_{1/2} assay. First, what is the optimal range of concentrations of ketoconazole to be used? Second, what is the relationship between the IC_{1/2} and the actual concentration of ketoconazole which must be achieved for fungistatic activity in human infections? Last, how does one apply this type of assay to miconazole, which itself causes visible turbidity above a concentration of 12.5 $\mu g/ml$ (C. Hughes and W. Beggs, unpublished data)? Additional work is needed to further standardize this promising method of testing for antifungal susceptibility.

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**LITERATURE CITED**


