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Multisite Reproducibility of Colorimetric Broth Microdilution Method for Antifungal Susceptibility Testing of Yeast Isolates

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MICs of fluconazole and amphotericin B were determined independently for 100 coded yeast isolates by each of six laboratories to determine reproducibility of results by using a colorimetric oxidation-reduction-based broth microdilution test. In addition, each site tested five quality control isolates on at least four different occasions during the study. Results agreed within a three-dilution range (mode ± 1 $\log_2$ dilution) for 96.2% of fluconazole tests and 92.7% of amphotericin B tests. Agreement among tests with the quality control isolates was 99.4% with fluconazole and 98.6% with amphotericin B. These results indicate that the colorimetric microdilution method is reproducible among laboratories.

Serious fungal infections are increasing rapidly in incidence (3, 15, 17, 18, 20, 21, 26, 29, 30). Yeast isolates may account for up to 10% of all nosocomial bloodstream infections and are a serious source of morbidity and mortality among some patient populations (3, 17, 20, 21, 29, 30).

Until recently, the selection of antifungal agents was limited largely to amphotericin B and 5-fluorocytosine (4, 26). Introduction of other agents, particularly the imidazole and triazole compounds, has increased the antifungal armamentarium markedly (2, 4, 5, 13, 26, 31). The increase in the number of antifungal agents, coupled with an apparent increase in infections caused by *Candida* species other than *C. albicans* and the variable susceptibilities of these organisms to the currently available antifungal agents, places a greater emphasis on in vitro susceptibility testing to guide therapeutic decisions (1–3, 5, 10, 12, 14, 16, 24, 25, 28, 31).

Methods for performing antifungal susceptibility testing have been in existence for many years, but these methods have suffered from lack of standardization and, consequently, poor reproducibility among laboratories (6, 8, 9–12, 24, 25). Through a consensus process, the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Tests has developed a proposed reference method for broth macrodilution testing of yeasts (NCCLS M27-P) (19). Because the macrodilution format is too cumbersome for use in most clinical laboratories, efforts have been focused recently on adapting the NCCLS M27-P method to a format that may be more practical and user friendly in the clinical laboratory (7, 8, 22–24). Recently, we have proposed the use of a colorimetric MIC method using an oxidation-reduction indicator (Alamar Biosciences, Inc., Sacramento, Calif.) to provide an objective, easy to read MIC in a microdilution format (22, 23). Excellent agreement (≥94%) was observed between MICs by the reference method and the colorimetric microdilution MICs when tested in a single laboratory (22, 23). The present multicenter study was performed to evaluate a prototype colorimetric microdilution antifungal panel produced by Alamar Biosciences, Inc., for its ability to provide reproducible MIC endpoints when used by multiple laboratories.

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MATERIALS AND METHODS

Test organisms. One hundred clinical yeast isolates were selected for testing. The collection included *C. albicans* (23 isolates), *Candida krusei* (10 isolates), *Candida lusitaniae* (14 isolates), *Candida parapsilosis* (22 isolates), *Candida tropicalis* (21 isolates), and *Torulopsis glabrata* (10 isolates). The test organisms were all clinical bloodstream isolates selected to maximize the number of genetically distinct strains that were obtained from patients hospitalized at the University of Iowa (9). The isolates were identified by standard methods (27) and were stored as water suspensions at ambient temperature. Each organism was coded numerically from 1 to 100, and identical sets were sent to each participating laboratory. All organisms were maintained on Sabouraud glucose agar slants at ambient temperature until used in the study. Prior to testing, each was passaged at least twice on Sabouraud glucose agar to ensure optimal growth.

Antifungal drugs and microdilution trays. Amphotericin B and fluconazole were obtained as reagent-grade powders from their respective manufacturers. Microdilution trays containing serial dilutions of the antifungal agents plus a colorimetric oxidation-reduction indicator were prepared by Alamar Biosciences, Inc. The final concentration ranges of the antifungal agents were 0.03 to 4.0 μg/ml for amphotericin B and 0.12 to 256 μg/ml for fluconazole. Each tray also contained a positive growth control well with indicator without an antifungal agent. The microdilution trays were dried and sealed in individual packages prior to shipment to the participating laboratories. The trays were stored at ambient temperature until used in the study.

Antifungal susceptibility test method. Broth microdilution testing was performed according to NCCLS proposed standard guidelines by the spectrophotometric method of inoculum preparation, 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 (American Biorganics, Inc., North Tonawonda, N.Y.) (19). The wells of each microdilution tray were reconstituted by the

TABLE 1. Colorimetric broth microdilution MICs of amphotericin B and fluconazole for QC strains reported by six separate laboratories

Drug	Control strain	n^a	MIC (μg/ml)	
			Mode	Range encompassing ≥95% of values
Amphotericin B	*S. cerevisiae* ATCC 9763	29	0.25	0.25–1.0 (100)[b]
	C. krusei ATCC 6258	36	1.0	0.25–1.0 (95)
	C. tropicalis ATCC 750	29	1.0	0.25–1.0 (100)
	C. albicans ATCC 24433	29	1.0	0.25–1.0 (100)
	C. parapsilosis ATCC 22019	23	1.0	0.5–2.0 (100)
Fluconazole	*S. cerevisiae* ATCC 9763	29	8.0	4.0–16 (100)
	C. krusei ATCC 6258	29	32	16–64 (100)
	C. tropicalis ATCC 750	29	1.0	0.5–2.0 (100)
	C. albicans ATCC 24433	29	1.0	0.5–2.0 (97)
	C. parapsilosis ATCC 22019	29	2.0	1.0–4.0 (100)

[a] *n*, total number of MIC determinations.
[b] Percentage of MICs in each three-dilution range.

addition of 100 μl of the inoculum suspension. The trays were incubated in air at 35°C for 24 h. The colorimetric MIC endpoints were read visually by observing a change in color from blue (negative) to red (positive). The MIC was defined as the lowest concentration of antifungal drug preventing the development of a red color (first blue well) (22, 23). Generally, the colorimetric endpoints were sharp; however, occasional isolates produced a purple color in transition from blue to red. In such instances, the endpoint was read as the concentration at which the color changed from purple to red.

QC. Quality control (QC) was ensured by testing the following strains at least four times throughout the course of the study: *Saccharomyces cerevisiae* ATCC 9763, *C. albicans* ATCC 24433, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750.

Study design and analysis. Six laboratories (referred to as laboratories 1 to 6) participated in the study. Each laboratory received individual subcultures of the test and QC isolates, each of which was identified by a coded number. In addition, the laboratories were sent dried microdilution trays and sufficient buffered RPMI 1640 test medium to perform the study. All laboratories tested each isolate once without retesting (a total of 600 tests per drug). QC isolates were tested a minimum of four times each in the six laboratories. Results were recorded on data sheets supplied to each laboratory and were submitted to a coordinating laboratory for analysis.

Interlaboratory agreement was determined by calculating the percentage of MICs within a three-dilution range for each isolate and of each antifungal agent. Generally, the three-dilution range constituted the mode ± 1 $\log_2$ dilution (96% of drug-organism combinations); however, for some isolates without a clear modal MIC, it was the three-dilution range that encompassed the largest number of MICs reported. The analysis of the MIC data included both MICs for which endpoints were obtained (on-scale results) and those which were off scale. The high off-scale MICs (>4.0 μg/ml for amphotericin B and >256 μg/ml for fluconazole) were converted to the next highest concentration (8.0 and 512 μg/ml, respectively), and the low off-scale MICs (≤0.03 and ≤0.12 μg/ml, respectively) were left unchanged. Overall, ≥95% of MICs were on scale.

RESULTS

QC and intralaboratory reproducibility. Each of the QC isolates was tested at least four times in each laboratory during the course of the study. Analysis of these MICs provides an estimate of intralaboratory reproducibility as well as interlaboratory agreement. A very tight distribution of MICs was observed for all isolates with both amphotericin B and fluconazole (Table 1). In the case of fluconazole, 174 of 175 (99.4%) MICs fell within a three-dilution range for the respective isolates. In every case, the range was defined by the modal MIC ± 1 $\log_2$ dilution. Results for amphotericin B were also highly reproducible: 174 of 176 (98.6%) MICs fell within a three-dilution range for the respective isolates. In contrast to fluconazole, the distribution of MICs around the amphotericin B mode was generally skewed to one side, depending on the isolate, and thus the three-dilution range for a given isolate was the one that encompassed the largest number of MICs (Table 1).

Variability among laboratories. The colorimetric microdilution MICs of amphotericin B and fluconazole for the 100 test isolates are shown in Table 2. These MICs were determined by using the modal MIC for each isolate. A broad distribution of MICs of each drug was obtained for the different species, with very few off-scale determinations.

TABLE 2. MICs of amphotericin B and fluconazole for 100 isolates tested in six laboratories

Species	n^a	Antifungal agent	MIC (μg/ml)[b]		
			Range	50%	90%
C. albicans	23	Amphotericin B	0.25–1.0	1.0	1.0
		Fluconazole	≤0.12–>256	0.5	8.0
C. krusei	10	Amphotericin B	0.25–1.0	1.0	1.0
		Fluconazole	2.0–32	32	32
C. lusitaniae	14	Amphotericin B	0.25–2.0	1.0	2.0
		Fluconazole	≤0.12–2.0	0.5	2.0
C. parapsilosis	22	Amphotericin B	0.06–1.0	0.5	1.0
		Fluconazole	0.25–2.0	0.5	1.0
C. tropicalis	21	Amphotericin B	0.5–1.0	1.0	1.0
		Fluconazole	1.0–>256	1.0	2.0
T. glabrata	10	Amphotericin B	0.12–2.0	1.0	1.0
		Fluconazole	0.25–32	8.0	8.0

[a] *n*, total number of isolates tested in each laboratory.
[b] Values determined with the modal MIC for each isolate. 50% and 90%, MICs of each agent required to inhibit 50 and 90% of the isolates tested, respectively.

TABLE 3. Interlaboratory agreement of MICs for 100 isolates tested in six laboratories

Species	n^a	% of replicate tests with MICs within a three-dilution range[b]	
		Amphotericin B	Fluconazole
C. albicans	138	94.9	97.1
C. krusei	60	98.3	96.7
C. lusitaniae	84	85.7	89.3
C. parapsilosis	132	82.6	97.7
C. tropicalis	126	100	96.0
T. glabrata	60	98.3	100
All isolates	600	92.7	96.2

[a] *n*, total number of MIC determinations for each antifungal agent.
[b] For most isolates (96%), the three-dilution range was the mode ± 1 dilution. For isolates without a clear modal MIC (4% of isolates), it was the range that encompassed the largest number of MICs.

The levels of interlaboratory agreement of MICs are shown in Table 3. Overall agreement among laboratories was 92.7% for amphotericin B and 96.2% for fluconazole. Regarding the individual species, only *C. lusitaniae* (amphotericin B and fluconazole) and *C. parapsilosis* (amphotericin B only) had levels of agreement of <94%. It was noted that one of the reasons for a lower level of agreement with *C. lusitaniae* and *C. parapsilosis* was that some of the laboratories reported very low 24-h MICs for amphotericin B and/or fluconazole, whereas other laboratories reported MICs that were four to five wells higher for the same strains. Because this was not observed with other species, one explanation could be poor or inconsistent growth of *C. lusitaniae* and *C. parapsilosis* in the RPMI test medium.

Further analysis of the basis for the lower agreement observed with *C. lusitaniae* and *C. parapsilosis* was undertaken with data from one of the participating laboratories (laboratory 4) from which both 24- and 48-h MICs were obtained. The effect of extending the duration of incubation to 48 h on amphotericin B and fluconazole MICs for *C. lusitaniae* and *C. parapsilosis* is shown in Table 4. For *C. lusitaniae*, the MICs of both amphotericin B and fluconazole increased substantially from very low at 24 h to mid-range or higher at 48 h. Likewise, for *C. parapsilosis*, an increase in amphotericin B MICs, but not fluconazole MICs, was observed with longer incubation. For comparison, the 24- and 48-h MICs of fluconazole and amphotericin B for *C. albicans* are also shown in Table 4. *C. albicans* grows very well in RPMI medium, and the interlaboratory agreement was excellent for this species (Table 3). In contrast to *C. lusitaniae* and *C. parapsilosis*, the MICs for *C. albicans* did not change substantially with extended incubation.

TABLE 4. Comparison of 24- and 48-h MICs for three *Candida* species[a]

Species	n^b	Antifungal agent	Time (h)[c]	MIC (μg/ml)[d]		
				Range	50%	90%
C. lusitaniae	14	Amphotericin B	24	≤0.03–1.0	≤0.03	1.0
			48	0.5–4.0	4.0	4.0
		Fluconazole	24	≤0.12–4.0	≤0.12	1.0
			48	1.0–8.0	4.0	4.0
C. parapsilosis	20	Amphotericin B	24	0.03–0.5	0.12	0.25
			48	0.5–2.0	1.0	2.0
		Fluconazole	24	0.25–2.0	0.5	1.0
			48	0.5–4.0	1.0	2.0
C. albicans	16	Amphotericin B	24	0.25–0.5	0.5	0.5
			48	0.5–2.0	1.0	1.0
		Fluconazole	24	0.25–4.0	0.5	4.0
			48	0.5–32	1.0	4.0

[a] Testing performed in one laboratory.
[b] *n*, total number of isolates tested.
[c] Duration of incubation.
[d] 50% and 90%, MICs of each agent required to inhibit 50 and 90% of the isolates tested, respectively.

DISCUSSION

The results reported herein extend our previous observations and document that excellent intra- and interlaboratory agreement can be obtained with the colorimetric microdilution method for determination of in vitro susceptibilities of *Candida* spp. to amphotericin B and fluconazole. By testing a panel of five QC isolates on several occasions throughout the study, we have documented excellent intralaboratory reproducibility and comparable performance with the colorimetric microdilution test among all six laboratories. The QC strains included in this study were not those recommended in M27-P (19). Alternative strains were selected for this study because three of the six isolates recommended in M27-P had target MICs that were either off scale or clustered at the low end of the concentration range (19). The strains used in the present study were selected to include a wider range of on-scale MICs, to represent a greater number of yeast species, and to avoid the inclusion of pathogenic species (e.g., *Cryptococcus neoformans*) as QC isolates. Further evaluation of these strains as candidate QC isolates is indicated.

The panel of 100 clinical isolates tested once in each laboratory allowed us to evaluate the feasibility of the colorimetric microdilution method for performing antifungal susceptibility tests and to determine the reproducibility of this method among laboratories. Determination of MIC endpoints after a 24-h incubation resulted in excellent agreement among laboratories for both amphotericin B and fluconazole. Previously, we have shown that the 24-h reading of colorimetric microdilution panels produced results comparable to 48-h readings of the macrodilution reference method (M27-P) for testing *C. albicans* and fluconazole (23). The present study extends these observations to include amphotericin B and other *Candida* species.

Clearly, an earlier MIC reading is more desirable clinically; however, we observed some problems with the 24-h endpoint when testing *C. lusitaniae* and *C. parapsilosis*. The agreement among laboratories was considerably lower for these two species than with other test isolates. Further investigation at one of the participating laboratories suggested suboptimal growth of *C. lusitaniae* and *C. parapsilosis* in RPMI medium at 24 h. Incubation for an additional 24 h resulted in increased growth and a significant increase in the MICs of fluconazole and amphotericin B (Table 4). Although confirmatory experiments were not performed in other laboratories, these data suggest that for certain species a longer incubation or an alternative medium supporting better growth may be necessary to provide optimal test conditions.

The increase in MICs of amphotericin B for several strains of *C. lusitaniae* from ≤0.03 μg/ml at 24 h of incubation to 4.0 μg/ml at 48 h (Table 4) is of particular interest given the suggestion by Rex et al. (25) that the proposed NCCLS method (M27-P) does not appear to be well suited to detect amphotericin B resistance. By extending incubation with the colorimetric microdilution method, the MICs of amphotericin B for *C. lusitaniae* were clearly higher than those for *C. albicans* or *C. parapsilosis* (Table 4). These findings suggest that by simple manipulations of the basic NCCLS-based broth dilution

method (e.g., colorimetric endpoint, microdilution format, extended incubation), the range of amphotericin B MICs may be expanded such that susceptible and resistant populations may be identified. The practical application of such strategies must be investigated in subsequent studies.

In conclusion, this feasibility study was designed to evaluate the potential of the Alamar colorimetric method as a means of antifungal susceptibility testing by determining the reproducibility of test results among laboratories. We found that intralaboratory reproducibility, as determined by repeat testing of QC isolates, was excellent: 99.4 and 98.6% for fluconazole and amphotericin B, respectively. Furthermore, single-trial tests of 100 clinical yeast isolates demonstrated a high level of interlaboratory agreement: 96.2% for fluconazole and 92.7% for amphotericin B. These results indicate that 24-h MIC testing of yeast isolates is potentially achievable; however, some isolates did not respond adequately in 24 h. Acceptable performance with these isolates was obtained by extending the incubation to 48 h, with the added benefit of expanding the MIC range for amphotericin B. The new colorimetric microdilution antifungal susceptibility test method shows good potential and, with some modification, should be capable of providing highly reproducible antifungal susceptibility results with only a 24-h incubation.

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