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Comparison of Visual and Spectrophotometric Methods of MIC Endpoint Determinations by Using Broth Microdilution Methods To Test Five Antifungal Agents, Including the New Triazole D0870

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A study to compare three different methods for reading MIC endpoints tested by the broth microdilution modification of the National Committee for Clinical Laboratory Standards (Villanova, Pa.) reference method was conducted. MICs of amphotericin B, flucytosine, fluconazole, itraconazole, and a new triazole, D0870, were determined for five reference yeast strains and 100 clinical isolates of *Candida* spp. MICs were read visually according to National Committee for Clinical Laboratory Standards guidelines from microdilution trays that had been (VS) and had not been (V) shaken. MICs were also determined spectrophotometrically (SP) at 492 nm. SP endpoints were determined as the concentrations resulting in a ≥50% inhibition of growth (flucytosine and azoles) and a ≥90% inhibition of growth (amphotericin B) relative to control growth. The five reference strains were tested nine times each against all five antifungal agents, and the MIC results for each reading method were compared with a 3-$\log_2$ dilution reference range determined by the macrodilution (M27-P) method. Overall, 84 to 100% of the MICs determined by V, 93 to 100% of those determined by VS, and 89 to 100% of those determined by SP fell within the 3-$\log_2$ dilution reference range for each reference strain and antifungal agent. Reproducibility was 99% for V and SP and 98% for VS. Agreement among the three methods of reading ranged from 97 to 99%. Excellent agreement among reading methods was also observed for all antifungal agents when tested against 100 clinical isolates. Agreement between the standard V method (no agitation) and VS ranged from 99 to 100%, and that between V and SP ranged from 89 to 99%. The VS and SP reading methods provided more definitive endpoints than the V method, which does not involve shaking.

Progress in standardizing antifungal susceptibility testing has been considerable and includes the development of a reference macrodilution method by the National Committee for Clinical Laboratory Standards (NCCLS), Villanova, Pa. (M27-P) (5–7, 14, 17, 19), the establishment of quality control and reference strains (9, 10), and the adaptation of NCCLS M27-P to a more user-friendly microdilution format (1–4, 12, 13, 15, 16, 18). More recent efforts have been directed at the problem of accurate determination of MIC endpoints, particularly with the azole antifungal agents tested against *Candida* spp. (1, 2, 8, 12, 13, 15, 18). These studies have focused primarily on the microdilution modification of NCCLS M27-P (7) and have included evaluations of both colorimetric (12, 13, 15) and spectrophotometric (1, 2, 16) endpoints.

Studies by Anaissie et al. (1) have shown that the visual determination of microdilution MIC endpoints may be aided by the agitation of microdilution trays prior to the reading of the MICs. Such agitation disperses the yeast cells within the wells, producing a homogeneous suspension, and minimizes the effects of trailing that plague the interpretation of azole susceptibility tests (1, 2, 8, 16). Agitation likewise facilitates the use of a spectrophotometer for determining MIC endpoints. The spectrophotometric approach to MIC endpoint determination provides an objective and rapid MIC reading and eliminates the subjective judgments concerning minimal turbidity and trailing that confound antifungal susceptibility testing (2, 8, 16). Recent studies by Barchiesi et al. (2) and by Price et al. (16) have shown that MIC results comparable to those obtained with the NCCLS M27-P macrodilution reference method could be obtained by microdilution testing involving agitation and spectrophotometric endpoint determination. Spectrophotometric MICs reflecting either a ≥50% inhibition or a ≥70% inhibition of growth relative to control growth provided good agreement with the NCCLS reference MICs for fluconazole and other azoles (1, 2, 16).

In general, the earlier studies evaluating microdilution testing involving agitation and spectrophotometric MIC determinations have involved a limited number of isolates of *Candida* spp. or have restricted their analysis to MIC results obtained with a single antifungal agent (e.g., fluconazole) (1, 2, 16). The purpose of the present study was to extend the evaluation of both visual and spectrophotometric readings of microdilution trays that had been agitated to include additional antifungal agents, such as the new triazole D0870 (12), and a broad spectrum of clinical yeast isolates and to further compare these methods with a reference microdilution method performed according to NCCLS guidelines.

MATERIALS AND METHODS

Test organisms. The test organisms included five American Type Culture Collection (ATCC) strains which have been proposed as either quality control strains (*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258) or reference strains (*Candida albicans* ATCC 90028, *C. albicans* ATCC 24433, and *Candida tropicalis* ATCC 750) by the NCCLS (7, 9, 10). These isolates have well-defined macrodilution MIC reference ranges for amphotericin B, flucytosine (5FC), and fluconazole and provide a means of comparing the various microdilution MICs with the NCCLS macrodilution reference method (9, 10).

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TABLE 1. Comparison of broth microdilution MICs read by the V, VS, and SP methods for five antifungal agents against five reference strains[a]

Organism	Antifungal agent	Reference range (μg/ml)[b]	Modal MIC (μg/ml) determined at:					
			24 h			48 h		
			V	VS	SP[c]	V	VS	SP[c]
C. albicans ATCC 90028	Amphotericin B	0.5–2.0	0.35	0.25	8.0	0.25	0.25	0.5
	5FC	0.5–2.0	0.25	0.25	0.25	1.0	1.0	1.0
	Fluconazole	0.25–1.0	0.25	0.25	0.25	0.25	0.25	0.25
	Itraconazole	NA[d]	0.03	0.03	0.03	0.03	0.03	0.06
	D0870	NA	0.007	0.007	0.007	0.007	0.007	0.007
C. albicans ATCC 24433	Amphotericin B	0.25–1.0	0.5	0.12	1.0	0.5	0.5	0.5
	5FC	1.0–4.0	0.5	0.5	0.5	1.0	1.0	1.0
	Fluconazole	0.25–1.0	0.25	0.25	0.25	0.5	0.5	0.5
	Itraconazole	NA	0.04	0.03	0.03	0.04	0.06	0.12
	D0870	NA	0.007	0.007	0.007	0.007	0.007	0.007
C. parapsilosis ATCC 22019	Amphotericin B	0.25–1.0	0.25	0.25	0.5	0.5	0.5	0.5
	5FC	0.12–0.5	0.25	0.25	0.25	0.25	0.25	0.25
	Fluconazole	2.0–8.0	1.0	1.0	1.0	2.0	2.0	2.0
	Itraconazole	NA	0.12	0.12	0.03	0.12	0.12	0.12
	D0870	NA	0.03	0.015	0.015	0.03	0.03	0.03
C. tropicalis ATCC 750	Amphotericin B	0.5–2.0	0.5	0.5	1.0	0.5	1.0	0.5
	5FC	≤0.12–0.25	0.25	0.25	0.25	0.25	0.25	0.25
	Fluconazole	1.0–4.0	0.5	0.5	0.25	1.0	1.0	1.0
	Itraconazole	NA	0.12	0.12	0.12	0.12	0.12	0.12
	D0870	NA	0.015	0.015	0.015	0.03	0.03	0.03
C. krusei ATCC 6258	Amphotericin B	0.5–2.0	0.5	0.5	0.5	1.0	1.0	1.0
	5FC	4.0–16	8.0	8.0	8.0	16	16	11
	Fluconazole	16–64	16	23	16	64	64	64
	Itraconazole	NA	0.25	0.25	0.12	0.25	0.5	0.5
	D0870	NA	0.5	0.5	0.25	0.5	1.0	0.5

[a] All isolates were tested nine times over 3 days by the microdilution modification of NCCLS M27-P.
[b] Reference ranges were established by the broth macrodilution method (NCCLS M27-P).
[c] SP endpoints were determined as concentrations resulting in a ≥50% inhibition of growth (5FC, fluconazole, itraconazole, and D0870) and a ≥90% inhibition of growth (amphotericin B) relative to that of the control. Endpoints were measured at 492 nm.
[d] NA, not available.

An additional 100 clinical yeast isolates were also selected for testing. The collection included 20 isolates of *C. albicans*, 15 of *C. tropicalis*, 19 of *C. parapsilosis*, 10 of *Candida lusitaniae*, 10 of *C. krusei*, 10 of *Candida guilliermondii*, 1 of *Candida lipolytica*, and 15 isolates of *Candida* (*Torulopsis*) *glabrata*. The isolates were all recent clinical isolates, the majority of which were from blood or normally sterile body fluids.

Initial isolation from blood was accomplished with either the BACTEC (Becton Dickinson Microbiology Systems, Cockeysville, Md.) or the Isolator (Wampole Laboratories, Cranbury, N.J.) blood culture system. Specimens from other sites were cultured routinely on blood agar and eosin-methylene blue agar plates (Remel, Lenexa, Kans.). Sabouraud dextrose agar and brain heart infusion agar (Remel) were added when fungal cultures were specifically requested. The isolates were identified to species level with either the Vitek Yeast Biochemical Card (bioMerieux Vitek Inc., Hazelwood, Mo.) or the API 20C yeast identification system (bioMerieux Vitek Inc.), and these identifications were supplemented with additional biochemical reactions and morphology evaluations (20) as needed. Isolates were stored as water suspensions at ambient temperature until used in the study. Prior to the testing, each isolate was passaged at least twice on Sabouraud dextrose agar to ensure optimal growth characteristics.

Antifungal drugs and microdilution trays. Amphotericin B (E. R. Squibb & Sons, Princeton, N.J.), fluconazole (Roerig-Pfizer, New York, N.Y.), 5FC (Hoffmann-LaRoche, Inc., Nutley, N.J.), itraconazole (Janssen Pharmaceutica, Piscataway, N.J.), and D0870 (Zeneca Pharmaceuticals, Macclesfield, Cheshire, England) were obtained as reagent-grade powders from their respective manufacturers. Microdilution trays containing serial dilutions of the antifungal agents in RPMI 1640 medium (Sigma, St. Louis, Mo.) were prepared in a single lot and were stored frozen at −70°C until used in the study.

Antifungal susceptibility test methods. Broth microdilution testing was performed according to NCCLS guidelines by the spectrophotometric method of inoculum preparation with an inoculum concentration of 0.5×10^3 to 2.5×10^3 cells per ml and RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) (4, 7, 11–13, 15, 18, 19). Yeast inocula (100 μl) were added to each well of microdilution trays containing 100 μl of antifungal drug solution (2× final concentration). Final concentrations of the antifungal agents were 0.03 to 16 μg/ml for amphotericin B, 0.016 to 256 μg/ml for fluconazole and 5FC, and 0.007 to 8.0 μg/ml for itraconazole and D0870. The trays were incubated in air at 35°C and were observed for the presence or absence of growth at 24 and 48 h.

(i) Reference MIC endpoint reading (V). The broth microdilution wells that had not been agitated were read visually (V) with the aid of a reading mirror; the growth in each well was compared with that in the growth control (drug-free) well. A numerical score, which ranged from 0 to 4, was given to each well according to the following scale recommended by NCCLS (4, 7, 11–13, 15, 18, 19): 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity; 3, slight reduction in turbidity; and 4, no reduction in turbidity. The MIC of amphotericin B was defined as the lowest concentration at which a score of 0 (complete absence of growth) was observed, and the MICs of 5FC, fluconazole, itraconazole, and D0870 were defined as the lowest concentrations at which scores of 2 (prominent decrease in turbidity) were observed (4, 7, 11–13, 15, 18, 19).

(ii) Visual MIC following agitation (VS). In order to assess alternative methods for the determination of MIC endpoints, the MIC for each drug-organism pair was read visually following agitation of the microdilution trays. Agitation was accomplished by first sealing the tops of the trays with clear tape (Titertek plate sealer tape; Flow Laboratories) and then vortexing the entire tray with a Vortex Genie mixer set at nine (Scientific Industries, Bohemia, N.Y.) until a homogeneous yeast suspension was obtained in each well (approximately 15 to 20 s). The MIC endpoints for the agitated trays (VS) were defined exactly as described above for the reference MICs for the V trays.

(iii) Spectrophotometric MIC endpoint reading (SP). Spectrophotometric readings (SP) of each well were performed with a Whittaker Bioproducts automated plate reader (model 2001) set at 492 nm after the wells had been agitated. SP MIC endpoints were determined as the first concentration of the antifungal agent at which turbidity in the well was ≥50% less (5FC, fluconazole, itraconazole, and D0870) or ≥90% less (amphotericin B) than that in the control well.

Study design and analysis. Reproducibility and quality control were ensured by testing each of the five reference strains a total of nine times by each of the microdilution methods (V, VS, and SP) throughout the course of the study. This also provided an estimate of the accuracy of the microdilution methods relative to that of the macrodilution reference method for the testing of amphotericin B, fluconazole, and 5FC. Each of the clinical isolates was tested in triplicate, and the MICs of the five antifungal agents were determined by the three methods. The geometric mean MIC for each drug-organism pair was calculated for each of the

TABLE 2. Comparison of MICs of five antifungal agents determined by three methods of endpoint reading against 100 clinical isolates of *Candida* species[a]

Antifungal agent	Endpoint reading method	MIC (μg/ml)		
		Range	50%[b]	90%[b]
Amphotericin B	V	0.03–1.0	0.5	1.0
	VS	0.06–1.0	0.5	1.0
	SP[c]	0.08–2.5	0.5	1.0
5FC	V	0.25–>256	0.25	16
	VS	0.25–>256	0.25	16
	SP[c]	0.25–>256	0.25	8.0
Fluconazole	V	0.12–128	1.0	64
	VS	0.12–128	1.0	64
	SP[c]	0.12–64	1.0	32
Itraconazole	V	0.015–>8.0	0.25	1.0
	VS	0.015–>8.0	0.25	1.0
	SP[c]	0.007–2.0	0.12	1.0
D0870	V	0.007–8.0	0.03	2.0
	VS	0.007–8.0	0.03	2.0
	SP[c]	0.007–4.0	0.015	1.0

[a] All isolates were tested in triplicate by the microdilution modification of NCCLS M27-P.

[b] MICs encompassing 50 and 90% of all isolates tested.

[c] SP endpoints were determined as concentrations resulting in a ≥50% inhibition of growth (5FC, fluconazole, itraconazole, and D0870) and a ≥90% inhibition of growth (amphotericin B) relative to that of the control. Endpoints were measured at 492 nm.

test methods. Both on-scale and off-scale results were included in the analysis. As with previous studies (4, 11–13, 15, 18), the high off-scale MICs (>16 μg/ml for amphotericin B, >256 μg/ml for fluconazole and 5FC, and >8.0 μg/ml for itraconazole and D0870) were converted to the next highest concentrations (32, 512, and 16 μg/ml, respectively) and the low off-scale MICs (≤0.03, ≤0.016, and ≤0.007 μg/ml, respectively) were left unchanged. Overall, ≥96% of the MICs were on scale. When skipped wells were present, the MIC endpoint was the higher drug concentration. Discrepancies among MIC endpoints of no more than two dilutions (two wells) were used to calculate the percent agreement.

RESULTS

The five reference strains were tested nine times each versus all five antifungal agents, and the MICs were determined by the V, VS, and SP methods of endpoint determination. The reproducibility of each method ranged from 87 (SP) to 97% (V) at 24 h of incubation and from 98 (VS) to 99% (V and SP) at 48 h of incubation.

Table 1 summarizes the in vitro susceptibility of the five reference strains to amphotericin B, 5FC, fluconazole, itraconazole, and D0870 as judged by the three methods of MIC endpoint determination. Agreement among the three methods of reading ranged from 89 (V and SP) to 99% (V and VS) at 24 h of incubation and from 97 (VS and SP) to 99% (V and VS and V and SP) at 48 h of incubation. After 24 h of incubation, only 62 to 71% of amphotericin B, 5FC, and fluconazole MICs determined by V, 49 to 71% of those determined by VS, and 47 to 60% of those determined by SP fell within the 3-$\log_2$ dilution reference range for each strain and antifungal agent. Performance was much better after a 48-h incubation, with 84 to 100% of MICs determined by V, 93 to 100% of those determined by VS, and 89 to 100% of those determined by SP falling within the macrodilution reference ranges for the three antifungal agents and five reference strains. On the basis of reproducibility, agreement among methods, and agreement with reference MICs, it was determined that the 48-h MIC readings for all three microdilution methods should be used for comparison among the clinical isolates tested in this study.

Table 2 summarizes the in vitro susceptibility of 100 clinical yeast isolates to amphotericin B, 5FC, fluconazole, itraconazole, and D0870 as judged by the V, VS, and SP methods of MIC endpoint determination following incubation for 48 h. A broad range of MICs was observed for each antifungal agent and method of endpoint determination. In general, MICs determined by SP were slightly lower for 5FC and the azoles than MICs determined by the visual methods (V and VS). D0870 and itraconazole were the most active of the five agents tested.

TABLE 3. Agreement between alternative methods for MIC endpoint determinations for broth microdilution testing of 100 clinical isolates of *Candida* species against five antifungal agents

Antifungal agent	Agreement (%) with MIC determined by V	
	VS	SP[a]
Amphotericin B	100	99
5FC	99	98
Fluconazole	100	93
Itraconazole	100	89
D0870	99	96
All	99	95

[a] SP endpoints were determined as concentrations resulting in a ≥50% inhibition of growth (5FC, fluconazole, itraconazole, and D0870) and a ≥90% inhibition of growth (amphotericin B) relative to that of the control. Endpoints were measured at 492 nm after 48-h incubations.

Excellent agreement among reading methods was observed for all antifungal agents when they were tested against the 100 clinical isolates (Table 3). Agreement between the standard V method (no agitation) and the VS method ranged from 99 to 100%, and that between the V and SP methods ranged from 89 to 99%. Agreement between the VS and SP methods ranged from 90 to 99% (data not shown). When observed, discrepancies between the methods were generally due to the lower MICs obtained by methods entailing agitation (both VS and SP) by comparison with those obtained by the standard V method.

DISCUSSION

The results of the present study confirm and extend the previous observations of Anaissie et al. (1), Barchiesi et al. (2), and Price et al. (16) regarding the usefulness of the methods involving agitation (VS and SP) for readings of microdilution trays in the performance of antifungal susceptibility testing. The initial studies of Anaissie et al. (1) demonstrated that agitation of microdilution trays resulted in clear-cut visual MIC endpoints for fluconazole and *C. albicans* that were reproducible and that correlated well with the degree of fungal inhibition as determined by spectrophotometry. Further studies by Barchiesi et al. (2) and Price et al. (16) documented the excellent agreement of SP MICs read following the agitation of microdilution trays with MICs determined by the NCCLS reference method. The SP approach was noted to give precise, objective MIC endpoints, particularly for the azole antifungals (2, 16).

In the present study, we first used five well-characterized reference strains of *Candida* spp. to establish the reproducibility of the V, VS, and SP microdilution methods and their levels of agreement with the M27-P reference method. Excellent results were obtained with all of the microdilution methods when readings were taken after 48 h of incubation. Although the V, VS, and SP readings of 24-h incubations were reproducible and in agreement with one another, it was necessary

for the incubations to be extended for an additional 24 h to achieve acceptable agreement with the macrodilution reference MICs. Extension of these studies to include clinical isolates demonstrated the excellent performance of all three microdilution methods for testing five different antifungal agents against a broad spectrum of clinical yeast isolates. The VS and SP methods provided a wide range of MIC endpoints that were easy to read and consistent with those determined by the V method (Table 2). Agitation of the microdilution trays by vortexing them produced homogeneous yeast suspensions rapidly within the wells and markedly reduced the trailing observed with some organism-drug combinations. This approach produced very clear-cut visual endpoints.

In addition to providing an entirely objective means of determining the MIC endpoint, the SP method also offers the potential for automation. Automation or instrument-assisted reading of antifungal agent MICs would make antifungal susceptibility testing much more practical and attractive for busy clinical and reference microbiology laboratories.

The level of agreement between either the VS or SP method and the standard V method (no agitation) of reading microdilution trays that had been incubated for 48 h was $\geq 95\%$ overall, with only the itraconazole MICs read by the SP method showing $<90\%$ agreement (Table 3). When discrepancies between the V method and either the VS or SP method occurred, the MICs obtained by the VS and SP methods were generally lower than those obtained by the V method. These differences reflect, in part, the influence of trailing on visual MICs read from trays that had not been agitated and underscore the usefulness of agitation in clarifying MIC endpoints.

Given these results, it appears that the VS and SP microdilution approaches to antifungal susceptibility testing are viable alternatives to the NCCLS reference method. The ease of MIC determination and the potential for automation provided by the SP method makes the approach particularly attractive; however, the simplicity and flexibility of visual determinations from trays that had been agitated (the VS method) make this method very practical and accessible for those clinical laboratories without access to a spectrophotometer. Additional studies will be required to further standardize the SP approach and to document the interlaboratory reproducibility of results by the VS and SP microdilution methods.

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