Microtiter Broth Dilution Method for Yeast Susceptibility Testing with Validation by Clinical Outcome

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There is no ideal laboratory procedure or culture medium in current use for susceptibility testing of pathogenic yeasts. Six candidate growth media (RPMI 1640 with L-glutamine, yeast nitrogen base, Casamino Acids medium, Mueller-Hinton broth, Sabouraud dextrose broth, and minimum essential medium-Eagle salts) were screened by spectrophotometric absorbance for nucleic acid and protein. From these, two media were selected: a chemically defined growth medium (RPMI 1640 with L-glutamine) and a chemically complex medium (Casamino Acids). MICs of four antifungal agents (5-fluorocytosine, miconazole, ketoconazole, and amphotericin B) for 84 clinical isolates of various Candida species were then determined with both media in agar dilution and microtiter broth dilution systems. The resultant MICs were correlated with clinical outcome for those isolates obtained from patients treated with single antifungal agents, and susceptibility cut points were calculated. Derived MIC cut points for susceptibility were validated in a murine model of systemic candidiasis. RPMI 1640 with L-glutamine was found to have the lowest absorbance values for both nucleic acid and protein, while Casamino Acids medium was highest in both categories. We found that RPMI 1640 with L-glutamine was superior to Casamino Acids medium in the yield of MICs which correlated with actual clinical and animal outcome data. While there were no significant differences in MICs when RPMI 1640 medium was used, the microtiter broth dilution technique was superior to agar dilution in efficiency and ease of performance. We conclude that a microtiter broth system containing RPMI 1640 medium with L-glutamine is a simple, precise, and economical technique for susceptibility testing of pathogenic Candida species. We also suggest that the validation of susceptibility cut points with patient and animal outcome data make this microtiter broth system a preferred method for yeast susceptibility testing.

Pathogenic yeasts are now recognized as frequent causes of serious opportunistic infections (10) in a broadening range of compromised hosts: cancer patients (25), premature infants (2), patients receiving hyperalimentation (17), patients with deep venous access catheters (18) or prosthetic vascular devices (27), and those with specific immunological defects (12). Even when such infection is suspected, the mycological diagnosis is often elusive (16, 23). Should the infecting organism fortuitously be isolated, yeast susceptibility testing, as a guide to specific therapy, is not widely available, and methods often differ significantly from one laboratory to another (4).

There is no consensus as to the optimal culture medium for yeast susceptibility testing. Instead, a variety of formulations have been evaluated: modified yeast nitrogen base, yeast morphology agar, Casamino Acids medium, synthetic amino acid medium (fungal), Mueller-Hinton agar, Sabouraud dextrose medium, modified minimum essential medium (Eagle salts), neopeptone medium, and RPMI 1640 medium with L-glutamine (5, 14, 19, 20, 21, 30, 33, 36, 42) (S. Adamson, T. C. Granade, and W. M. Artis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C238, p. 351). Various investigators have proposed criteria for antifungal susceptibility cut points based on the distribution of growth in these media, but without any clinical validation (11, 14, 20, 35, 36, 38). The two studies which utilized animal models of Candida albicans infection as a means of clinical correlation indicated that the predictive value of susceptibility testing with 5-fluorocytosine was questionable (31, 39). Neither study sought any comparison with outcome data in humans. Despite published recommendations (36), these deficiencies lead some commentators to conclude that a valid standardization of methodology is not possible at the present time (E. L. Chan, Clin. Microbiol. News. 7:84–86, 1985).

To formulate a validatable yeast susceptibility test procedure, we processed a number of Candida isolates recovered from human infections for susceptibilities to the four standard antifungal agents: 5-fluorocytosine, amphotericin B, miconazole, and ketoconazole. In each instance, two fungal media were compared: a chemically complex medium (Casamino Acids) used consistently for years by the Mycological Reference Laboratory, Public Health Laboratory Service, United Kingdom (33), and a chemically defined medium (RPMI 1640) advocated for its advantageous nutritional characteristics and consistent formulation (Adamson et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1983). Agar dilution and microtiter broth dilution techniques were also compared. Susceptibility cut points were then determined from outcome data for human infections treated with a single antifungal agent, as well as for induced infection in an animal model.

MATERIALS AND METHODS

Antifungal agents. The four antifungal agents used in the susceptibility testing procedure were 5-fluorocytosine (Hoffman-LaRoche, Inc., Nutley, N.J.), miconazole (Sigma Chemical Co., St. Louis, Mo.), ketoconazole (Janssen Pharmaceuticals, Piscataway, N.J.), and amphotericin B (E. R. Squibb & Sons, Princeton, N.J.). While amphotericin B was supplied as the pharmacological compound for patient use

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that of 0.2 and wells contained dilutions of media for miconazole (6 wk). Prepared in this manner, a volume of 0.1 ml of the final broth dilution contained approximately 10^5 CFU of yeast, as determined by quantitative plating onto Sabouraud dextrose agar. For comparison, a McFarland 0.5 standard contains 10^7 to 10^8 CFU/ml. A 0.1-ml portion of broth (10^6 CFU) was used as the standard inoculum in both the agar dilution and microtiter broth techniques. Agar dilution and microtiter broth dilution plates were incubated at 35°C in 5% CO_2 for 24 h. Isolates were then tested in duplicate on two different days.

MIC. The MIC was defined as that concentration of the antifungal agent contained in the microtiter well or in the agar dilution plate in which the absence of visual turbidity (colonies) was first observed. A microtiter plate reader (Minireader II; Dynatech Laboratories, Inc.) was used to assess growth in the RPMI microtiter plates, and the results were compared with the independent visual endpoint determinations made by six medical technologists.

Incubation temperature. Paired inocula of 20 isolates, drawn at random from the panel of test organisms, were incubated in parallel at 30 and 35°C in RPMI broth to determine the effect of temperature on growth and endpoint determination.

Spectrophotometry. Six media employed in yeast susceptibility testing at other centers were compared for absorbance characteristics at 260 and 280 nm on a dual-beam spectrophotometer (model 635; Varian Techtron, Downey, Calif.) to estimate the presence of nucleic acids and proteins. The media chosen for this comparison were RPMI 1640, yeast nitrogen base, Casamino Acids, Mueller-Hinton broth, Sabouraud dextrose broth, and modified minimal essential medium (Eagle salts).

Clinical isolates. The Candida isolates tested were all recovered from human infections, and included 78 from blood cultures, four from oral swabs, and two from urine cultures. Fifty blood isolates were kindly provided by S. Mirrett (University of Colorado Health Sciences Center, Denver), two oral isolates were furnished by C. Kirkpatrick (National Jewish Hospital, Denver, Colo.), and one blood isolate was kindly supplied by J. Dick (Johns Hopkins

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Diagnosis</th>
<th>Organism</th>
<th>Therapy</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3</td>
<td>Endocarditis</td>
<td>C. albicans</td>
<td>Ketoconazole (4 wk)</td>
<td>Dissemination</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>Systemic candidiasis</td>
<td>C. tropicalis</td>
<td>5-Fluorocytosine (3 wk)</td>
<td>No improvement</td>
</tr>
<tr>
<td>C</td>
<td>36</td>
<td>Mucocutaneous candidiasis</td>
<td>C. albicans</td>
<td>Ketoconazole (6 wk)</td>
<td>No improvement</td>
</tr>
<tr>
<td>D</td>
<td>22</td>
<td>Mucocutaneous candidiasis</td>
<td>C. albicans</td>
<td>Ketoconazole (6 wk)</td>
<td>No improvement</td>
</tr>
<tr>
<td>E</td>
<td>19</td>
<td>Mucocutaneous candidiasis</td>
<td>C. albicans</td>
<td>Ketoconazole (4 wk)</td>
<td>Recovery</td>
</tr>
<tr>
<td>F</td>
<td>15</td>
<td>Urinary tract infection</td>
<td>C. lusitaniae</td>
<td>Amphotericin B (66 days)</td>
<td>Renal candidiasis at autopsy</td>
</tr>
<tr>
<td>G</td>
<td>30</td>
<td>Systemic candidiasis</td>
<td>C. albicans</td>
<td>5-Fluorocytosine</td>
<td>Recovery</td>
</tr>
</tbody>
</table>

**Inoculum.** C. albicans (Table 1), Candida tropicalis (Table 1), and Saccharomyces cerevisiae ATCC 9763 were used as controls. These strains, and all other clinical isolates tested, were grown in parallel in each of the following media: brain-heart infusion, Sabouraud dextrose broth, Casamino Acids medium, and RPMI 1640 with L-glutamine. After 24 h of incubation at 35°C in a 5% CO_2 atmosphere, the isolates were then diluted 1:100 in normal saline, followed by a 1:100 dilution in either Casamino Acids medium or RPMI 1640 broth. Prepared in this manner, a volume of 0.1 ml of the final broth dilution contained approximately 10^5 CFU of yeast, as determined by quantitative plating onto Sabouraud dextrose agar. For comparison, a McFarland 0.5 standard contains 10^7 to 10^8 CFU/ml. A 0.1-ml portion of broth (10^6 CFU) was used as the standard inoculum in both the agar dilution and microtiter broth techniques. Agar dilution and microtiter broth dilution plates were incubated at 35°C in 5% CO_2 for 24 h. Isolates were then tested in duplicate on two different days.

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Medical Institutions, Baltimore, Md.). All other isolates were recovered from patients admitted to The Children's Hospital, Denver, Colo. It was possible in seven instances to obtain adequate follow-up on the clinical outcome of patients infected with a Candida species and treated with only a single antifungal agent. Clinical deterioration, fungal dissemination, or failure to improve either clinically or microbiologically were all considered to indicate clinical resistance of the organism to the antifungal agent used. Improvement, recovery, and microbiological cure were accepted as clinical indications of fungal susceptibility.

**Murine treatment model.** To validate further the laboratory definition of resistance, an animal model of systemic candidiasis was used. Seventy adult CD-1 mice (Charles River Laboratories, Wilmington, Mass.) were given intracardiac injections with a 27-gauge needle. A total of 10 mice received an injection of 0.1 ml of sterile saline; 30 animals were injected with 0.1 ml of Candida albicans (clinical strain A) suspended at a concentration of 10^8 CFU/ml in brain heart infusion broth; and 30 received an equal volume of C. tropicalis (clinical strain B) at a similar concentration in brain heart infusion broth. Strain A was an isolate of C. albicans considered susceptible to 5-fluorocytosine but resistant to ketoconazole by both clinical and laboratory criteria; strain B was an isolate of C. tropicalis considered to be resistant to 5-fluorocytosine but susceptible to ketoconazole by the same criteria. The mice that were injected with saline alone received oral water and no antifungal medication. The 30 mice in each cohort given intracardiac injections of yeast were divided into three groups of 10 mice: one group that received oral water only; another group that received oral 5-fluorocytosine; and a third that received oral ketoconazole. Both oral antifungal agents were dissolved in water and administered orally as a 0.1-ml volume after the mice had been deprived of water for 12 h. The test animals were treated according to the dosing schedule recommended for human subjects: 150 mg of 5-fluorocytosine daily per kg of body weight divided into 6-h intervals (42), and 3 mg of ketoconazole per kg of body weight as a single daily dose (9). Treatment was continued for 5 days, and cumulative mortality was determined over a 30-day period. Neither necropsies nor postmortem cultures were performed.

**RESULTS**

**Patient parameters.** Of the 84 isolates of Candida species tested, 7 were from patients who had been treated with a single antifungal agent and whose clinical course and follow-up were known. Data for these patients include age, clinical diagnosis, organism, antifungal agent used, length of therapy, and outcome (Table 1).

| TABLE 2. Spectrophotometric absorbance of growth media used for yeast susceptibility testing |
|---------------------------------|---------|---------|
| **Medium**                      | **A_{600}** | **A_{500}** |
| RPMI 1640                       | 0.53     | 0.57     |
| Yeast nitrogen base             | 1.32     | 1.50     |
| Casamino Acids medium           | 6.12     | 5.70     |
| Mueller-Hinton broth            | 1.90     | 1.80     |
| Sabouraud dextrose broth        | 1.67     | 1.66     |
| Minimum essential medium-Eagle salts | 1.59     | 1.72     |

* Nucleic acid.
* Protein.

**Yeast susceptibility testing.** (i) Medium composition. The spectrophotometric absorbance values of six media commonly used for yeast susceptibility testing are presented in Table 2. There are marked differences among the media, notably with respect to nucleic acid as well as protein content. RPMI 1640 had the lowest absorbance values in both categories.

(ii) Inoculum preparation, incubation temperature, and MIC endpoint determination. All primary growth media tested (brain heart infusion broth, Sabouraud dextrose broth, Casamino Acids medium, and RPMI 1640) provided equivalent growth conditions for preparation of the inoculum. An incubation temperature of 35°C was found to be superior to 30°C, both visually and spectrophotometrically, for determination of fungal growth and endpoints. The endpoints for organisms grown at 35°C were considered superior in 34 of 42 observations made by six evaluating technologists (P = 0.0001 by Cochran chi-square analysis). Visual and spectrophotometric endpoint values agreed within one drug dilution, and in most cases values were identical.

(iii) Concordance of results between medium types and assay techniques. Precision of successive runs within each medium type and assay method was excellent, with either identical endpoints or a variation of no more than one tube dilution in all cases.

In 32 randomly selected isolates (22 C. albicans and 10 C. tropicalis), MICs obtained with the agar dilution assay technique were compared with those from the microtiter broth method (Table 3). Little variability was seen when RPMI 1640 medium was used. However, discordance between the two techniques was greater than two tube dilutions in 9.3% of the assays for imidazole susceptibility testing when Casamino Acids medium was used. For the same 32 isolates, Casamino Acids medium and RPMI 1640 were compared for agreement of susceptibility results obtained by microtiter broth dilution (Table 4). A significant rate of discordance (>fourfold difference in MICs) was seen in 55.4% of isolates exposed to 5-fluorocytosine and in 38.3% of isolates tested against amphotericin B. On the other hand, there was agreement in 96% of isolates tested for imidazole.

| TABLE 3. Agar dilution compared with microtiter broth dilution for determination of susceptibilities of 32 Candida isolates |
|-----------------|-------|--------|--------|-------|
| **Discordance** | 5-F \( insensitive \) Fluorocytosine | Miconazole | Ketoconazole | Amphotericin B |
| (tube dilution) | % of isolates | % of isolates | % of isolates | % of isolates |
| <1              | 100/100a | 87.6/100 | 87.6/96.9 | 93.8/100 |
| 2               | 3.1     | 3.1/3.1 | 6.2     | 6.2     |
| 3               | 6.2     | 6.2     | 6.2     | 6.2     |
| ≥4              | 3.1     | 3.1     | 3.1     | 3.1     |

* Percentage of all isolates tested: Casamino Acids medium/RPMI 1640.

| TABLE 4. Microtiter broth dilution susceptibilities of 32 Candida isolates: Casamino Acids medium compared with RPMI 1640 |
|-----------------|-------|--------|--------|-------|
| **Discordance** | 5-F \( insensitive \) Fluorocytosine | Miconazole | Ketoconazole | Amphotericin B |
| (tube dilution) | % of isolates | % of isolates | % of isolates | % of isolates |
| <1              | 34.0   | 68.0   | 78.7   | 8.5   |
| 2               | 4.2    | 17.0   | 12.8   | 10.6  |
| 3               | 6.4    | 10.6   | 8.5    | 42.6  |
| ≥4              | 55.4   | 4.4    | 38.3   | 38.3  |

* Nucleic acid.
* Protein.
susceptibility. It was also noted that all 32 isolates tested against 5-fluorocytosine in Casamino Acids medium uniformly displayed high MICs (>8 μg/ml), whereas the use of RPMI 1640 medium resulted in a more characteristic spectrum of values. However, RPMI 1640 tended to yield MICs for amphotericin B that were higher than those achieved in Casamino Acids medium but were in no case at a level considered indicative of resistance. If the microtiter plates were examined after 36 h, endpoint determinations for the imidazole became difficult to read owing to loss of the sharp transition between presence and absence of growth. This difficulty was most pronounced when ketoconazole was being tested.

In vivo treatment model. Cumulative mortality data for mice infected with C. albicans (strain A: MIC of 5-fluorocytosine, 2.0 μg/ml; MIC of ketoconazole, >32 μg/ml by microtiter susceptibility testing in RPMI 1640) and C. tropicalis (strain B: MIC of ketoconazole, 0.5 μg/ml; MIC of 5-fluorocytosine, >16.0 μg/ml) are presented in Table 5. The 10 control mice given an intracardiac injection of sterile saline all survived the 30-day observation period. Of the mice infected with strain A (5-fluorocytosine susceptible, ketoconazole resistant by microtiter susceptibility testing in RPMI 1640), all animals treated with 5-fluorocytosine survived for 30 days, while all the mice treated with ketoconazole died. In the mice infected with strain B (ketoconazole susceptible, 5-fluorocytosine resistant), all animals treated with 5-fluorocytosine died, while 9 of 10 given ketoconazole survived. All animals given only sterile water and no antifungal agent died in both groups.

Susceptibility cut point determination. MICs of the four standard antifungal agents were calculated for the 84 clinical isolates assayed in the microtiter broth dilution system containing RPMI 1640 medium with 1-glutamine (Fig. 1 to 4). Superimposed on each histogram are the clinical outcome data for the seven patients presented in Table 1. Based on the distribution of MICs and the outcome of treated infections, cut points for susceptibility and resistance were determined. Susceptibility was defined as the highest MIC which still displayed documented clinical efficacy in treated patients (Table 1). Resistance was defined as the lowest MIC which still resulted in treatment failure. MICs between these two cut points were considered indeterminate, since there were no clinical outcome data on which to judge the likelihood of therapeutic success or failure. In the case of miconazole, no clinical isolates from patients successfully treated with this drug only were available, and the susceptibility cut point was based on the bimodal distribution of MICs in our population of isolates (Table 2) and on analyzing with cut points determined for ketoconazole (Table 3).

![FIG. 1. 5-Fluorocytosine MICs for 84 Candida isolates, with superimposed clinical outcome.](http://jcm.asm.org/)

![FIG. 2. Miconazole MICs for 84 Candida isolates, with superimposed clinical outcome.](http://jcm.asm.org/)
Confirmatory validation was obtained from the murine candidiasis model reported above. The derived cut points are reported in Table 6.

**DISCUSSION**

Successful therapy of serious infections requires accurate information regarding the susceptibility of the infecting organism to antimicrobial agents (29). The application of this principle to infections caused by pathogenic yeasts has been hampered by a lack of uniformity in laboratory susceptibility testing procedures and the absence of correlative clinical data to validate cut points (4, 13). When a paucity of antifungal agents had been available, and universal susceptibility to such agents had been assumed, the necessity for laboratory susceptibility testing had been less imperative. Now, a choice of antifungal agents exists (9), and clinically resistant Candida species have been recovered from human infections both in this current study (Table 1) and in others (6, 22, 26). Consequently, when a pathogenic yeast is isolated, the blind choice of an antifungal agent in the absence of reliable susceptibility data may result in inadequate therapy and a poor outcome (25).

The characteristics of an ideal yeast susceptibility testing technique have already been suggested (19, 20, 21, 42). Both an ideal growth medium and an ideal protocol are necessary. An ideal medium is one which is widely available, economical, well-standardized, supports rapid growth of isolates, is appropriate for all antifungal agents, has a stable pH, and contains no interfering substances which might alter antifungal activity. Many media used for yeast susceptibility testing run afool of one or more of these criteria as in the following cases.

(i) Chemically complex and undefined media, such as the Sabouraud dextrose medium and brain heart infusion broth, contain either purine or pyrimidine bases or nucleosides which antagonize the activity of 5-fluorocytosine and result in an erroneously high MIC (19, 32). We now demonstrate that this is true also of Casamino Acids medium, in which MICs against 5-fluorocytosine of greater than 64 μg/ml were seen in the majority of the 84 isolates.

(ii) At a physiological pH, buffered yeast nitrogen base results in a prolonged doubling time for inoculated Candida species (3). This poor growth support has been thought to result in less sharp zones of inhibition when an agar dilution procedure is used (20) and could possibly interfere with the use of rapid methods of susceptibility testing, such as turbidimetric measurement, release of radioactive markers, or inhibition of radioactive base pair uptake (15). On the other hand, unbuffered yeast nitrogen base, with a pH below 5.0, is unsuitable for testing polynucleotide antimicrobial agents or ketoconazole, which are partially inactivated in an acid medium (24, 28).

(iii) Synthetic amino acid medium-fungal (SAAMF) has been promoted as a totally defined product that is free of interfering agents and has a stable pH (19). Although this medium is now commercially available (GIBCO Laboratories, Grand Island, N.Y.), it is quite expensive and has not acquired a wide following. Furthermore, the use of the MOPS-Tris buffer system in the current formulation has been shown to interfere with 5-fluorocytosine activity (3).

RPMI 1640 medium with L-glutamine (pH 7.2) was chosen as a possible culture medium after Adamson and colleagues successfully used it for ketoconazole susceptibility testing (Adamson et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1983). The advantages of this medium are that it is readily available, economical, quality controlled, chemically defined, simple to prepare, well buffered (carbonate and phosphate), and contains a pH indicator (phenol red). In RPMI 1640, growth of all strains of Candida species in our study was equivalent to that in other standard media. Spectrophotometric absorbance comparisons of the six media show that RPMI 1640 also had the lowest recorded values at both 260

![Graph showing MICs for 84 Candida isolates, with superimposed clinical outcome.](http://jcm.asm.org/)

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**TABLE 6. Microtiter broth dilution susceptibility cut points**

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>MIC (μg/ml)</th>
<th>Susceptible</th>
<th>Indeterminate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Fluorocytosine</td>
<td>&lt;1.0</td>
<td>1.0–16.0</td>
<td>&gt;16.0</td>
<td></td>
</tr>
<tr>
<td>Miconazole</td>
<td>&lt;8.0</td>
<td>8.0–32.0</td>
<td>&gt;32.0</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>&lt;8.0</td>
<td>8.0–32.0</td>
<td>&gt;32.0</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>≤0.8</td>
<td>1.6–3.2</td>
<td>&gt;6.4</td>
<td></td>
</tr>
</tbody>
</table>
and 280 nm, which suggests relative freedom from interfering nucleic acids and protein residues (Table 2).

Direct comparisons of RPMI 1640 and Casamino Acids medium were made. The latter medium was chosen because of its exclusive use in susceptibility testing for many years at the Mycological Reference Laboratory, Public Health Laboratory Services, Colindale, England, where the bulk of yeast susceptibility testing for Great Britain is carried out (D. W. R. MacKenzie, personal communication). There is no comparable experience with a single medium or single procedure in this country (4). When the microtiter broth dilution method was followed, the rate of discordance (>fourfold difference in MIC) between the two media was high for both 5-fluorocytosine (55.4%) and amphotericin B (38.3%). These differences in MICs for amphotericin B would not have led to the false categorization of any isolate as resistant. However, the use of Casamino Acids medium would have resulted in a uniformly erroneous impression that most isolates were resistant to 5-fluorocytosine, including isolates that were shown to be susceptible both in an animal model and in clinical infections (Tables 1 and 5). Susceptibility testing performed in RPMI 1640 medium agreed with animal and clinical outcome data in all cases.

The characteristics of an ideal susceptibility testing protocol have been suggested (35). These include the requirements that the protocol be easy to perform, need little unique or expensive equipment, be economical of time and money, have clear endpoint determinations, be reproducible, and have good clinical correlation. Consequently, the use of different media for different antifungal agents, a laborious broth dilution technique, the use of special incubators, the necessity for freshly prepared antifungal solutions, the reliance on a spectrophotometer, and the need for prolonged incubation are all characteristics which would make a susceptibility protocol unappealing. The goal is to make the procedure for yeast susceptibility testing resemble that for testing the susceptibilities of aerobic bacteria (35).

Microtiter broth dilution for yeast susceptibility testing offers several advantages (1, 11). When compared with the standard broth dilution procedure (36), this method obviously saves space, media, and time in processing a large number of isolates. The microtiter plates with antifungal agents in the correct dilutions can be made in advance and frozen until required (11). Endpoint determinations (turbidity) are accurate; our study shows complete agreement between visually determined endpoints and those assessed with a spectrophotometer. Furthermore, our method yields close agreement in MICs when compared with other methods. Ellis and colleagues showed agreement between standard broth and microtiter broth dilution techniques, especially when a chemically defined culture medium was used (11). Our study showed that a microtiter broth dilution method agreed with MICs derived from an agar dilution system. However, some investigators have cautioned against the use of agar dilution in ketoconazole susceptibility testing because of the variability in cation content among lots of bacteriological agar (19).

There is also a theoretical advantage which would favor use of the microtiter broth dilution technique when testing for susceptibility to ketoconazole. The primary mode of action of ketoconazole is likely to be inhibition of \(C. \text{albicans} \) mitochondrial respiration (41). It has been well demonstrated that ketoconazole is inactive against yeasts under anaerobic conditions (40). Thus, the standard broth dilution method, without some modification, may not be accurate in testing ketoconazole (36), because of the anaerobic conditions which may often exist in the butt of the dilution tubes with some media. With its larger surface-to-volume ratio, an anaerobic milieu is rarely found in microtiter wells.

The ultimate validation of any susceptibility procedure is treatment outcome. However, the predictive value of yeast susceptibility testing in clinical infections has not been established (30). Still et al. were unable to predict outcome based on 5-fluorocytosine susceptibility testing in a murine candidiasis model and concluded that there was a continuous spectrum of susceptibilities rather than a distinct subset of resistant isolates (39). Persson and colleagues concluded that isolates of \(C. \text{albicans} \) resistant to 5-fluorocytosine in tests were more virulent in untreated mice, but they did not attempt to treat the mice with the antifungal agent to assess outcome (31). Perfect and Durack found a lack of correlation between in vitro activity of the polyene antifungal agents tested and in vivo response in a \(C. \text{albicans} \) endocarditis model (30). On the one hand, Ryley et al. have shown some correlation between susceptibility designation and outcome for several ketoconazole-resistant strains of \(C. \text{albicans} \) in murine infections (34). On the other hand, in a trial of ketoconazole in humans, there was no relationship between in vitro testing and clinical response (7).

Our data show consistent agreement between the determination of antifungal susceptibility in a microtiter broth dilution system and actual clinical outcome in seven patients infected with a \(C. \text{species} \) and treated with a single antifungal agent (Table 1). Furthermore, a murine candidiasis model verified this predictive accuracy for two of the \(C. \text{isolates} \) (Table 5). Our human cases are few. However, it is difficult to find patients with severe \(C. \) infections who are treated with single antifungal agents; combination therapy, especially with amphotericin B and 5-fluorocytosine, is the current recommendation for significant infection (37). In this instance outcome data are always somewhat suspect, since survival in invasive candidiasis may depend equally on host factors and not entirely on susceptibility to antifungal agents (10). Nevertheless, we know of no other study which has attempted this type of systematic clinical correlation.

Conclusion. We tested a microtiter broth dilution method for determining yeast susceptibilities to antifungal agents. This method includes RPMI 1640 medium with L-glutamine, a well inoculum of \(10^7 \) CFU, and 24-h incubation at 35°C (in a 5% CO₂ atmosphere to prevent the medium from becoming alkaline). We have also established susceptibility cut points for MICs, based on experimental and clinical correlations. This is a departure from the recommendations of the Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards (13), which favors the broth dilution procedure outlined in the Manual of Clinical Microbiology (36) as an interim reference method. Nevertheless, simplicity, precision, economy, and most important, clinical validation, suggest our microtiter technique as preferable.

LITERATURE CITED