Antifungal Susceptibility Testing of Yeasts: Evaluation of Technical Variables for Test Automation

F. C. ODDS,* L. VRANCKX, AND F. WOESTENBORGHS

Department of Bacteriology and Mycology, Janssen Research Foundation, B-2340 Beerse, Belgium

The technical parameters for antifungal susceptibility testing with *Candida* species were reexamined to determine the optimal conditions for testing with semiautomated preparations of broth microdilution cultures, automated spectrophotometric readings of the cultures, and dose-response and endpoint determinations by means of a computer spreadsheet. Tests were based on proposed standard method M27P of the National Committee for Clinical Laboratory Standards for antifungal agents. RPMI 1640 broth with extra glucose to a final concentration of 2% gave higher and more reproducible drug-free control readings without affecting susceptibility endpoint readings. An inoculum of 8×10^4 yeasts per ml prepared from a carbon-limiting broth culture without further standardization was found to give optimal control readings after 48 h of incubation at 37°C. For flucytosine, fluconazole, itraconazole, and ketoconazole, endpoints based on 50% growth inhibition (50% inhibitory concentration) gave the minimum variation with inoculum size and the fewest endpoint differences with RPMI 1640 medium obtained from two different suppliers. The 50% inhibitory concentration was also the optimal endpoint for fluconazole and ketoconazole susceptibilities in comparison with broth macrodilution MICs determined by the method of the National Committee for Clinical Laboratory Standards. Intralaboratory reproducibility was determined by retrospective analysis of replicate results for isolates retested at random over a 2-year period. This approach showed less favorable reproducibility than has been reported from purpose-designed, prospective antifungal susceptibility studies, but it may better reflect real-life test reproducibility. Susceptibility data for 616 clinical isolates of yeasts, representing 16 *Candida* and *Saccharomyces* spp., confirmed the tendency of *Candida lusitaniae* isolates to show relatively low susceptibilities to amphotericin B, the tendency of *Candida krusei* isolates to show low flucytosine and fluconazole susceptibilities, and the presence of some isolates in the species *Candida albicans*, *Candida glabrata*, and *Candida tropicalis* with low susceptibilities to azole derivative antifungal agents. The study demonstrates the value of automation and standardization in all stages of yeast susceptibility testing, from plate preparation to data analysis.

Interest in antifungal susceptibility testing has increased in recent years, as witnessed by the growing number of published articles in this field. Most investigations have focused on tests with fluconazole against pathogenic yeasts, particularly since reports have appeared suggesting that *Candida* isolates with reduced fluconazole susceptibilities are being encountered clinically after long-term fluconazole treatment (4, 5, 19, 24, 27, 31, 32, 34, 35, 41). Current methods for antifungal susceptibility testing need to conform to three considerations, which overlap mutually. They should give results that correspond to the clinical outcome of antifungal treatment, that show some correlation to those obtained with the reference broth dilution method M27P published by the National Committee for Clinical Laboratory Standards (NCCLS) (14), and that are reproducible. Additional desiderata for an antifungal susceptibility test are that the test should be rapid and easy to perform.

The publication of the NCCLS reference method for antifungal testing should have led rapidly to a convergence of test protocols in different laboratories. However, this method is based on a tube macrodilution protocol that is more cumbersome to perform than tests done in microdilution plates, and it does not lend itself readily to the production of commercial test systems. There also remain doubts concerning the reliability of the NCCLS method for the detection of amphotericin B-resistant yeasts (30, 31). For these reasons susceptibility

studies based on methods other than the reference standard continue to appear (5, 7, 8, 22, 29, 33, 34, 36, 37, 39, 40, 42) and commercial susceptibility test systems such as the E test (9, 38), Alamar Blue (21, 23, 25), Autobac (29), ATBfungus (6, 28), and API (26) have been introduced and evaluated.

Direct downscaling of the NCCLS reference method to a broth microdilution system is possible. The results of broth microdilution tests show fair to good agreement with those obtained by the original method (3, 10, 11, 13, 23, 38), depending on the agent tested. In general, these studies showed that the correlations were better for water-soluble agents such as fluconazole and flucytosine than for relatively insoluble agents such as itraconazole and ketoconazole.

The factors that influence outcome with yeast antifungal susceptibility determinations are well known: they include choice of medium, inoculum size, incubation temperature and pH, reading time, and the nature of the endpoint chosen (31). Some investigators have measured the optical densities of growth turbidity in broth microdilution assays as a means of establishing MIC endpoints (5, 7, 8, 15, 27, 29, 33, 36). Such measurements, based on dose-response curve data, theoretically offer an endpoint determination in microdilution assays that is of higher precision than the subjective visual assessment of turbidity on a 1+ to 4+ scale used by others (3, 10–12, 21–23, 33, 34, 38, 39). However, the various investigators who used spectrophotometric measurements have not consistently used either the same growth conditions for the broth microdilution assay or the same endpoint. For azole antifungal agents data based on spectrophotometric reduction of growth below 50% of control values (50% inhibitory concentration [IC_{50}])

* Corresponding author. Mailing address: Department of Bacteriology and Mycology, Janssen Research Foundation, B-2340 Beerse, Belgium. Phone: 32-14-603004. Fax: 32-14-605403.

have been published (5, 29, 33, 36) and below 25 to 30% of control growth (IC_{70}) (7, 8, 15, 27). Yet, the endpoint proposed in the NCCLS protocol is a reduction of growth below 20% of that of the control (IC_{80}) (14).

The present study was undertaken to reexamine the parameters for antifungal susceptibility determination in the context of a semiautomated broth microdilution system based on the method recommended by NCCLS. Dose-response curves and endpoint readings were derived automatically by computer analysis of the raw data for clinically important yeasts tested against five systemically active antifungal agents. The test results were evaluated for reproducibility and agreement with published results as well as with results from the reference NCCLS broth macrodilution method.

MATERIALS AND METHODS

Yeast isolates. The 616 yeast isolates studied came from our stock collection of isolates, and most were first tested within 6 weeks of receipt. The cultures were not a cross-sectional representation from any defined clinical group. Approximately one-half were unselected isolates grown directly from clinical material received in the course of epidemiologic studies or clinical trials of antifungal agents. The remainder were selected isolates submitted specifically for susceptibility testing or other investigations, often because of a patient's failure to respond to antifungal therapy. The species represented were *Candida albicans* (272 isolates), *Candida famata* (12 isolates), *Candida glabrata* (134 isolates), *Candida guilliermondii* (25 isolates), *Candida humicola* (1 isolate), *Candida inconspicua* (2 isolates), *Candida kefyr* (10 isolates), *Candida krusei* (41 isolates), *Candida lambica* (1 isolate), *Candida lusitaniae* (25 isolates), *Candida norvegensis* (6 isolates), *Candida parapsilosis* (29 isolates), *Candida pelliculosa* (6 isolates), *Candida tropicalis* (43 isolates), *Candida utilis* (2 isolates), and *Saccharomyces cerevisiae* (7 isolates). These species identifications were reconfirmed before susceptibility testing and were based on morphological criteria plus assimilation profiles on ID 32C strips (BioMérieux sa., Marcy l'Étoile, France). Isolates of *Cryptococcus neoformans* were specifically excluded from the study because they were found to give variable and generally low levels of growth in the test medium used.

The geographical sources of the isolates were unknown in 56 cases; the rest of the isolates came from Belgium (151 isolates), Canada (10 isolates), France (32 isolates), Germany (264 isolates), Japan (1 isolate), The Netherlands (7 isolates), the Philippines (1 isolate), Rwanda (2 isolates), the United Kingdom (39 isolates), and the United States (53 isolates). Of the 616 yeasts, 335 came from patients known to have human immunodeficiency virus infection, 36 were from patients seen in dermatology practices, and 107 were from patients seen in gynecology practices: the remainder were from miscellaneous or unknown clinical backgrounds. Most of the isolates came from superficial anatomical sites. Only 3 were from blood cultures, while 377 were oral isolates, 62 were vaginal isolates, and 34 were nail isolates. The remainder were cultured from feces or anal swabs (10 isolates), urine (5 isolates), skin (4 isolates), veterinary sources (2 isolates), and bronchial aspirate, milk, and urethra (1 isolate each). The anatomical sources of the isolates were unknown in 111 instances; the remaining 5 isolates were reference laboratory strains obtained from the United Kingdom and Belgium.

The yeasts were maintained on Sabouraud agar (Oxoid, Basingstoke, United Kingdom) stored at 4°C for up to 6 weeks or as suspensions in dilute casein hydrolysate-yeast extract-glucose (CYGi) broth (18) containing 10% (vol/vol) glycerol at −70°C for longer periods.

For each experiment a selection of the whole set of yeast isolates was included. The number included varied according to the experimental design and the practical limits on the numbers of plates that could be handled within a suitable time. Isolates were not all chosen at random; an effort was usually made to include strains known to show high-, low-, and mid-range susceptibilities to the agents chosen as well as to include a representative selection of the various yeast species. Preference was generally given to recently received isolates to avoid the overuse of isolates that had been conditioned by repeated laboratory subculture. Some isolates were included in several experiments so that data from multiple repeated tests with these isolates could be analyzed. Many isolates were tested at least in duplicate and often in triplicate because they were included as random choices in experiments that involved large numbers of different yeasts. No isolates were prospectively tested to assess the reproducibility of the results; the reproducibility data presented therefore represent the real-life repeatability of data in which replicate testing occurred by chance rather than by design.

Inoculum preparation. Except where otherwise noted, yeast inocula were prepared in 5-ml volumes of CYGi broth (18) incubated at 30°C for 18 to 24 h with rotation at 20 rpm at an angle of 5° from the horizontal (17). This procedure yielded suspensions containing 4×10^7 yeasts per ml without the need for further standardization (18; see also results below). The suspensions were diluted in

susceptibility testing culture medium to yield the desired initial cell concentration for each experiment.

Antifungal agents and stock solutions. Amphotericin B was purchased as the Fungizone intravenous preparation in sodium deoxycholate (Bristol-Myers Squibb Belgium S.A., Brussels, Belgium). Preliminary experiments showed no difference in the results obtained with this commercial preparation and those obtained with pure amphotericin B (Bristol Myers-Squibb). Flucytosine was purchased from ACROS Chimica (Geel, Belgium), fluconazole was obtained from Pfizer UK (Sandwich, United Kingdom), and itraconazole and ketoconazole were obtained from the Janssen Research Foundation (Beerse, Belgium). Stock solutions were prepared as follows: amphotericin B by resuspension of a Fungizone vial in 10 ml of water; flucytosine by dissolution in water at 6.0 mg/ml; and fluconazole, itraconazole, and ketoconazole by dissolution in dimethyl sulfoxide (DMSO) at 1.5 mg/ml. The stock solutions were stored frozen at −20°C for a maximum of 2 months.

Microdilution plate susceptibility testing. For preparation of test microdilution plates the stock solution of amphotericin B was first further diluted to a concentration of 6 mg/ml (273 μl in 2 ml of water); the remaining compounds were used directly from their stock solutions. A series of 10 twofold dilutions was prepared from the stock solutions in the same solvent used for the stock solutions; thus, amphotericin B and flucytosine were serially diluted in water and the three azole antifungal agents were serially diluted in DMSO. Each antifungal dilution was then diluted with 19 volumes of water, bringing their concentrations to three times the desired final concentrations. These solutions were pipetted in 50-μl volumes in rows of dilutions in the wells of flat-bottom microdilution plates (Falcon 3072; Becton Dickinson, Lincoln Park, N.J.). (Other plate types were evaluated in preliminary experiments; see Results.) Water alone was used as a control since the inclusion of DMSO made no difference to control optical density (OD) readings in pilot experiments. The plates were stored at −20°C for up to 6 months. The final concentrations of amphotericin B ranged from 10 to 0.01 μg/ml, the final concentrations of flucytosine and fluconazole ranged from 100 to 0.1 μg/ml, and the final concentrations of itraconazole and ketoconazole ranged from 25 to 0.025 μg/ml, all in twofold dilution steps.

For all experiments the basal test medium was RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinophosphonyl sulfate (MOPS). This was prepared by admixture of 10×-concentrated RPMI 1640 medium without bicarbonate or glutamine (ICN Biomedicals, Asse-Relegem, Belgium), 1.65 M MOPS (ACROS Chimica) adjusted to pH 7.2 and sterilized by filtration, 200 mM glutamine (ICN), and sterile water in appropriate volumes to achieve the desired final pH and concentration of ingredients. Experimental medium additives were similarly prepared as concentrates. The medium was first diluted to a concentration of 1.5 times the final concentration, the appropriate volume of the CYGi inoculum suspension was added (in most experiments, 20 μl in 10 ml of medium), and 100-μl volumes of inoculated broth were added to the 50-μl dilutions of antifungal agents. This last step brought all reagents and the inoculum to the desired final concentration.

The plates were sealed with plastic stickers and were incubated for 48 h at 37°C. Preliminary experiments revealed no substantial difference between data obtained at 37°C and those obtained at the 35°C recommended by NCCLS (14). After incubation, the stickers were removed and the turbidity in each well was measured at 405 nm with a microdilution plate reader (model 3550; Bio-Rad, Richmond, Calif.).

Analysis of results from broth microdilution tests. The raw OD readings were analyzed with Microsoft Excel software. Adjacent to the location of the raw data in the spreadsheet, a calculation matrix was created to convert OD readings into measurements of growth as percentages of control readings, corrected for the background OD. Next to this calculation matrix, a display of growth inhibition endpoints was created. A further, optional area of the spreadsheet contained graphic displays of dose-response curves.

Fixed background OD measurements for each concentration of antifungal agent were established after preliminary experimentation with uninoculated plates. The values have been rescrutinized at intervals in the course of 2 years of testing and have not been found to require readjustment. Minor run-to-run variations in background OD values did not significantly affect the percent growth calculation.

The calculation matrix comprised two spreadsheet cells displaying the mean and the percent coefficient of variation (CV) for corrected OD readings from the set of drug-free control cultures and an array of cells displaying growth as a percentage of the control growth for each antifungal agent and concentration tested. Since the validity of each experimental datum depended heavily on the accuracy of the calculated mean control OD, the calculated control mean and CV were scrutinized for each microdilution plate to ensure validity. All isolates that gave corrected mean OD readings less than 0.25 were rejected from further analysis (89% of the yeast isolates gave corrected mean OD readings of 0.5 or greater; the mean control OD value for the 616 isolates was 0.776 in the RPMI 1640 medium with 2% glucose that was used in most experiments). When the CV exceeded 10%, the raw data for the control cultures were scrutinized for the source of the variation. If one of the control OD readings was self-evidently substantially lower or higher than the other six readings, it was regarded as an artifactual outlier and was removed from the calculation. When the variation could not be attributed to such outlying readings and the CV for the mean control OD exceeded 15%, the results were rejected and the test was repeated.

MICs were calculated automatically in single spreadsheet cells for each antifungal agent on the basis of a logical function that took account of specific endpoint criteria (IC_{50} based on growth reduction below 50% of control, IC_{70} below 30% of control, IC_{80} below 20% of control). The IC was expressed as being greater than the highest concentration tested or less than or equal to the lowest concentration tested if the growth at the four highest concentrations exceeded the endpoint criterion or the growth at the four lowest concentrations was below the endpoint criterion. On-scale readings were expressed by a subtractive algorithm that related the antifungal agent concentration to the percentage of control growth. IC results were expressed to two significant figures.

In instances in which the dose-response curve was not smooth (the theoretical curve for any agent should progress from readings of 100% of control growth at low concentrations to lower readings at high concentrations), an aberrant endpoint calculation sometimes resulted. This was almost always the result of a single maverick experimental reading. Such results were indicated automatically by an evaluation function in the spreadsheet cell adjacent to each IC endpoint that showed the word "fault" when the calculated IC did not lie on the scale of concentrations tested. When the fault indication appeared in the spreadsheet, the IC endpoint was decided by scrutiny of the full dose-response curve. This approach to IC determination approximated more closely the usual manual procedure for dealing with artifactually high or low skipped tubes in a dilution series than mathematical manipulation of the dose-response data. Curve-fitting procedures would automatically smooth maverick readings into a new curve and endpoint that might grossly distort the IC determination by comparison with visual evaluation of the growth in the wells.

Susceptibility testing by the NCCLS reference method. In tests done by the NCCLS broth macrodilution protocol, the antifungal agent stock solutions and dilutions and the yeast inocula were prepared exactly as described in NCCLS protocol M27P (14). Incubation was for 48 h at 35°C, and endpoints were read according to the NCCLS criteria.

RESULTS

Selection of well cross-section and spectrophotometric reading method for broth microdilution susceptibility tests. In preliminary experiments the influence of microdilution plate well shape (flat bottom, U-shaped bottom, or V-shaped bottom) and the effect of shaking the plates to resuspend growth before spectrophotometric reading were studied. In total, 48 yeast isolates (15 isolates of *C. albicans*; 6 isolates each of *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*; and 3 isolates each of *C. famata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, and *C. lusitaniae*) were inoculated in quadruplicate microdilution plate wells in RPMI 1640 medium with 2% glucose (see below). Plates with flat bottoms and U and V shapes were set up in parallel and were incubated at 37°C for 48 h, and the OD at 405 nm (OD_{405}) of the resulting growth in the wells was measured with a plate reader without prior shaking of the plates. The overall means of all of the OD readings for the 48 isolates were 0.930, 0.837, and 0.964 for the flat-bottom, U-bottom, and V-bottom plates, respectively. For each set of four readings per yeast isolate, the CV was determined. The average CVs for the 48 yeasts tested were 2.9, 5.6, and 3.7% for the flat-bottom, U-bottom, and V-bottom plates, respectively.

Others have reported that shaking of the microdilution plates to resuspend the yeasts gives better reproducibility for susceptibility determinations (1). To reappraise the value of shaking for spectrophotometric readings, the 48 yeast cultures in quadruplicate were resealed and resuspended by vigorous shaking on a vortex mixer. After resuspension of the yeasts by shaking, the global mean OD readings were 0.947, 1.036, and 1.138 for the flat-bottom, U-bottom, and V-bottom plates, respectively. The respective mean CVs were 2.9, 2.4, and 4.3%. Shaking of the microdilution plates therefore increased the OD values and reduced the variability between replicate wells in U-bottom and V-bottom microdilution plate wells. However, yeast cultures in flat-bottom wells gave similar OD readings and CVs with and without shaking. Consideration of the extra work and biohazard involved in shaking large numbers of microdilution plates led to the conclusion that unshaken microdilution plates with flat-bottom wells gave reliable readings. Moreover, it was noted that plates with flat-bottom wells were less prone to visible spattering of reagents from the wells during preparative additions from microtip pipettes. On the basis of all these considerations, microdilution plates with flat-bottom wells were used in all further experiments.

Effect of glucose concentration in growth medium on susceptibility test results. Buffered RPMI 1640 medium normally contains 2 g of glucose per liter, a concentration lower than that commonly used for cultures of *Candida* spp. Rodriguez-Tudela and Martinez-Suarez (33) showed that supplementation of the medium with glucose to a final concentration of 20 g/liter resulted in higher control turbidity readings without major changes in fluconazole MICs. To study further the influence of the glucose concentration of RPMI 1640 medium on susceptibility test outcome, seven *Candida* isolates were inoculated in duplicate microdilution plate susceptibility tests with RPMI 1640 medium containing different glucose concentrations. The mean ± standard deviation (SD) control OD_{405} readings for the seven isolates were 0.35 ± 0.10, 0.52 ± 0.16, 0.76 ± 0.17, and 0.88 ± 0.20 in RPMI 1640 medium with final glucose concentrations of 1, 5, 10, and 20 g/liter, respectively. The averages of the individual CVs between the duplicate control ODs for the seven isolates were 11.7, 19.5, 7.7, and 4.4%, respectively, with increasing glucose concentration. The highest glucose concentration therefore gave the highest and the most reproducible control OD readings.

Discrepancies between susceptibility endpoints for amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole in duplicate tests with the same isolates rarely exceeded ±1 drug dilution, regardless of the glucose concentration or choice of endpoint reading (IC_{50}, IC_{70}, or IC_{80}). The extent of duplicate variation was, however, slightly higher at the two lowest glucose concentrations compared with that at the two highest concentrations. On the basis of these results, a final glucose concentration of 20 g/liter (supplementation of the medium with 18 g/liter) was used in all subsequent experiments. This medium is referred to as RPMI–MOPS–2% glucose (RMG medium).

Preparation of yeast inoculum. Preliminary experiments with seven isolates of *Candida* spp. indicated that the following variables in inoculum preparation had little or no effect on the outcomes of the susceptibility tests in RMG medium (data not shown): inoculum grown in CYGi medium rotated at 30°C and 20 rpm (18) versus inoculum grown on Sabouraud glucose agar and resuspended to 4×10^7 cells per ml (hemocytometer count) in sterile water, preparation of inoculum at 30°C versus preparation of one at 37°C in CYGi medium or on Sabouraud agar, and use of an inoculum 18- to 24-h old versus one 72-h old (in CYGi broth).

Preparation of yeast inocula by incubation for 18 to 24 h at 30°C in CYGi medium with constant rotation of the culture at 20 rpm (18) was confirmed as a suitable standard method for susceptibility testing without the necessity for yeast cell enumeration. The OD_{530} of the yeast suspensions after incubation in CYGi medium was determined for 197 isolates of *Candida* spp. (107 *C. albicans* isolates, 41 *C. glabrata* isolates, 16 *C. krusei* isolates, 17 *C. tropicalis* isolates, and 16 isolates representing 7 other species). The arithmetic mean OD (SD) was 1.037 (0.160), the geometric mean was 1.020, the lowest reading was 0.257, and the highest reading was 1.445; these measurements generally matched previously published results (18). Four (2.0%) of the 197 yeasts gave an OD_{530} of less than 0.5 and 9 (4.6%) gave an OD_{530} of less than 0.7. Converted to CFU per milliliter by reference to a calibration curve determined with 5 yeast isolates, the OD_{530} data from the 197 isolates showed a yeast concentration of between 8.6×10^6 and

4.9×10^7/ml, a range sufficiently narrow for the purposes of providing a consistent susceptibility test inoculum.

Effect of initial yeast cell concentration on susceptibility test results. The initial yeast concentration in the susceptibility test cultures substantially influenced the endpoint measurements for flucytosine and the azole antifungal agents but made little difference to the outcomes of tests with amphotericin B. The experiments were done with 29 yeast isolates (13 *C. albicans*, 4 *C. glabrata*, 3 *C. krusei*, 2 *C. lusitaniae*, 1 *C. parapsilosis*, 5 *C. tropicalis*, and 1 *C. utilis* isolate) diluted from CYGi cultures to yield initial concentrations of 10^3, 10^4, 10^5, and 10^6 yeast cells per ml in RMG medium. After 48 h of incubation at 37°C, the median value of the mean control OD_{405} readings for triplicate microdilution cultures of each isolate rose progressively with increasing initial yeast concentration from 0.46 (10^3/ml) to 0.86 (10^6/ml), while the SDs for those mean control OD readings fell progressively from 0.07 (10^3/ml) to 0.03 (10^6/ml).

The variations in susceptibility endpoint readings with the initial yeast concentration differed among the five antifungal agents tested. With amphotericin B, a 10,000-fold variation of the initial yeast concentration from 10^3 to 10^6 cells per ml had little influence on the IC, regardless of the endpoint criterion used. All except 1 of the 29 isolates gave endpoints in the narrow range of 0.16 to 1.3 μg/ml; 1 isolate gave IC_{70}s and IC_{80}s of 2.5 μg/ml in tests with an initial yeast concentration of 10^3 cells per ml. None of the 29 isolates tested showed amphotericin B endpoint variations exceeding 2 dilution steps with changes in inoculum size whether it was measured by using an endpoint of IC_{50}, IC_{70}, or IC_{80}.

By contrast, the flucytosine susceptibility test outcome varied considerably with the initial yeast concentration. At least 10 of the 29 isolates tested showed inoculum size-dependent variations in ICs that exceeded 2 dilution steps. This vulnerability of the flucytosine IC to inoculum size was lowest for the IC_{50} endpoints and was highest for the IC_{80} endpoints. By IC_{80} measurements, 8 of the 29 isolates tested gave flucytosine susceptibility endpoints that rose over 4 or more twofold dilutions with increasing initial cell concentration. Three of the 29 isolates showed unequivocal and consistent flucytosine resistance, irrespective of the endpoint criterion (all endpoint values were >100 μg/ml) at initial cell concentrations of 10^5 and 10^6/ml.

The three azole antifungal agents also showed a lower tendency to IC variation when IC_{50} endpoints were used compared with the variation when IC_{70} and IC_{80} measurements were used. With IC_{80}s, at least half of the yeast isolates gave >2-dilution-step variations with increasing inoculum size. For at least six of the isolates, the IC_{80} rose by 6 or more dilution steps when the initial yeast concentration rose from 10^3 to 10^6 cells per ml. It was notable that for all three azole derivatives, the lowest initial yeast concentration resulted in more endpoint readings at the high end of the drug concentration range tested than were evident with higher initial cell concentrations.

The frequency with which automated calculation of susceptibility endpoints generated a "fault" condition in the analysis spreadsheet varied considerably with the initial yeast cell concentration. The fault condition resulted from maverick percent growth readings, equivalent to skipped tubes (both overgrown and undergrown) in manually read broth dilution tests. Among the 435 endpoint determinations made (29 isolates, five test compounds, three types of endpoint reading), faults occurred most often with cultures starting at 10^3 yeasts per ml (34 faults) and 10^6 cells per ml (24 faults) and least often at initial concentrations of 10^4 cells per ml (8 faults) and 10^5 cells per ml (9 faults).

The lowest (10^3/ml) and highest (10^6/ml) initial cell concen-

trations were rejected for subsequent use since these gave the poorest endpoint reproducibilities and the most fault conditions in automatic endpoint calculations. Initial concentrations of 10^4 and 10^5 yeasts per ml both gave a reasonably broad spread of susceptibility endpoints for all antifungal agents except amphotericin B, with minimal fault conditions in the automated calculations of ICs. At 10^5 cells per ml the median (background-corrected) control OD reading of 0.80 was substantially higher than the mean reading of 0.62 from an initial concentration of 10^4 yeasts per ml. The distribution of SDs for mean control OD readings was also narrower at 10^5 cells per ml than at 10^4 cells per ml. For subsequent experiments, an initial yeast concentration of 8×10^4 cells per ml was therefore chosen as the best compromise (20 μl of inoculum suspension diluted into 10 ml of medium).

Comparison of microdilution plate test results with results from NCCLS reference method. A panel of 100 clinical yeast isolates (50 *C. albicans*, 21 *C. glabrata*, 1 *C. guilliermondii*, 1 *C. kefyr*, 4 *C. krusei*, 3 *C. lusitaniae*, 9 *C. parapsilosis*, and 11 *C. tropicalis* isolates) was used to make a comparison between the results obtained by the semiautomated microdilution plate susceptibility assay and those obtained by the NCCLS reference broth macrodilution test read visually according to the criteria defined by NCCLS (14). Because the dilution series used in the two test systems were not identical, endpoints were compared in terms of differences in $\log_2$ instead of in numbers of dilutions. The data are summarized in Table 1.

With amphotericin B there was a slight tendency toward higher readings for the NCCLS broth macrodilution MICs than for any of the IC endpoints used with the microdilution test (Table 1). Nevertheless, the agreement between MICs and IC endpoints was usually excellent. The microdilution IC_{80} agreed with the NCCLS MIC within ±1 $\log_2$ for 83 of the 100 isolates tested. For all IC endpoints, the results obtained by the two methods agreed within ±2 $\log_2$ for 97% of the isolates. With flucytosine, agreement between the NCCLS MICs and the microdilution IC endpoints was also extremely good; for more than 90 of the 100 isolates tested there was agreement between the two methods, regardless of which IC endpoint was used in the microdilution test (Table 1).

With the three azole antifungal agents, agreement between the NCCLS macrobroth MICs and the microdilution ICs was less good (Table 1). For fluconazole and ketoconazole precise agreement between the two methods was best obtained with the IC_{50}s, since for more than 50 of the 100 test isolates IC_{50}s matched NCCLS MICs within ±1 $\log_2$. When the less stringent criterion of agreement within ±2 $\log_2$ was used, the IC_{70} reading for fluconazole showed the best agreement with the NCCLS MICs, since results for 75 isolates agreed at this level, whereas results for 69 isolates agreed with the IC_{50} and results for 70 isolates agreed with the IC_{80}. With ketoconazole the IC_{50} remained the endpoint that showed the best agreement with the NCCLS MICs: for 67 of the 100 isolates the IC_{50} agreed with the MIC within ±2 $\log_2$, whereas for 65 isolates the IC_{70} agreed and for 61 isolates the IC_{80} agreed.

Itraconazole readings showed the greatest disparity between NCCLS data and microdilution plate data. Fewer than 50% of the test isolates showed agreement between the NCCLS MIC and any of the microdilution IC endpoints within ±2 $\log_2$, and for as many as 48% of the isolates NCCLS MICs were 4 or more $\log_2$ greater than the microdilution IC_{50}. This high level of difference may have been partly species related, since 39 (81%) of the 48 isolates for which IC_{50} and NCCLS MIC disagreements were 4 or more $\log_2$ were *C. albicans*, whereas only 11 (21%) of the remaining 52 isolates were *C. albicans*.

Two yeast strains, *C. parapsilosis* ATCC 22019 and *C. krusei*

TABLE 1. Comparison of microdilution IC_{50}, IC_{70}, and IC_{80} readings with MIC determined visually by the NCCLS reference broth macrodilution susceptibility test[a]

Difference measured as no. of log₂ between NCCLS and microdilution methods	No. of isolates														
	Amphotericin B			Flucytosine			Fluconazole			Ketoconazole			Itraconazole		
	IC_{50}	IC_{70}	IC_{80}	IC_{50}	IC_{70}	IC_{80}	IC_{50}	IC_{70}	IC_{80}	IC_{50}	IC_{70}	IC_{80}	IC_{50}	IC_{70}	IC_{80}
NCCLS < microdilution															
≤4 log₂	0	0	0	2	2	4	2	3	5	2	4	5	1	3	7
<3 log₂	0	0	1	0	0	1	3	4	7	0	1	4	0	2	0
<2 log₂	0	0	0	0	2	0	5	6	10	4	8	10	1	1	3
<1 log₂	0	1	2	4	11	14	6	14	19	6	10	16	2	5	7
NCCLS = microdilution	64	77	83	85	78	77	54	52	46	56	52	43	19	27	25
NCCLS > microdilution															
>1 log₂	33	19	12	2	4	2	9	9	5	5	3	2	14	9	9
>2 log₂	2	2	1	4	2	1	13	5	4	15	11	12	10	7	10
>3 log₂	0	0	0	2	0	0	1	0	1	3	3	1	5	7	12
≥4 log₂	1	1	1	1	1	1	7	7	3	9	8	7	48	39	27

[a] Data are the number of *Candida* isolates (of 100 tested) for which NCCLS MICs were either less than the microdilution endpoint by the extent (number of log₂) shown (NCCLS < microdilution) or fell within ±1 log₂ of the same reading (NCCLS = microdilution) or exceeded the microdilution endpoint by the extent (number of log₂) shown (NCCLS > microdilution).

ATCC 6258, have been provisionally selected by the NCCLS Subcommittee on Antifungal Susceptibility Tests as reference strains for susceptibility test quality control purposes (20). Table 2 summarizes the results obtained with these strains in NCCLS broth macrodilution tests and microdilution IC endpoints compared with the acceptable ranges specified by NCCLS. For the three antifungal agents for which acceptable MIC ranges have been specified, the NCCLS broth macrodilution results fell within the designated quality control limits for both isolates. By the broth microdilution method, the IC_{70} and IC_{80} results for amphotericin B lay closest to the midpoints of the specified ranges. For flucytosine the IC_{50}, IC_{70}, and IC_{80} readings for the two yeast isolates all fell within the acceptable limits. With fluconazole the IC readings at all endpoints were identical and within range for ATCC 6258, but only the IC_{80} reading for ATCC 22019 fell within the specified range. For both yeasts, microdilution ICs of itraconazole and ketoconazole were all lower than the readings obtained by the broth macrodilution method, although the extent of the difference was less for ketoconazole than for itraconazole.

Reproducibility of the microdilution method. For assessment of the reproducibility of the microdilution susceptibility test method, data from two panels of *Candida* spp. isolates

were analyzed. The first panel comprised 51 yeast isolates (23 *C. albicans*, 13 *C. glabrata*, 3 *C. krusei*, 1 *C. inconspicua*, 1 *C. lusitaniae*, 1 *C. parapsilosis*, and 9 *C. tropicalis* isolates) that had been tested twice against the five antifungal agents. The second panel comprised 47 isolates (20 *C. albicans*, 8 *C. glabrata*, 1 *C. kefyr*, 4 *C. krusei*, 4 *C. lusitaniae*, 2 *C. parapsilosis*, and 8 *C. tropicalis* isolates) that had been tested on three or more occasions (several isolates had been tested five or more times in the course of a 2-year period). For each of these isolates, the maximum difference between replicate IC_{50}, IC_{70}, and IC_{80} susceptibility endpoints was calculated in terms of the numbers of twofold dilutions. Thus, for a strain for which IC_{50}s were, for example, 0.2, 0.4, and 0.8 µg/ml in three replicate tests, an IC_{50} variation of 2 dilutions was recorded. For a strain for which IC_{70}s were 0.05 and 1.6 µg/ml in two replicate tests, an IC_{70} variation of 5 dilutions was recorded. (Off-scale values were scored as 1 twofold dilution higher than the highest or lower than the lowest actual test concentrations for these calculations.)

The results of this analysis are summarized in Table 3. For all five antifungal agents the replicate variation was greater when the tests had been repeated three or more times than when tests were repeated twice. With amphotericin B, repli-

TABLE 2. IC endpoints, NCCLS broth macrodilution MICs, and reference MICs for two yeast isolates selected for quality control purposes

Yeast isolate	Antifungal agent	Acceptable MIC range (µg/ml)	Endpoint (µg/ml) by the following method:			
			NCCLS broth macrodilution	Broth microdilution		
				IC_{50}	IC_{70}	IC_{80}
C. krusei ATCC 6258	Amphotericin B	0.5–2.0	1	0.32	0.63	0.63
	Flucytosine	4–16	8	6.3	6.3	13
	Fluconazole	16–64	32	50	50	50
	Itraconazole	Not specified	0.125	0.1	0.2	0.2
	Ketoconazole	Not specified	0.25	0.4	0.4	0.4
C. parapsilosis ATCC 22019	Amphotericin B	0.25–1.0	0.5	0.32	0.63	0.63
	Flucytosine	0.12–0.5	0.25	0.2	0.4	0.4
	Fluconazole	2–8	2	1.6	1.6	3.2
	Itraconazole	Not specified	0.064	0.1	0.1	0.1
	Ketoconazole	Not specified	0.064	0.05	0.05	0.05

TABLE 3. Reproducibility of microdilution antifungal susceptibility test in repeat experiments[a]

Antifungal agent	No. of test repetitions	No. (%) of isolates					
		IC$_{50}$ variation		IC$_{70}$ variation		IC$_{80}$ variation	
		≤2 dilutions	>2 dilutions	≤2 dilutions	>2 dilutions	≤2 dilutions	>2 dilutions
Amphotericin B	2	49 (96)	2 (4)	48 (94)	3 (6)	49 (96)	2 (4)
	≥3	43 (91)	4 (9)	40 (85)	7 (15)	41 (87)	6 (13)
Flucytosine	2	44 (86)	7 (14)	42 (82)	9 (18)	39 (76)	12 (24)
	≥3	38 (81)	9 (19)	31 (66)	16 (34)	26 (55)	21 (45)
Fluconazole	2	44 (86)	7 (14)	42 (82)	9 (18)	42 (82)	9 (18)
	≥3	28 (60)	19 (40)	29 (62)	18 (38)	29 (62)	18 (38)
Itraconazole	2	44 (86)	7 (14)	39 (76)	12 (34)	37 (73)	14 (27)
	≥3	29 (62)	18 (38)	29 (62)	18 (38)	24 (51)	23 (49)
Ketoconazole	2	40 (78)	11 (22)	36 (71)	15 (29)	38 (75)	13 (25)
	≥3	29 (62)	18 (38)	32 (68)	15 (32)	27 (57)	20 (43)

[a] The data show the numbers (and percentages) of the isolates tested that gave endpoint results that varied only within a 2-dilution range or over 3 or more dilutions in the course of repeated testing. The data are stratified according to whether isolates were tested twice or on three or more occasions.

cate variation was generally small. Of the 98 isolates in total that underwent replicate testing, at least 85% showed replicate variation within a 2-dilution range, regardless of which IC endpoint was used. For flucytosine, variation between replicates was least with the IC$_{50}$ endpoint and greatest with the IC$_{80}$ endpoint. IC$_{50}$ readings for more than 80% of the isolates agreed within a 2-dilution variation. Less consistent results were obtained with IC$_{70}$ and IC$_{80}$ readings (Table 3).

With the azole antifungal agents, the best agreement was seen with IC$_{50}$s and with isolates that had been tested twice. More than 85% of the duplicate IC$_{50}$ readings for fluconazole and itraconazole were the same within a 2-dilution range, and almost 80% were within a 2-dilution range for ketoconazole. However, with IC$_{70}$ and IC$_{80}$ endpoints fewer than 80% of the isolates tested in duplicate with itraconazole and ketoconazole showed results at this level of consistency, and for all three azole antifungal agents the extent of variation beyond 2 dilutions was considerable for isolates tested on three or more occasions.

Effects of culture media from different suppliers on susceptibility endpoint. As a final test of the inherent reproducibility of the semiautomated microdilution susceptibility test method described in this report, a panel of 58 clinical yeast isolates (51 *C. albicans* and 7 *C. tropicalis* isolates), chosen on the basis of existing susceptibility data to represent a diverse spread of IC results, was retested by the microdilution method but with RPMI 1640 medium from two suppliers (ICN and Gibco).

Control OD$_{405}$ readings after 48 h of incubation differed between the two media (mean ± SD ODs corrected for background absorbance of ICN and Gibco RPMI 1640 media were 0.566 ± 0.186 and 0.708 ± 0.212, respectively). Susceptibility endpoints for amphotericin B were the same within 2 test dilutions in terms of the IC$_{70}$ and IC$_{80}$ (Table 4). However, endpoints for 2 of the 58 yeasts tested varied between the media by more than 2 dilutions in terms of the IC$_{50}$. With flucytosine either 2 or 3 of the 58 yeasts showed endpoint variation greater than 2 dilutions between ICN and Gibco RPMI 1640 media, regardless of the endpoint measurement used (Table 4). With the three azole-derivative antifungal agents, up to 12 (21%) of the isolates tested showed significant endpoint variations between ICN and Gibco RPMI 1640 me-

dia; the least variation was evident in terms of the IC$_{50}$, and the most variation was evident in terms of the IC$_{80}$ (Table 4).

Distributions of ICs for 616 yeast isolates. A total of 616 yeast isolates, each from a different patient or from different sites within the same patient, were tested to assess the outcomes of susceptibility testing in microdilution plates against five antifungal agents. For isolates that had been tested on more than one occasion, a single dose-response curve was constructed from the mean values of the percent growth data at each drug concentration.

The IC data for each of seven clinically important *Candida* spp. are summarized in Table 5. IC$_{80}$ readings are given for amphotericin B, and IC$_{50}$ readings are given for the other antifungal agents. The data suggest that most isolates of *C. parapsilosis* were susceptible to all five antifungal agents. For *C. lusitaniae* isolates comparatively high ICs of amphotericin B and flucytosine were sometimes obtained, but they were susceptible to all three azole derivatives tested. Isolates of *C. guilliermondii* were generally susceptible to most of the agents, although for some of these isolates fluconazole ICs were high. *C. krusei* was comparatively insusceptible to fluconazole but not to the other two azole derivatives. For *C. glabrata* isolates ICs of all three azole antifungal agents tended to be high, but the *C. glabrata* isolates were normally susceptible to amphotericin B and flucytosine, while *C. tropicalis* isolates showed a strong tendency toward insusceptibility to all of the agents

TABLE 4. Number of yeast isolates of 58 tested that gave an endpoint difference greater than 2 dilutions in microdilution tests done in RPMI 1640 medium supplied by ICN and Gibco

Antifungal agent	No. of yeasts giving endpoint difference of >2 dilutions by:		
	IC$_{50}$	IC$_{70}$	IC$_{80}$
Amphotericin B	2	0	0
Flucytosine	3	2	3
Fluconazole	9	11	12
Itraconazole	5	7	12
Ketoconazole	2	7	9

TABLE 5. ICs of five antifungal agents for isolates among the 616 clinical yeast isolates tested determined by semiautomated microplate susceptibility test

Species	Antifungal agent	Endpoint	MIC (μg/ml)[a]		
			Range	50%	90%
C. albicans ($n = 272$)	Amphotericin B	IC_{80}	0.08–5.0	0.32	0.63
	Flucytosine	IC_{50}	0.05->100	0.2	0.8
	Fluconazole	IC_{50}	0.05->100	6.3	50
	Itraconazole	IC_{50}	≤0.025->25	0.1	0.8
	Ketoconazole	IC_{50}	≤0.025->25	0.1	0.8
C. glabrata ($n = 134$)	Amphotericin B	IC_{80}	0.16->10	0.32	0.63
	Flucytosine	IC_{50}	≤0.1->100	0.05	0.8
	Fluconazole	IC_{50}	6.3->100	50	>100
	Itraconazole	IC_{50}	≤0.025->25	3.2	13
	Ketoconazole	IC_{50}	≤0.025-13	3.2	6.3
C. guilliermondii ($n = 25$)	Amphotericin B	IC_{80}	0.32–1.3	0.32	0.63
	Flucytosine	IC_{50}	0.05–0.2	0.05	0.05
	Fluconazole	IC_{50}	1.6–25	6.3	13
	Itraconazole	IC_{50}	0.05–0.8	0.4	0.4
	Ketoconazole	IC_{50}	0.05–0.2	0.1	0.2
C. krusei ($n = 41$)	Amphotericin B	IC_{80}	0.32–1.3	0.63	1.3
	Flucytosine	IC_{50}	0.2–3.2	1.6	3.2
	Fluconazole	IC_{50}	≤0.1–100	50	100
	Itraconazole	IC_{50}	≤0.025–0.8	0.4	0.8
	Ketoconazole	IC_{50}	0.05–1.6	0.8	0.8
C. lusitaniae ($n = 25$)	Amphotericin B	IC_{80}	0.32–2.5	0.63	2.5
	Flucytosine	IC_{50}	≤0.1->100	0.05	25
	Fluconazole	IC_{50}	≤0.1->100	0.8	3.2
	Itraconazole	IC_{50}	≤0.025–0.8	0.05	0.4
	Ketoconazole	IC_{50}	≤0.025–3.2	0.05	0.1
C. parapsilosis ($n = 29$)	Amphotericin B	IC_{80}	0.32–1.3	0.32	1.3
	Flucytosine	IC_{50}	≤0.1–0.8	0.2	0.4
	Fluconazole	IC_{50}	0.2–1.6	0.8	1.6
	Itraconazole	IC_{50}	≤0.025–0.2	0.05	0.1
	Ketoconazole	IC_{50}	≤0.025–0.1	0.05	0.05
C. tropicalis ($n = 43$)	Amphotericin B	IC_{80}	0.32–2.5	0.63	1.3
	Flucytosine	IC_{50}	≤0.1->100	0.2	50
	Fluconazole	IC_{50}	≤0.1->100	13	>100
	Itraconazole	IC_{50}	≤0.025->25	0.4	3.2
	Ketoconazole	IC_{50}	≤0.025–6.3	0.2	3.2
Other yeasts ($n = 47$)	Amphotericin B	IC_{80}	0.08–2.5	0.16	0.32
	Flucytosine	IC_{50}	≤0.1–50	0.05	0.05
	Fluconazole	IC_{50}	≤0.1–100	6.3	6.3
	Itraconazole	IC_{50}	≤0.025–13	0.8	0.8
	Ketoconazole	IC_{50}	≤0.025–3.2	0.2	0.4

[a] 50% and 90%, MICs at which 50 and 90% of strains tested were inhibited, respectively.

tested except amphotericin B, although for two *C. tropicalis* isolates already designated as putatively resistant (30), amphotericin B readings were 2.5 μg/ml at all three endpoint measurements. Among the other species listed in Table 5, for isolates of *C. utilis* and *S. cerevisiae* ICs of flucytosine were often comparatively high.

DISCUSSION

Spectrophotometric turbidity readings of broth microdilution series have been in use for more than 10 years for determination of the susceptibilities of yeasts to antifungal agents. A spectrophotometric reading is a more objective measurement of yeast growth in the presence of inhibitory agents than sub-

jective visual assessment, and construction of dose-response curves, with or without automatic determination of inhibition endpoints, affords a simpler and more reliable basis for data analysis than visual scoring of turbidities on a 1+ to 4+ scale, as has been used elsewhere for both broth macrodilution and broth microdilution determinations (10, 12, 21–23, 39). The earliest example of the approach, by Johnson et al. (15) in 1984, was a method that differed little from the one used in the present study. However, like most publications describing the spectrophotometric-broth microdilution approach, this early use of the technique paid little heed to the known effects of variable test parameters on endpoints. We therefore considered that these variables required reexamination in the context of developing broth microdilution tests for full automation.

In the present study, reagents and inocula were added by hand pipetting, but with the currently available technology, these steps could be performed by automatic fluid handling devices, with a potential quantitative improvement in the results as well as a reduction in the labor necessary for susceptibility testing. Spectrophotometric reading of the microdilution plates and analysis of the data were done automatically by means of computer-assisted devices and spreadsheet templates. Automation improves the ease and speed of setting up tests with multiple isolates and multiple antifungal agents; it also makes data analysis and endpoint reading truly quantitative and objective and more flexible than endpoint determination by eye. It is therefore regrettable that automation does not appear to overcome the inherent vulnerability of yeast susceptibility determinations, particularly with azole derivatives, to variations that are apparently related to the yeast strain tested and the culture conditions.

In the present study, in establishing the selected test method, we attempted to pay particular attention to fine points of technique that are commonly overlooked or that are regarded as too trivial for comment. Our experiments have shown that microdilution cultures in flat-bottom wells are superior for quantitation than those in wells of other shapes and that agitation of such cultures before spectrophotometric determinations is unnecessary.

Our microplate method is based on the standard broth macrodilution yeast susceptibility test method proposed by NCCLS (14). It differs from that method in just three important respects: it is performed on a broth microdilution scale, the inoculum size of 8×10^4 cells per ml is 80 times greater than that recommended by NCCLS, and the glucose concentration of the medium is 10 times higher than that in unmodified RPMI 1640 medium. These modifications all confer advantages in terms of optimization of the spectrophotometric readings that are obtained. The higher glucose concentration and the higher inoculum level both serve to maximize the differential between uninhibited and inhibited yeast growth and to reduce quantitative variations in OD readings for drug-free control cultures. Some previous investigators have also used higher glucose concentrations in the culture medium (33, 36) and higher inoculum levels (7, 27, 29, 33, 36, 39) than those recommended by NCCLS in broth microdilution adaptations of yeast susceptibility tests.

Other investigators have reported agreement within a twofold dilution range for 90% or more of amphotericin B and flucytosine MICs determined by the NCCLS method and by broth microdilution adaptations of the method (3, 10, 21), and similar results were obtained in the present study. However, the agreement between the ICs of fluconazole in the present microdilution test and the NCCLS broth macrodilution test was less impressive (a maximum 75% of isolates showing agreement within a twofold dilution range) than the agreement found by others, which was never lower than 80% (3, 10, 21) and which sometimes exceeded 90% (23, 38). The poor agreement between broth macrodilution and broth microdilution endpoints for ketoconazole and itraconazole found in the present study mirrors data published by others, who obtained lower levels of agreement with these two relatively insoluble azole-derivative antifungal agents (3, 10). Itraconazole and ketoconazole have consistently been the source of the lowest interlaboratory agreement in collaborative evaluations of antifungal susceptibility tests (12, 39). The reason why *C. albicans* isolates showed the greatest variation between NCCLS endpoints and microdilution plate endpoints is unknown. In a previous study a high level of discrepancy was similarly noticed

for a single yeast species, but on that occasion the species concerned was *C. glabrata* (27).

The intralaboratory reproducibility of the present test system was less favorable than has been reported in published studies of yeast susceptibility tests. However, such studies are normally done under circumstances in which a panel of yeast isolates is set up purposefully, prospectively, and exceptionally for testing in two replicate experiments that are done in close succession to each other. Our approach to the evaluation of reproducibility was to include isolates for retesting on a semi-random basis over a period of 2 years. This led to many isolates being tested twice, while others were tested repeatedly on three or more occasions. The reproducibility of the data was then assessed retrospectively. In this way no special or conscious effort was being made to achieve a high intralaboratory reproducibility of the susceptibility determinations. We therefore believe that the results give a more realistic impression than is usual of the level of test variability expected to be encountered in replicate assays. As with the comparison between NCCLS broth macrodilution and the new, semiautomated microdilution tests, the level of reproducibility was highest with amphotericin B; this was followed by flucytosine and fluconazole, with itraconazole and ketoconazole showing the poorest reproducibility.

The NCCLS recommendations for antifungal susceptibility endpoints differ for different types of antifungal agents (14). For amphotericin B, complete growth inhibition (absence of visual turbidity) is specified, but reduction of turbidity to 20% of control growth (nominally equivalent to the IC_{80} endpoint in the present study) is specified for flucytosine, fluconazole, and ketoconazole. Broth microdilution adaptations of the NCCLS method have similarly used 1+ and 2+ visual turbidity endpoints for determination of flucytosine and fluconazole MICs but 0 turbidity for amphotericin B (21–23, 38, 39). The results from the present study also indicate a different optimal endpoint for amphotericin B susceptibility compared with those for the other antifungal agents tested. With amphotericin B the data were generally robust and varied little between IC_{50}, IC_{70}, and IC_{80} endpoints. However, in the comparison between broth microdilution endpoints and NCCLS broth macrodilution MICs (Table 1), greater agreement was obtained with the IC_{80} endpoint than with the other two endpoints. In the comparison between data obtained with ICN and Gibco RPMI 1640 media, some disagreement was seen between IC_{50}s but not between IC_{70} and IC_{80} readings (Table 5). On the basis of these findings, the IC_{80} endpoint is proposed as optimal for yeast susceptibility determinations with amphotericin B by the method described in this report.

With flucytosine the IC_{50} endpoint is selected as optimal for susceptibility determinations. This endpoint was the least vulnerable to inoculum size variation and showed the least variability between replicate tests (Table 3). No preference for endpoint could be determined on the basis of comparisons with NCCLS broth macrodilution MICs or differences in readings between tests done with RPMI 1640 medium from different suppliers.

IC_{50} readings also appear to be the optimal choice for the three azole antifungal agents on the basis of three of the four criteria that could be assessed in the present study. While all of the endpoints showed similar degrees of correlation for fluconazole in terms of replicate variability (Table 3), the IC_{50} reading was consistently superior to the IC_{70} and IC_{80} readings when the extent of variation with inoculum size, comparison with NCCLS MICs (Table 1), and variation between RPMI 1640 broths from different suppliers (Table 4) were considered. With ketoconazole, the same considerations also favored the

IC_{50} when a choice was possible between IC_{50}, IC_{70}, and IC_{80}. With itraconazole the IC_{50} was the optimum in terms of robustness to inoculum size variation, replicate variability (Table 3), and variation with different RPMI 1640 broths (Table 4). It is possible that the IC_{50}s of azole-derivative antifungal agents give generally higher levels of reproducibility than the more stringent inhibition endpoints because some yeast isolates show a tendency toward trailing endpoints. When this phenomenon arises, growth is reduced to a certain level, usually below 50% of the control, but it then shows little further inhibition until very high antifungal concentrations are reached (16, 31).

The azole-derivative susceptibility data for different *Candida* spp. in Table 5 represent a high level of agreement with the findings published by others within the last 2 years (3, 8, 21, 29). The data show that species such as *C. glabrata* and *C. tropicalis* as well as *C. albicans* contain strains with generally low susceptibilities to azole-derivative antifungal agents, while *C. krusei* isolates have an inherently low susceptibility, particularly to fluconazole and flucytosine. Some *C. albicans* isolates showed apparent cross-resistance between fluconazole, itraconazole, and ketoconazole, while others did not (data not shown), a finding in accordance with the results of a study by Barchiesi et al. (2). For *C. lusitaniae* isolates ICs of amphotericin B were often higher than those for other yeast species. However, as has been observed elsewhere (30, 31), the extent to which the current, RPMI 1640 medium-based methodology revealed a usefully large quantitative differential between amphotericin B-susceptible isolates and potentially resistant strains was limited and probably not dependable for clinical interpretation.

The interlaboratory reproducibility of the data was not investigated in the present study since the intention was to establish optimal conditions for subsequent automated susceptibility determinations, including future studies of interlaboratory reproducibility.

Tests were not conducted for incubation periods shorter than 48 h, even though some investigators have published data from 24-h yeast susceptibility tests (7, 8, 10, 21–23, 25, 27, 33, 34, 36). The concept of determining yeast susceptibilities in a time less than 2 days is inherently useful from a clinical standpoint, and future studies with automated systems should include earlier reading times as a parameter for evaluation. The consistency and reproducibility of the data are just as important as speed of measurement; for some antifungal agents the less than impressive reproducibility obtained even with a semiautomated, objectively read test system for some of the antifungal agents tested in the present study reemphasizes the need for caution in terms of accuracy of determinations. Since the quantitative results for susceptibility testing obtained in the present study showed variations even between tests done with the same, defined, synthetic broth medium obtained from two different suppliers (Table 4), it is still too early to assume that all the problems of standardization of antifungal susceptibility testing with yeasts have been solved. Complete automation, perhaps with commercialization, of the technical aspects of plate preparation and inoculation, combined with automatic determination of susceptibility endpoints and a well-managed system of laboratory quality control, should facilitate future efforts toward a standard test system.

REFERENCES

1. **Anaissie, E., V. Paetznick, and G. P. Bodey.** 1991. Fluconazole susceptibility testing of *Candida albicans*: microtiter method that is independent of inoculum size, temperature, and time of reading. Antimicrob. Agents Chemother. **35:**1641–1646.
2. **Barchiesi, F., A. L. Colombo, D. A. McGough, A. W. Fothergill, and M. G. Rinaldi.** 1994. In vitro activity of itraconazole against fluconazole-susceptible and -resistant *Candida albicans* isolates from oral cavities of patients infected with human immunodeficiency virus. Antimicrob. Agents Chemother. **38:**1530–1533.
3. **Barchiesi, F., A. L. Colombo, D. A. McGough, and M. G. Rinaldi.** 1994. Comparative study of broth macrodilution and microdilution techniques for in vitro antifungal susceptibility testing of yeasts by using the National Committee for Clinical Laboratory Standards' proposed standard. J. Clin. Microbiol. **32:**2494–2500.
4. **Cameron, M. L., W. A. Schell, S. Bruch, J. A. Bartlett, H. A. Waskin, and J. R. Perfect.** 1993. Correlation of in vitro fluconazole resistance of *Candida* isolates in relation to therapy and symptoms of individuals seropositive for human immunodeficiency virus type 1. Antimicrob. Agents Chemother. **37:**2449–2453.
5. **Chavanet, P., J. Lopez, M. Grappin, A. Bonnin, M. Duong, A. Waldner, M. Buisson, P. Camerlynck, and H. Portier.** 1994. Cross-sectional study of the susceptibility of *Candida* isolates to antifungal drugs and *in vitro-in vivo* correlation in HIV-infected patients. AIDS **8:**945–950.
6. **Demontclos, M., H. Demontclos, and J. P. Flandrois.** 1992. A 4-agent comparative study of 2 kits for determining the susceptibility of yeasts to antifungal agents. Pathol. Biol. **40:**495–499.
7. **Dermoumi, H.** 1992. *In vitro* susceptibility of yeast isolates from the blood to fluconazole and amphotericin B. Chemotherapy (Basel) **38:**112–117.
8. **Dermoumi, H.** 1994. *In vitro* susceptibility of fungal isolates of clinically important specimens to itraconazole, fluconazole and amphotericin B. Chemotherapy (Basel) **40:**92–98.
9. **Espinel-Ingroff, A.** 1994. E test for antifungal susceptibility testing of yeasts. Diagn. Microbiol. Infect. Dis. **19:**217–220.
10. **Espinel-Ingroff, A., C. W. Kish, Jr., T. M. Kerkering, R. A. Fromtling, K. Bartizal, J. N. Galgiani, K. Villareal, M. A. Pfaller, T. Gerarden, M. G. Rinaldi, and A. Fothergill.** 1992. Collaborative comparison of broth macrodilution and microdilution antifungal susceptibility tests. J. Clin. Microbiol. **30:**3138–3145.
11. **Espinel-Ingroff, A., L. Steele-Moore, and J. N. Galgiani.** 1994. Evaluation of 80% inhibition standards for the determination of fluconazole minimum inhibitory concentrations in three laboratories. Diagn. Microbiol. Infect. Dis. **20:**81–86.
12. **Fromtling, R. A., J. N. Galgiani, M. A. Pfaller, A. Espinel-Ingroff, K. F. Bartizal, M. S. Bartlett, B. A. Body, C. Frey, G. Hall, G. D. Roberts, F. B. Nolte, F. C. Odds, M. G. Rinaldi, A. M. Sugar, and K. Villareal.** 1993. Multicenter evaluation of a broth macrodilution antifungal susceptibility test for yeasts. Antimicrob. Agents Chemother. **37:**39–45.
13. **Galgiani, J. N.** 1993. Susceptibility testing of fungi—current status of the standardization process. Antimicrob. Agents Chemother. **37:**2517–2521.
14. **Galgiani, J. N., M. S. Bartlett, A. Espinel-Ingroff, R. A. Fromtling, M. A. Pfaller, and M. G. Rinaldi.** 1992. Reference method for broth dilution antifungal susceptibility testing of yeasts; proposed standard. NCCLS publication M27P. National Committee for Clinical Laboratory Standards, Villanova, Pa.
15. **Johnson, E. M., M. D. Richardson, and D. W. Warnock.** 1984. In-vitro resistance to imidazole antifungals in Candida albicans. J. Antimicrob. Chemother. **13:**547–558.
16. **Odds, F. C.** 1985. Laboratory tests for the activity of imidazole and triazole antifungal agents in vitro. Semin. Dermatol. **4:**260–270.
17. **Odds, F. C.** 1992. Relative growth of *Candida* species at single antifungal concentrations: a new approach to antifungal susceptibility testing. Antimicrob. Agents Chemother. **36:**1727–1737.
18. **Odds, F. C.** 1993. Effects of temperature on anti-*Candida* activities of antifungal antibiotics. Antimicrob. Agents Chemother. **37:**685–691.
19. **Odds, F. C.** 1993. Resistance of yeasts to azole-derivative antifungals. J. Antimicrob. Chemother. **31:**463–471.
20. **Pfaller, M. A., M. Bale, B. Buschelman, M. Lancaster, A. Espinel-Ingroff, J. H. Rex, and M. G. Rinaldi.** 1994. Selection of candidate quality control isolates and tentative quality control ranges for in vitro susceptibility testing of yeast isolates by National Committee for Clinical Laboratory Standards proposed standard methods. J. Clin. Microbiol. **32:**1650–1653.
21. **Pfaller, M. A., and A. L. Barry.** 1994. Evaluation of a novel colorimetric broth microdilution method for antifungal susceptibility testing of yeast isolates. J. Clin. Microbiol. **32:**1992–1996.
22. **Pfaller, M. A., B. Dupont, G. S. Kobayashi, J. Müller, M. G. Rinaldi, A. Espinel-Ingroff, S. Shadomy, P. F. Troke, T. J. Walsh, and D. W. Warnock.** 1992. Standardized susceptibility testing of fluconazole: an international collaborative study. Antimicrob. Agents Chemother. **36:**1805–1809.
23. **Pfaller, M. A., C. Grant, V. Morthland, and J. Rhine-Chalberg.** 1994. Comparative evaluation of alternative methods for broth dilution susceptibility testing of fluconazole against *Candida albicans*. J. Clin. Microbiol. **32:**506–509.

24. **Pfaller, M. A., J. Rhine-Chalberg, S. W. Redding, J. Smith, G. Farinacci, A. W. Fothergill, and M. G. Rinaldi.** 1994. Variations in fluconazole susceptibility and electrophoretic karyotype among oral isolates of *Candida albicans* from patients with AIDS and oral candidiasis. J. Clin. Microbiol. **32:** 59–64.

25. **Pfaller, M. A., Q. Vu, M. Lancaster, A. Espinel-Ingroff, A. Fothergill, C. Grant, M. R. McGinnis, L. Pasarell, M. G. Rinaldi, and L. Steele-Moore.** 1994. Multisite reproducibility of colorimetric broth microdilution method for antifungal susceptibility testing of yeast isolates. J. Clin. Microbiol. **32:** 1625–1628.

26. **Philpot, C. M., and D. Charles.** 1993. Determination of sensitivity to antifungal drugs—evaluation of an API kit. Br. J. Biomed. Sci. **50:**27–30.

27. **Price, M. F., M. T. LaRocco, and L. O. Gentry.** 1994. Fluconazole susceptibilities of *Candida* species and distribution of species recovered from blood cultures over a 5-year period. Antimicrob. Agents Chemother. **38:**1422–1424.

28. **Quindós, G., R. Salesa, A. J. Carrillo-Muñoz, V. Lipperheide, L. Jáudenes, R. San Millán, J. Torres-Rodríguez, and J. Pontón.** 1994. Multicenter evaluation of ATB fungus: a standardized micromethod for yeast susceptibility testing. Chemotherapy (Basel) **40:**245–251.

29. **Regli, P., A. Blancard, M. Goudard, J. Moulin-Traffort, J. M. Sarzier, and M. Quilici.** 1992. *In vitro* evaluation of the susceptibility to fluconazole of yeasts recovered from clinical specimens. Pathol. Biol. **40:**500–506.

30. **Rex, J. N., C. R. Cooper, Jr., W. G. Merz, J. N. Galgiani, and E. J. Anaissie.** 1995. Detection of amphotericin B-resistant *Candida* species in a broth-based system. Antimicrob. Agents Chemother. **39:**906–909.

31. **Rex, J. H., M. A. Pfaller, M. G. Rinaldi, A. Polak, and J. N. Galgiani.** 1993. Antifungal susceptibility testing. Clin. Microbiol. Rev. **6:**367–381.

32. **Rex, J. H., M. G. Rinaldi, and M. A. Pfaller.** 1995. Resistance of *Candida* species to fluconazole. Antimicrob. Agents Chemother. **39:**1–8.

33. **Rodriguez-Tudela, J. L., and J. V. Martinez-Suarez.** 1994. Improved medium for fluconazole susceptibility testing of *Candida albicans*. Antimicrob. Agents Chemother. **38:**45–48.

34. **Ruhnke, M., A. Eigler, I. Tennagen, B. Geiseler, E. Engelmann, and M. Trautmann.** 1994. Emergence of fluconazole-resistant strains of *Candida albicans* in patients with recurrent oropharyngeal candidosis and human immunodeficiency virus infection. J. Clin. Microbiol. **32:**2092–2098.

35. **Sandven, P., A. Bjørneklett, A. Maeland, and the Norwegian Yeast Study Group.** 1993. Susceptibilities of Norwegian *Candida albicans* strains to fluconazole: emergence of resistance. Antimicrob. Agents Chemother. **37:**2443–2448.

36. **Scheven, M.** 1993. Testing susceptibility of fungi to fluconazole. Eur. J. Clin. Microbiol. Infect. Dis. **12:**393–395.

37. **Sekhon, A. S., A. K. Garg, and Z. Hamir.** 1991. Effect of culture media on *in vitro* susceptibility testing of fluconazole against some yeasts. Mycoses **34:** 319–322.

38. **Sewell, D. L., M. A. Pfaller, and A. L. Barry.** 1994. Comparison of broth macrodilution, broth microdilution, and E test antifungal susceptibility tests for fluconazole. J. Clin. Microbiol. **32:**2099–2102.

39. **Shawar, R., V. Paetznick, Z. Witte, L. G. Ensign, E. Anaissie, and M. LaRocco.** 1992. Collaborative investigation of broth microdilution and semi-solid agar dilution for in vitro susceptibility testing of *Candida albicans*. J. Clin. Microbiol. **30:**1976–1981.

40. **Tellier, R., M. Krajden, G. A. Grigoriew, and I. Campbell.** 1992. Innovative endpoint determination system for antifungal susceptibility testing of yeasts. Antimicrob. Agents Chemother. **36:**1619–1625.

41. **Warnock, D. W.** 1992. Azole drug resistance in *Candida* species. J. Med. Microbiol. **37:**225–226.

42. **Weber, S., and A. Polak.** 1992. Susceptibility of yeast isolates from defined German patient groups to 5-fluorocytosine. Mycoses **35:**163–171.