

Quantitation of Ergosterol Content: Novel Method for Determination of Fluconazole Susceptibility of *Candida albicans*

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MIC end points for the most commonly prescribed azole antifungal drug, fluconazole, can be difficult to determine because its fungistatic nature can lead to excessive “trailing” of growth during susceptibility testing by National Committee for Clinical Laboratory Standards broth macrodilution and microdilution methods. To overcome this ambiguity, and because fluconazole acts by inhibiting ergosterol biosynthesis, we developed a novel method to differentiate fluconazole-susceptible from fluconazole-resistant isolates by quantitating ergosterol production in cells grown in 0, 1, 4, 16, or 64 μg of fluconazole per ml. Ergosterol was isolated from whole yeast cells by saponification, followed by extraction of nonsaponifiable lipids with heptane. Ergosterol was identified by its unique spectrophotometric absorbance profile between 240 and 300 nm. We used this sterol quantitation method (SQM) to test 38 isolates with broth microdilution end points of ≤ 8 $\mu\text{g}/\text{ml}$ (susceptible), 16 to 32 $\mu\text{g}/\text{ml}$ (susceptible dose-dependent [SDD]), or ≥ 64 $\mu\text{g}/\text{ml}$ (resistant) and 10 isolates with trailing end points by the broth microdilution method. No significant differences in mean ergosterol content were observed between any of the isolates grown in the absence of fluconazole. However, 18 susceptible isolates showed a mean reduction in ergosterol content of 72% after exposure to 1 μg of fluconazole/ml, an 84% reduction after exposure to 4 $\mu\text{g}/\text{ml}$, and 95 and 100% reductions after exposure to 16 and 64 μg of fluconazole/ml, respectively. Ten SDD isolates showed mean ergosterol reductions of 38, 57, 73, and 99% after exposure to 1, 4, 16, and 64 μg of fluconazole/ml, respectively. In contrast, 10 resistant isolates showed mean reductions in ergosterol content of only 25, 38, 53, and 84% after exposure to the same concentrations of fluconazole. The MIC of fluconazole, by using the SQM, was defined as the lowest concentration of the drug which resulted in 80% or greater inhibition of overall mean ergosterol biosynthesis compared to that in the drug-free control. Of 38 isolates which gave clear end points by the broth microdilution method, the SQM MIC was within 2 dilutions of the broth microdilution MIC for 33 (87%). The SQM also discriminated between resistant and highly resistant isolates and was particularly useful for discerning the fluconazole susceptibilities of 10 additional isolates which gave equivocal end points by the broth microdilution method due to trailing growth. In contrast to the broth microdilution method, the SQM determined trailing isolates to be susceptible rather than resistant, indicating that the SQM may predict clinical outcome more accurately. The SQM may provide a means to enhance current methods of fluconazole susceptibility testing and may provide a better correlation of in vitro with in vivo results, particularly for isolates with trailing end points.

Rapid and reliable antifungal susceptibility testing has become particularly important in recent years because of the increased incidence of serious fungal infections and the concomitant emergence of antifungal-drug resistance (20, 28). The National Committee for Clinical Laboratory Standards (NCCLS) recently published an approved broth macrodilution method (document M27-A) for in vitro testing of the susceptibilities of *Cryptococcus neoformans* and *Candida* species to amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole (14). This reference method, and simplified microdilution adaptations of it, have significantly improved the inter- and intralaboratory reproducibility of antifungal susceptibility testing for most isolates. However, in tests with the most commonly used azole drug, fluconazole, some isolates do not give a clear-cut end point and exhibit a “trailing” growth effect, making interpretation of test results difficult (21). For isolates with trailing end points, MICs of less than 1 $\mu\text{g}/\text{ml}$ at 24 h and of 64 $\mu\text{g}/\text{ml}$ or greater at 48 h are usually observed (21). Therefore, these isolates would be considered resistant by

NCCLS M27-A methodologies which recommend reading results after 48 h of growth (14). Clinical outcomes for human immunodeficiency virus-infected patients with oropharyngeal candidiasis (21), as well as in vivo animal model data from our laboratory (4) and from others (23), have demonstrated that infections caused by organisms which produce trailing growth in vitro typically respond to low doses of fluconazole, suggesting that the lower MICs at 24 h better reflect host responsiveness to therapy.

We therefore sought to improve the correlation of in vitro susceptibility testing results with in vivo therapeutic outcomes by developing a novel in vitro test which involves the quantitation of membrane sterols (the sterol quantitation method [SQM]) to determine the MICs of fluconazole for clinical isolates of *Candida albicans*. This test measures the sensitivity of ergosterol biosynthesis in *C. albicans* isolates to the effects of fluconazole by quantitation of steady-state amounts of ergosterol following growth of the organism in various concentrations of fluconazole (2). The primary mechanism of action by which azole antifungal drugs inhibit yeast cell growth is through disruption of the normal sterol biosynthetic pathway, leading to a reduction in ergosterol biosynthesis (10). Ergosterol is the major sterol component of the yeast cell membrane and is responsible for maintaining cell integrity and function

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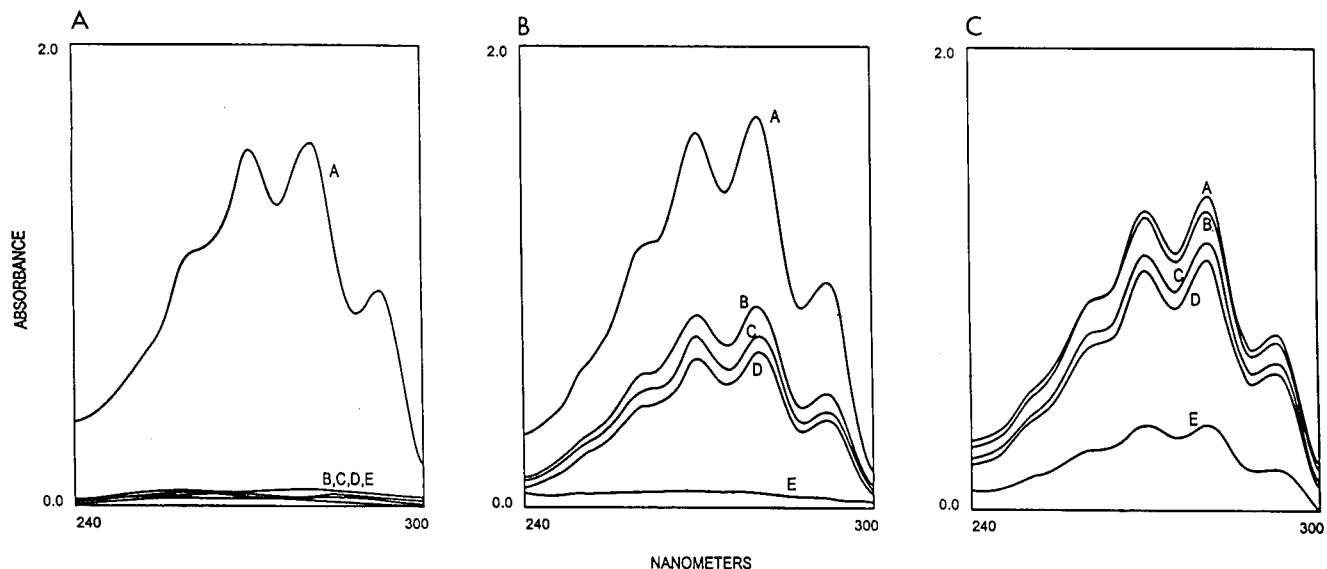


FIG. 1. UV spectrophotometric sterol profiles of representative fluconazole-susceptible (A), -SDD (B), and -resistant (C) *C. albicans* isolates. Isolates were grown for 16 h in Sabouraud dextrose broth containing 0 (curve A), 1 (curve B), 4 (curve C), 16 (curve D), or 64 (curve E) μg of fluconazole per ml, sterols were extracted from cells, and spectral profiles between 240 and 300 nm were determined.

(5, 24). Therefore, disruption of this pathway by azole drugs leads to fungistasis.

The SQM takes advantage of the unique spectral absorption pattern produced between 240 and 300 nm by extracted sterols, which is indicative of the ergosterol and 24(28)dehydroergosterol [24(28)DHE, a late sterol pathway intermediate] content. Both ergosterol and 24(28)DHE absorb at 281.5 nm, whereas only 24(28)DHE shows an intense spectral absorption band at 230 nm. Therefore, the amount of ergosterol can be determined by calculating the total ergosterol-plus-24(28)DHE content and then subtracting from the total the amount of absorption due to 24(28)DHE only (6). Ergosterol content determined by the SQM is an absolute measurement, eliminating the need for subjective determination of growth inhibition, as required for broth-based susceptibility testing methods. Therefore, in this regard, this method should be more objective and reproducible than standard NCCLS methods.

Because decreased susceptibility to fluconazole is correlated with the ability of *C. albicans* isolates to produce ergosterol even in the presence of azole drugs, we were able to determine fluconazole susceptibility by quantitating total intracellular ergosterol production in cells grown in increasing concentrations of fluconazole and to assign unequivocal MIC end points to organisms which exhibit trailing growth during standard broth microdilution drug susceptibility testing. We compared MIC results obtained by the broth microdilution drug susceptibility method to those obtained by the SQM, using a panel of isolates determined by the broth microdilution method to be susceptible, susceptible dose-dependent (SDD), or resistant to fluconazole, or classified as trailers.

MATERIALS AND METHODS

Isolates. A total of 48 oral or vaginal *C. albicans* isolates, 38 without trailing characteristics (Tables 1 through 3) and 10 with trailing characteristics (Table 4) were tested. Isolates were obtained from David A. Stevens (Stanford University, Palo Alto, and Santa Clara Valley Medical Center, San Jose, Calif.) and Dora Warren (Division of Reproductive Health, Centers for Disease Control and Prevention). Isolates were identified to the species level by the API 20C (Analytab Products, Plainview, N.Y.) yeast identification system. Two reference

strains, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258, were included each day of broth microdilution testing to ensure quality control.

Isolates were retrieved from storage at -70°C and were subcultured twice on Sabouraud dextrose agar plates (BBL, Cockeysville, Md.) to ensure optimal growth. Prior to testing, subcultures on Sabouraud dextrose agar plates were incubated at 35°C for 24 h.

Broth microdilution method. Broth microdilution was performed according to the guidelines of NCCLS document M27-A (14). Analytical-grade powder of fluconazole was obtained as a gift from Pfizer (Groton, Conn.). A stock solution of fluconazole was prepared in sterile distilled water, diluted with RPMI-1640 medium (with L-glutamine but without bicarbonate) (Sigma Chemical Co., St. Louis, Mo.), and buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS; Sigma). The final concentration range for fluconazole was 0.125 to 64 $\mu\text{g}/\text{ml}$.

Testing was performed in 96-well round-bottom microtitration plates. Cell suspensions were prepared in RPMI-1640 medium and were adjusted to give a final inoculum concentration of 0.5×10^3 to 2.5×10^3 cells/ml. The plates were incubated at 35°C and were read after 48 h. The MIC of fluconazole was defined as the lowest concentration at which there was 80% inhibition of growth compared with that in a drug-free control.

SQM. Total intracellular sterols were extracted as reported by Breivik and Owades (6) with slight modifications. Briefly, a single *C. albicans* colony from an overnight Sabouraud dextrose agar plate culture was used to inoculate 50 ml of Sabouraud dextrose broth (Difco, Detroit, Mich.) containing 0, 1, 4, 16, or 64 μg of fluconazole per ml. The cultures were incubated for 16 h with shaking at 35°C . The stationary-phase cells were harvested by centrifugation at 2,700 rpm (model TJ-6 centrifuge; Beckman Instruments, Palo Alto, Calif.) for 5 min and washed once with sterile distilled water. The net wet weight of the cell pellet was determined. Three milliliters of 25% alcoholic potassium hydroxide solution (25 g of KOH and 35 ml of sterile distilled water, brought to 100 ml with 100% ethanol), was added to each pellet and vortex mixed for 1 min. Cell suspensions were transferred to 16- by 100-mm sterile borosilicate glass screw-cap tubes and were incubated in an 85°C water bath for 1 h. Following incubation, tubes were allowed to cool to room temperature. Sterols were then extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of *n*-heptane followed by vigorous vortex mixing for 3 min. The heptane layer was transferred to a clean borosilicate glass screw-cap tube and stored at -20°C for as long as 24 h. Prior to analysis, a 20- μl aliquot of sterol extract was diluted fivefold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm with a Gilford Response Spectrophotometer (Ciba Corning Diagnostics Corp., Gilford Systems, Oberlin, Ohio). The presence of ergosterol and the late sterol intermediate 24(28)DHE in the extracted sample resulted in a characteristic four-peaked curve (Fig. 1). The absence of detectable ergosterol in extracts was indicated by a flat line. A dose-dependent decrease in the height of the absorbance peaks was evident and corresponded to decreased ergosterol concentration.

Ergosterol content was calculated as a percentage of the wet weight of the cell by the following equations: % ergosterol + % 24(28)DHE = $[(A_{281.5}/290) \times F]/\text{pellet weight}$, % 24(28)DHE = $[(A_{230}/518) \times F]/\text{pellet weight}$, and

TABLE 1. MICs of fluconazole for *C. albicans* isolates as determined by the NCCLS broth microdilution method and SQM

Classification of isolates ^a	MIC ($\mu\text{g/ml}$) as determined by:						% Agreement
	Microdilution			SQM			
	Range	50%	90%	Range	50%	90%	
Susceptible ($n = 18$)	0.25–8	1	8	<1–18	1	16	100
SDD ($n = 10$)	16–32	16	32	3–29	17	52	70
Resistant ($n = 10$)	64–>64	64	>64	14–190	51	183	80

^a By the NCCLS broth microdilution assay.

% ergosterol = [% ergosterol + % 24(28)DHE] – % 24(28)DHE, where F is the factor for dilution in ethanol and 290 and 518 are the E values (in percentages per centimeter) determined for crystalline ergosterol and 24(28)DHE, respectively. The wet weight of the cell pellet ranged from 1.09 ± 0.14 g for organisms grown in 0 μg of fluconazole per ml to 0.97 ± 0.12 g for organisms grown in 64 μg of fluconazole per ml ($n = 48$; $P > 0.05$). The MIC of fluconazole was defined as the concentration of fluconazole which caused an 80% reduction in the total cellular ergosterol content compared to that in the drug-free control. MICs which fell between two fluconazole concentrations (i.e., less than 80% reduction at one concentration but more than 80% reduction at the next-higher concentration) were mathematically extrapolated based on the amount of reduction at the fluconazole concentration which gave results closest to an 80% reduction end point.

Analysis of results. Breakpoints for fluconazole susceptibility have been established for isolates of *Candida* spp. tested according to NCCLS guidelines. Organisms are classified as susceptible if the fluconazole MIC is ≤ 8 $\mu\text{g/ml}$, as SDD if it is 16 to 32 $\mu\text{g/ml}$, and as resistant if it is ≥ 64 $\mu\text{g/ml}$ (14). Interpretive breakpoints for the SQM were based on those defined for the NCCLS method. MICs which fell between two susceptibility categories were assigned to the next-closest category (i.e., organisms with MICs of 14, 15, and 21 $\mu\text{g/ml}$ were classified as SDD, and those with MICs of 52 and 61 $\mu\text{g/ml}$ were classified as resistant; Table 3). The SQM MICs were compared with the microdilution MICs by using both on-scale and off-scale results. The high off-scale MICs were converted to the next-highest concentration, and the low off-scale MICs were left unchanged. SQM MICs were considered to be in agreement with the NCCLS microdilution MICs if they differed by no more than 2 drug dilutions.

Statistical analysis. Differences between means were analyzed by Student's t test, and P values of <0.05 were considered to represent statistically significant differences. Correlations between MICs and reductions in ergosterol levels were analyzed by Pearson's correlation coefficient.

RESULTS

Correlation between susceptibilities determined by the broth microdilution method and the SQM. Table 1 summarizes the in vitro susceptibilities of 38 nontrailing isolates of *C. albicans* to fluconazole as measured by the broth microdilution method and the SQM. The data are reported as MIC ranges and MICs required to inhibit 50 and 90% of the isolates (MIC₅₀ and MIC₉₀, respectively). In each day of testing, MICs of fluconazole for the two quality control strains were within the accepted limits defined by the NCCLS (14) (data not shown). The overall agreement between the results of the two methods was 87% for 38 isolates which gave unequivocal end points by the broth microdilution method (Table 1). Overall, agreement between methods was 100% for 18 fluconazole-

susceptible isolates, 70% for 10 fluconazole-SDD isolates, and 80% for 10 fluconazole-resistant isolates.

Table 2 summarizes the effect of fluconazole on ergosterol biosynthesis in fluconazole-susceptible, -SDD, and -resistant *C. albicans* isolates. The total ergosterol content was determined for each isolate grown in 0, 1, 4, 16, or 64 μg of fluconazole per ml. No significant differences in the mean amount of ergosterol produced by strains grown in the absence of fluconazole were observed regardless of the degree of fluconazole susceptibility ($P > 0.05$). In contrast, a dose-dependent decrease in ergosterol production was observed when isolates were grown in the presence of fluconazole (Table 2). The degree of sensitivity of the ergosterol biosynthetic pathway to the effects of fluconazole decreased as the broth microdilution MIC of fluconazole increased (inverse correlation at 1 μg of fluconazole/ml, $r = 0.82$; at 4 $\mu\text{g/ml}$, $r = 0.95$; at 16 $\mu\text{g/ml}$, $r = 1.0$; and at 64 $\mu\text{g/ml}$, $r = 0.60$). As shown in Table 2, the mean decrease in total cellular ergosterol content for susceptible isolates ranged from 72% for cells grown in 1 μg of fluconazole/ml to 100% for cells grown in 64 $\mu\text{g/ml}$. The mean decrease in total cellular ergosterol content for SDD isolates ranged from 38% after exposure to 1 μg of fluconazole/ml to 99% after exposure to 64 $\mu\text{g/ml}$. In contrast, for resistant isolates, the mean decrease in total cellular ergosterol content ranged from 25% after exposure to 1 μg of fluconazole/ml to 84% after exposure to 64 $\mu\text{g/ml}$. The less susceptible the *C. albicans* isolate was to fluconazole, the less sensitive ergosterol biosynthesis was to the inhibitory effects of the drug. Incubation times from 16 to 24 h and inoculum sizes from 10^5 to 10^7 cells/ml were also tested and did not alter the SQM MICs (data not shown).

The capacity of the SQM to discriminate more clearly the degrees of fluconazole resistance among nontrailing, resistant isolates relative to the broth microdilution method is presented in Table 3. For the 10 isolates tested, the broth microdilution MICs were ≥ 64 $\mu\text{g/ml}$, while the SQM differentiated these isolates into three distinct groups. Specifically, three isolates (CA23, CA24, and CA28) classified as resistant by broth microdilution (MICs of ≥ 64 $\mu\text{g/ml}$) were SDD by the SQM (MICs of 14, 15, and 21 $\mu\text{g/ml}$), and two isolates (CA29 and CA30) for which the broth microdilution MICs were ≥ 64

TABLE 2. Inhibition of ergosterol biosynthesis in *C. albicans* isolates by fluconazole

Classification of isolates ^a	Mean ergosterol content ^b of cells grown with fluconazole at a concn ($\mu\text{g/ml}$) of:				
	0	1	4	16	64
Susceptible ($n = 18$)	1.4 ± 0.07	0.4 ± 0.1 (72) ^c	0.23 ± 0.06 (84) ^c	0.07 ± 0.03 (95) ^c	0 ± 0 (100) ^c
SDD ($n = 10$)	1.6 ± 0.14	1.0 ± 0.11 (38)	0.69 ± 0.17 (57)	0.43 ± 0.13 (73) ^c	0.005 ± 0.003 (99) ^c
Resistant ($n = 10$)	1.6 ± 0.13	1.2 ± 0.09 (25)	1.0 ± 0.13 (38)	0.8 ± 0.13 (53)	0.2 ± 0.13 (84) ^c

^a By the NCCLS broth microdilution assay.

^b Expressed as a percentage of the wet weight of the cell \pm the standard error of the mean (followed in parentheses by the percent reduction in the mean cellular ergosterol content compared with that of control cells grown without fluconazole).

^c Significant reduction compared with controls ($P < 0.05$).

TABLE 3. Stratification of fluconazole-resistant *C. albicans* isolates by the SQM versus the broth microdilution method

Isolate	MIC determined by:		Ergosterol content ^b (reduction ^c) of cells grown with fluconazole at a concn (μg/ml) of:		
	Microdilution ^a	SQM			
			4	16	64
CA23	64	14 ^d	0.7 (65)	0.2 (92)	0 (100)
CA28	≥64	15 ^d	0.5 (66)	0.2 (87)	0 (100)
CA24	64	21 ^d	0.8 (66)	0.7 (62)	0 (100)
CA25	64	51	1.2 (0)	0.7 (42)	0 (100)
CA26	64	51	1.6 (16)	1.2 (37)	0 (100)
CA21	64	52	1.7 (26)	1.3 (44)	0.02 (99)
CA22	64	52	0.9 (40)	0.9 (40)	0.03 (98)
CA27	≥64	61	0.7 (42)	0.3 (75)	0.2 (84)
CA29	≥64	183 ^e	1.2 (33)	1.4 (23)	1.3 (28)
CA30	≥64	190 ^e	0.8 (27)	0.8 (27)	0.8 (27)

^a After 48 h of incubation.

^b Expressed as a percentage of the wet weight of the cell.

^c Percent reduction in ergosterol content compared with that of control cells grown without fluconazole.

^d SDD by SQM.

^e Highly resistant by SQM.

μg/ml were strikingly more resistant to fluconazole (28 and 27% inhibition of ergosterol biosynthesis when these isolates were grown in 64 μg of fluconazole/ml, respectively) than the other isolates for which broth microdilution MICs were ≥64 μg/ml (Table 3). The SQM further stratified isolates within the resistant category into "resistant" (MICs, 51 to 61 μg/ml) and "highly resistant" (MICs, 183 to 190 μg/ml) subcategories, compared to the microdilution method, where ≥64 μg/ml is traditionally the highest MIC reported.

Using the SQM to differentiate fluconazole-resistant isolates from fluconazole-susceptible isolates which exhibit trailing by the broth microdilution method. Ten *C. albicans* isolates which exhibited trailing growth in fluconazole, making end point determinations ambiguous by the broth microdilution method, and which were not included in the above analyses, were examined. Table 4 summarizes the in vitro susceptibilities of the isolates to fluconazole as measured by the broth microdilution and SQM methods. There was no agreement between broth microdilution MICs and SQM MICs for these isolates. By the NCCLS broth microdilution method, all were

TABLE 4. Ergosterol contents of 10 *C. albicans* isolates with trailing MIC end points by the microdilution method

Isolate	Broth microdilution MIC at:		Ergosterol content ^a (reduction ^b) of cells grown with fluconazole at a concn (μg/ml) of:			SQM MIC
	24 h	48 h				
			0	1	4, 16, or 64	
CA2	0.2	64	1.0	0 (100)	0 (100)	<1.0
CA4	0.5	64	1.6	0 (100)	0 (100)	<1.0
CA5	0.5	64	2.1	0 (100)	0 (100)	<1.0
CA31	0.5	64	2.0	0.05 (98)	0 (100)	<1.0
CA32	0.5	64	1.3	0 (100)	0 (100)	<1.0
CA33	0.5	64	1.8	0 (100)	0 (100)	<1.0
CA35	1.0	64	1.6	0 (100)	0 (100)	<1.0
CA36	1.0	64	1.1	0 (100)	0 (100)	<1.0
CA37	1.0	≥64	1.0	0.7 (33)	0 (100)	2.0
CA38	1.0	≥64	1.5	0 (100)	0 (100)	<1.0

^a Expressed as a percentage of the wet weight of the cell.

^b Percent reduction in ergosterol content compared with that of control cells grown without fluconazole.

TABLE 5. Interlaboratory variability in MIC end points for three *C. albicans* isolates which exhibit trailing growth when tested by the broth microdilution method

Isolate	Broth microdilution MIC (μg/ml)				SQM MIC
	Lab A	Lab B	Lab C	Lab D	
CA2	0.25	0.25	>64	1.0–2.0	<1.0
CA4	0.5	0.5	>64	2.0–4.0	<1.0
CA5	0.5	0.5	>64	1.0–2.0	<1.0

susceptible (MIC ≤ 1.0 μg/ml) to fluconazole at 24 h and resistant (MIC ≥ 64 μg/ml) at 48 h. By the SQM, all 10 isolates were determined to be susceptible to fluconazole (MICs ≤ 2 μg/ml). Preliminary results using spectrophotometric MIC₈₀ end point determination of the broth microdilution assay (13) did not improve agreement between the two methods (data not shown).

Interlaboratory reproducibility of MIC end point determinations for organisms which exhibit trailing. Table 5 summarizes the results of an interlaboratory comparison of fluconazole susceptibility test results. Three of the 10 isolates exhibiting trailing by the broth microdilution method were retested in three other laboratories. The MICs of fluconazole for these isolates ranged from 0.25 to >64 μg/ml depending on the laboratory conducting the susceptibility testing (Table 5). Three of the four laboratories reported all of the isolates to be susceptible to fluconazole, and one laboratory reported all of the isolates to be resistant. SQM results revealed all three isolates to be susceptible to fluconazole, supporting the findings of all but one of the four laboratories conducting broth microdilution antifungal susceptibility testing.

DISCUSSION

The incidence of invasive fungal diseases and antifungal drug resistance has increased in recent years, making the development of reliable antifungal drug susceptibility tests more important (20). Substantial efforts have been made by the NCCLS first to standardize and then to simplify antifungal susceptibility testing, resulting in the publication of the M27-A guidelines and the acceptance of a standard broth microdilution format (14). Simplified broth microdilution adaptations of the M27-A method have been developed and have been shown to be useful (7, 8). However, problems with end point interpretation, particularly for isolates with trailing end points, remain (21, 23).

Commercial companies have developed alternative antifungal susceptibility testing tools which offer simple and rapid approaches to antifungal susceptibility testing. Whereas the E test (AB Biodisk, Solna, Sweden) is an agar diffusion test, and the YeastOne (AccuMed International, Westlake, Ohio) and PASCO (Becton Dickinson, Pasco Division, Wheat Ridge, Colo.) tests are broth dilution systems, all of these methods rely on visual detection of growth inhibition as an indicator of drug susceptibility (1, 3, 16). Thus, they can be influenced by variables such as inoculum size, incubation time, cell culture medium, and subjective end point determination (17, 18).

Recently, efforts have been made to determine end points more objectively by reading broth microdilution plates with a spectrophotometer (13, 25). Unfortunately, this method still does not eliminate ambiguous end point determinations for trailing isolates. Adoption of a MIC₅₀ rather than a MIC₈₀ end point value may improve the correlation of in vitro susceptibility testing results with in vivo outcomes (4a). Alternatively,

adoption of a 24- rather than a 48-h end point reading for trailing isolates may achieve the same goal.

New methods using flow cytometric techniques for determining the antifungal susceptibilities of *Candida* species have also been described (19) and have been shown to be rapid and sensitive alternatives to broth dilution methods. However, this approach requires costly equipment and the use of hazardous compounds, such as ethidium bromide (30).

Three general mechanisms of azole resistance have been described for *Candida* spp. The first is alteration in the target enzyme, 14 alpha-demethylase, leading to its overexpression and/or reduced susceptibility to azole inhibition (12, 26, 31). Decreased drug accumulation, mediated by either diminished uptake or increased efflux of the drug, is the second mechanism (15, 27). The third is a deficiency in C5(6) sterol desaturase, which suppresses the accumulation of toxic sterol intermediates, as a result of azole-mediated 14 alpha-demethylase inhibition (9, 11). The SQM is capable of detecting increased resistance due to any of the above mechanisms based on its ability to detect intracellular ergosterol following the exposure of the organisms to fluconazole.

The SQM provides definitive MIC end points in 18 h (16 h of incubation plus 2 h to complete the assay), uses common laboratory equipment (shaking incubator, tabletop centrifuge, water bath, and UV spectrophotometer), is simple to perform, and shows excellent agreement with the NCCLS broth microdilution method for nontrailing isolates. Preliminary data collected by our laboratory have suggested that the SQM may be equally useful for the determination of the susceptibilities of other *Candida* species to fluconazole and other azoles (unpublished data). Ultimately, the best use of the SQM may be for the determination of the antifungal-drug susceptibilities of filamentous fungi, where determination of a visual or spectrophotometric end point may be problematic (8a).

In the design of the prototype SQM test, four concentrations of fluconazole were chosen to represent the different NCCLS-determined fluconazole susceptibility categories (susceptible, 1 and 4 µg/ml; SDD, 16 µg/ml; resistant, 64 µg/ml), thus simplifying a comparison of results with those of the broth microdilution method. Unlike a physical or chemical measurement, such as the determination of a drug level, a MIC determination by standardized broth dilution methodology is a function of the conditions selected by the tester (22). Variations in any condition can produce slight to dramatic variations in the measured MIC (8, 29). The utility of the SQM as an index of antifungal drug susceptibility is that it is a physical measurement of total cellular ergosterol content. Stationary-phase cells are used so that steady-state levels of ergosterol are measured for all drug concentrations tested, making the assay far less sensitive to factors such as inoculum size and incubation time. Furthermore, determination of an exact numerical value eliminates subjective interpretation of MIC end points when trailing growth occurs.

Trailing growth has been shown to be a major cause of interlaboratory variability in antifungal susceptibility testing (21, 23). This phenomenon complicates MIC end point determination and often leads to misclassification of susceptible isolates (susceptibility based on animal models of candidiasis) as resistant (21, 23). Because the SQM is a direct measurement of total intracellular ergosterol content, MIC end point determination is unequivocal. Although three of the four laboratories participating in our study determined that trailing isolates were susceptible by the broth microdilution method, one laboratory (25% of our sample) gave a significantly different end point interpretation (i.e., resistant). Because this laboratory is

one of the most experienced and widely used in the United States, this discrepancy is particularly significant.

For the isolates tested in this study, the intracellular ergosterol contents of resistant and susceptible isolates grown in the absence of fluconazole were not significantly different. This observation suggests that increased resistance to fluconazole in these isolates is not due to a stable genetic change in an ergosterol biosynthetic gene leading to altered ergosterol content but rather that these isolates are capable of reducing intracellular drug concentrations so that ergosterol biosynthesis is inhibited less by the presence of fluconazole.

Finally, the SQM offers the advantage of further differentiating isolates within a given susceptibility category based on their individual percentages of ergosterol inhibition. Thus, subtle changes leading to decreased fluconazole susceptibility of an isolate would be detected by the SQM even if the change was not large enough to shift the isolate to the next category of drug resistance. For example, isolates which were resistant to fluconazole by the broth microdilution method (MIC ≥ 64 µg/ml) demonstrated distinct degrees of resistance by the SQM. In addition, MIC end points which fall between the drug concentrations routinely tested by the broth microdilution method can be determined by the SQM without the need to test the organisms against additional fluconazole concentrations. This feature allows for additional stratification of degrees of susceptibility within the NCCLS-established categories of SDD and resistant. Adaptation of the SQM to a kit format would increase the usefulness of this test for clinical laboratory testing. Efforts are currently under way in our laboratory to accomplish this task.

In summary, the SQM demonstrated good agreement with the broth microdilution method for *C. albicans* isolates with unequivocal end points and gave clear MICs for isolates which trail in the broth microdilution test format. The SQM offered the additional advantage of enhanced discrimination of isolates within fluconazole-SDD and -resistant categories. The clinical impact of dividing isolates into "degrees" of susceptibility or resistance will require further analysis using animal models of candidiasis. Such studies will determine if the SQM offers increased clinical correlation and improved therapeutic decision making compared with standard antifungal susceptibility testing methods.

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