JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1994, p. 1992–1996
0095-1137/94/$04.00+0

Vol. 32, No. 8

Evaluation of a Novel Colorimetric Broth Microdilution Method for Antifungal Susceptibility Testing of Yeast Isolates

M. A. PFALLER[1]* AND A. L. BARRY[2]

Department of Pathology, University of Iowa College of Medicine, Iowa City, Iowa,[1] and The Clinical Microbiology Institute, Tualatin, Oregon[2]

Received 22 February 1994/Returned for modification 1 April 1994/Accepted 23 April 1994

A comparative evaluation of two broth microdilution methods for antifungal susceptibility testing of 600 clinical yeast isolates (*Candida* spp., *Torulopsis glabrata*, and *Cryptococcus neoformans*) against amphotericin B, fluconazole, and flucytosine (5FC) was conducted. Microdilution testing was performed according to National Committee for Clinical Laboratory Standards (NCCLS) recommendations (NCCLS document M27-P). By using the growth control for comparison, reference microdilution MIC endpoints for amphotericin B were scored as the lowest concentration at which a score of 0 (complete absence of growth) was observed, and those for 5FC and fluconazole were scored as the lowest concentration at which a score of 2 (prominent decrease in turbidity) (MIC-2) was observed. The second microdilution method employed a colorimetric endpoint using an oxidation-reduction indicator (Alamar Biosciences, Inc., Sacramento, Calif.) and was assessed independently of the reference microdilution MICs. The MICs for the two microdilution test systems were read after 24 and 48 h of incubation. Excellent agreement between the reference and colorimetric microdilution MICs was observed. Overall agreement was ≥95% for all three drugs at 24 h. At 48 h, agreement was ≥98% for amphotericin B and 5FC but dropped to 84% for fluconazole. Given these results it appears that the colorimetric microdilution approach to antifungal susceptibility testing may be a viable alternative to the NCCLS reference method for testing yeasts.

Over the past decade considerable effort has been expended by groups such as the National Committee for Clinical Laboratory Standards (NCCLS) to define the conditions necessary to achieve reproducible in vitro susceptibility testing of antifungal agents (1–7, 9, 10, 13–15). As a result of many collaborative studies, consensus within the NCCLS Subcommittee on Antifungal Susceptibility Tests has been achieved and a standardized reference method for broth dilution antifungal susceptibility testing of yeasts has been proposed (NCCLS document M27-P) (7). Despite this progress, problems regarding the determination of MIC endpoints still remain. Partial inhibition of fungal growth in vitro often takes place over a range of antifungal concentrations (3, 4, 6–9, 12, 13, 15). This is particularly a problem with flucytosine (5FC) and the azole antifungal agents and can make endpoint determinations both difficult and subjective (6–8, 10, 12, 13, 15).

In the NCCLS proposed standard, document M27-P, macrodilution endpoints are determined by visually grading turbidity relative to the amount of growth in the growth control tube (no antifungal agent) (7). For amphotericin B the MIC is defined as the lowest concentration that inhibits growth completely. For 5FC and the azoles (e.g., fluconazole), a less stringent endpoint allowing for slight turbidity above the MIC is recommended and the MIC is defined as the lowest drug concentration that inhibits growth by 80% relative to that of the growth control. Recent reports have described the adaptation of the NCCLS reference method to a microdilution format (2, 3, 11, 13). Microdilution endpoints are scored on a scale of 0 (no growth) to 4 (growth equal to that of the drug-free control). Microdilution endpoints for amphotericin B are defined as the lowest concentration that completely inhibits growth (score of 0 of a possible score of 4), and microdilution endpoints for the azoles and 5FC are defined as the lowest concentration at which a prominent decrease in turbidity (score of 2 of a possible score of 4) is observed compared with that of the growth control (MIC-2). Reports of comparative studies have documented excellent agreement between the NCCLS reference macrodilution method and microdilution testing and suggest that the microdilution test is an adequate tool for antifungal susceptibility testing when performed by following NCCLS standards for macrodilution susceptibility testing of yeasts (3, 11).

In a previous investigation of alternative methods of endpoint determination, we proposed the use of a colorimetric endpoint using an oxidation-reduction indicator (Alamar Biosciences, Inc., Sacramento, Calif.) to obtain an objective, easy-to-read MIC by using a microdilution format (11). Those studies were limited to testing fluconazole against *Candida albicans* but showed excellent agreement with standard broth macro- and microdilution testing performed according to NCCLS guidelines (94 and 97%, respectively). The purposes of the present study were to extend the evaluation of the colorimetric method to include additional antifungal agents and a broad spectrum of clinical yeast isolates and to further compare the method with a reference microdilution method performed according to NCCLS guidelines.

MATERIALS AND METHODS

Test organisms. Six hundred clinical yeast isolates were selected for testing. The collection included 533 isolates of *Candida* species (263 *C. albicans* isolates), 63 *Torulopsis glabrata* isolates, and 4 *Cryptococcus neoformans* isolates. The isolates were all recent clinical isolates contributed by 41 different medical institutions. The majority were from blood or normally sterile body fluids. The isolates were identified by standard methods (16) and were stored as water suspensions at

* Corresponding author. Mailing address: Department of Pathology, 273 MRC, University of Iowa College of Medicine, Iowa City, IA 52242. Phone: (319) 335-8170. Fax: (319) 335-8348.

ambient temperature until used in the study. Prior to testing, each isolate was passaged at least twice on Sabouraud dextrose agar (Prepared Media Laboratories, Tualatin, Oreg.) 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.), and 5FC (Hoffmann-La Roche, Inc., Nutley, N.J.) were obtained as reagent-grade powders from their respective manufacturers. A research lot of microdilution trays containing serial dilutions of the antifungal agents with and without the oxidation-reduction indicator (Alamar Blue) was prepared by Alamar Biosciences, Inc. The microdilution trays were dried and sealed in individual packages prior to being shipped to the test laboratory. The trays were stored at ambient temperature until used in the study.

Antifungal susceptibility test methods. Broth microdilution testing was performed according to NCCLS guidelines by using 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 Tonawanda, N.Y.) (7). The wells of each microdilution tray were reconstituted by the addition of the inoculum suspension. Final concentrations of the antifungal agents were 0.03 to 16 μg/ml for amphotericin B, 0.016 to 512 μg/ml for fluconazole, and 0.016 to 512 μg/ml for 5FC. The trays were incubated in air at 35°C and were observed for the presence or absence of growth at 24 and 48 h. Because of the slow growth of *C. neoformans*, the MICs for these isolates were read at 48 h but not at 24 h.

(i) Reference MIC endpoint reading. The broth microdilution wells were scored 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: 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity; and 4, no reduction in turbidity (3, 7, 11). 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 and fluconazole were defined as the lowest concentration at which a score of 2 (prominent decrease in turbidity) (MIC-2) was observed.

(ii) Colorimetric MIC endpoint reading. In order to assess alternative methods for the determination of MIC endpoints, each isolate was tested in duplicate by the reference microdilution method and the colorimetric microdilution method. The colorimetric MIC endpoints were read with the aid of a reading mirror. Growth in each well was indicated by a color change from dark blue to red. The colorimetric MIC was defined as the lowest concentration of antifungal drug preventing the development of a red color (first blue well) (11).

QC. Quality control (QC) was ensured by testing the following strains recommended by NCCLS document M27-P (7): *C. albicans* ATCC 90028 and ATCC 90029, *C. parapsilosis* ATCC 90018, and *T. glabrata* ATCC 90030.

Analysis of results. The colorimetric MICs were compared with the reference microdilution MICs read at 24 and 48 h. Both on-scale and off-scale results were included in the analysis. As with previous studies (3, 10, 11, 14), the high off-scale MICs (>16 μg/ml for amphotericin B and >512 μg/ml for fluconazole and 5FC) were converted to the next highest concentrations (32 and 1,024 μg/ml, respectively) and the low off-scale MICs (≤0.03 and ≤0.016 μg/ml, respectively) were left unchanged. Overall, ≥94% of MICs were on-scale (94% for fluconazole, 96% for 5FC, and 100% for amphotericin B). 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

Table 1 summarizes the in vitro susceptibilities of 600 yeast isolates to amphotericin B, fluconazole, and 5FC as judged by the colorimetric microdilution method. A broad range of MICs was observed with each antifungal agent. As expected, MICs increased with increasing duration of incubation for all three drugs tested. Amphotericin B was most active (MIC for 90% of the isolates tested ≤ 1.0 μg/ml) against *C. albicans*, *C. guilliermondii*, *C. rugosa*, and *C. neoformans* and least active against *C. lusitaniae* (48-h MIC for 90% of the isolates tested ≤ 4.0 μg/ml). Fluconazole was most active against *C. lusitaniae* and *C. parapsilosis* and least active (48-h MIC for 90% of the isolates tested ≥ 16 μg/ml) against *C. krusei*, *C. tropicalis*, *C. rugosa*, and *T. glabrata*. 5FC was most active against *C. guilliermondii*, *C. parapsilosis*, *C. rugosa*, and *T. glabrata* and least active against *C. lusitaniae* and *C. krusei*.

QC determinations were performed on at least 15 different occasions with each of the four QC isolates recommended by the NCCLS (7). MICs were within control limits for each of the antifungal agents tested (data not shown).

The overall agreement between the reference and colorimetric MICs was ≥95% for all three drugs at 24 h (Table 2). At 48 h, the agreement was ≥98% for amphotericin B and 5FC but dropped to 84% for fluconazole. Regarding individual yeast species, the agreement was ≥90% for all drugs and all species at 24 h of incubation with the exception of fluconazole and *C. tropicalis* (84% agreement). In general, the discrepancies between reference and colorimetric MICs were due to higher colorimetric fluconazole MICs for *C. tropicalis*. Although the agreement at 48 h of incubation was lower for fluconazole and *C. albicans*, *C. lusitaniae*, and *C. tropicalis*, it remained ≥90% for all other drug-yeast combinations. The discrepancies for fluconazole and *C. albicans* were generally due to higher reference MICs, whereas the discrepancies for fluconazole and *C. lusitaniae* and *C. tropicalis* were due to higher colorimetric fluconazole MICs.

DISCUSSION

The results of the present study confirm and extend our previous observations regarding the usefulness of the colorimetric approach to antifungal susceptibility testing (11). Our earlier studies using this approach focused on fluconazole and *C. albicans* and showed excellent (94 to 97%) agreement between the colorimetric microdilution approach and reference macro- and microdilution MIC testing. The results reported herein demonstrate excellent performance of the colorimetric method for testing three different classes of antifungal agents against a broad spectrum of clinical yeast isolates. The method provides a wide range of MIC endpoints that are easy to read and consistent with those determined by the reference method. An additional advantage of the colorimetric microdilution format is the potential for automation of MIC readings. QC results were within accepted limits for all three drugs against four QC strains, providing additional evidence that the colorimetric method performed in a manner comparable to that of the NCCLS reference method.

The level of agreement between colorimetric and reference microdilution MICs following 24 h of incubation was ≥95% overall, with only the combination of fluconazole and *C. tropicalis* showing <90% agreement (Table 2). When discrep-

TABLE 1. Antifungal susceptibility of clinical yeast isolates as determined by a colorimetric broth microdilution method

Organism (*n*)	Antifungal agent	Incubation time (h)	MIC (μg/ml)[a]		
			Range	50%	90%
C. albicans (263)	Amphotericin B	24	0.06–2.0	0.5	0.5
		48	0.25–2.0	1.0	1.0
	Fluconazole	24	0.25–>512	0.5	2.0
		48	0.25–>512	1.0	64
	5FC	24	0.03–>512	0.25	1.0
		48	0.12–>512	0.5	4.0
C. guilliermondii (10)	Amphotericin B	24	0.03–1.0	0.25	1.0
		48	0.25–2.0	1.0	1.0
	Fluconazole	24	1.0–32	4.0	8.0
		48	4.0–64	4.0	64
	5FC	24	≤0.016–0.25	0.06	0.12
		48	0.06–1.0	0.25	0.5
C. krusei (24)	Amphotericin B	24	0.25–4.0	0.5	1.0
		48	0.5–4.0	2.0	2.0
	Fluconazole	24	4.0–>512	32	64
		48	8.0–>512	64	128
	5FC	24	0.12–64	8.0	16
		48	0.12–64	16	32
C. lusitaniae (35)	Amphotericin B	24	0.06–2.0	0.5	1.0
		48	0.12–8.0	0.5	4.0
	Fluconazole	24	0.03–4.0	1.0	2.0
		48	0.06–128	2.0	4.0
	5FC	24	≤0.016–>512	1.0	>512
		48	≤0.016–>512	1.0	>512
C. parapsilosis (105)	Amphotericin B	24	0.06–1.0	0.25	0.5
		48	0.5–>2.0	1.0	2.0
	Fluconazole	24	0.25–>512	0.5	2.0
		48	0.25–>512	2.0	8.0
	5FC	24	≤0.016–2.0	0.12	0.5
		48	0.12–256	0.5	1.0
C. rugosa (10)	Amphotericin B	24	0.5–1.0	0.5	1.0
		48	0.5–2.0	1.0	1.0
	Fluconazole	24	1.0–16	4.0	16
		48	2.0–32	4.0	16
	5FC	24	0.06–1.0	0.12	1.0
		48	0.12–2.0	0.5	1.0
C. tropicalis (86)	Amphotericin B	24	0.12–1.0	0.5	1.0
		48	0.25–4.0	1.0	2.0
	Fluconazole	24	0.25–>512	2.0	128
		48	0.25–>512	16	>512
	5FC	24	0.06–>512	0.25	1.0
		48	0.12–>512	0.5	4.0
T. glabrata (63)	Amphotericin B	24	0.25–2.0	0.5	1.0
		48	0.5–2.0	1.0	2.0
	Fluconazole	24	0.5–128	4.0	16
		48	1.0–512	16	32
	5FC	24	≤0.016–0.5	0.06	0.125
		48	0.06–2.0	0.25	0.5
C. neoformans (4)	Amphotericin B	48	0.25–1.0	0.25	
	Fluconazole	48	1.0–8.0	2.0	
	5FC	48	0.5–8.0	2.0	
Total (600)	Amphotericin B	24	0.03–4.0	0.5	1.0
		48	0.03–8.0	1.0	2.0
	Fluconazole	24	0.03–>512	1.0	16
		48	0.06–>512	2.0	256
	5FC	24	≤0.016–>512	0.25	1.0
		48	≤0.016–>512	0.5	4.0

[a] 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

TABLE 2. Percent agreement of colorimetric and reference MICs[a]

Organism (*n*)	Antifungal agent	% Agreement at[b]: 24 h	48 h
C. albicans (263)	Amphotericin B	100	100
	Fluconazole	96	89
	5FC	99	99
C. guilliermondii (10)	Amphotericin B	90	90
	Fluconazole	100	90
	5FC	100	90
C. krusei (24)	Amphotericin B	100	100
	Fluconazole	100	100
	5FC	96	100
C. lusitaniae (35)	Amphotericin B	100	100
	Fluconazole	97	77
	5FC	100	94
C. parapsilosis (105)	Amphotericin B	99	98
	Fluconazole	98	94
	5FC	100	99
C. rugosa (10)	Amphotericin B	100	100
	Fluconazole	100	100
	5FC	100	100
C. tropicalis (86)	Amphotericin B	100	100
	Fluconazole	84	57
	5FC	98	99
T. glabrata (63)	Amphotericin B	100	100
	Fluconazole	100	94
	5FC	100	100
C. neoformans (4)	Amphotericin B		100
	Fluconazole		100
	5FC		100
Total (600)	Amphotericin B	99	99
	Fluconazole	95	84
	5FC	99	98

[a] Percentage of colorimetric microdilution MICs within 2 dilutions of the reference microdilution MICs.
[b] Percent agreement of MICs following 24 and 48 h of incubation.

ancies between the reference and colorimetric fluconazole MICs for *C. tropicalis* occurred, the colorimetric MICs were generally higher than the reference MICs at both the 24- and 48-h readings (data not shown). A similar tendency was observed for fluconazole and *C. lusitaniae* at 48 h. Interestingly, just the opposite was observed for fluconazole and *C. albicans* at 48 h (reference MICs were higher than colorimetric MICs when discrepancies occurred). The reasons for these species-specific discrepancies between fluconazole colorimetric and reference endpoints are unclear at present. It should be noted that in previous comparative studies of fluconazole susceptibility testing, the best agreement between the reference macrodilution method (according to which the MIC is defined as the lowest drug concentration that inhibits growth by 80% relative to that of the growth control, read at 48 h) and the microdilution method was obtained when the microdilution MICs (either the MIC-2 or the colorimetric MIC) were read after 24 h of incubation (3, 11). The 24-h reading minimizes the effect of partial growth inhibition, or "trailing" of MIC endpoints, and provides the best overall agreement between the reference and colorimetric fluconazole MICs.

Given these results it appears that the colorimetric microdilution approach to antifungal susceptibility testing is a viable alternative to the NCCLS reference method. The ease of MIC endpoint determination and the potential for automation provided by the colorimetric method make this approach particularly attractive for use in the busy clinical microbiology laboratory. Additional studies for the purpose of documenting interlaboratory reproducibility are indicated, as are studies designed to establish clinical correlation.

ACKNOWLEDGMENTS

This study was partially supported by a grant from Pfizer Pharmaceuticals, Roerig Division, and by Alamar Biosciences, Inc.

REFERENCES

1. **Calhoun, D. L., G. D. Roberts, J. N. Galgiani, J. E. Bennett, D. S. Feingold, J. Jorgensen, G. S. Kobayashi, and S. Shadomy.** 1986. Results of a survey of antifungal susceptibility tests in the United States and interlaboratory comparison of broth dilution testing of flucytosine and amphotericin B. J. Clin. Microbiol. **23**:298–301.
2. **Espinel-Ingroff, A., T. M. Kerkering, P. R. Goldson, and S. Shadomy.** 1991. Comparison study of broth macrodilution and microdilution antifungal susceptibility tests. J. Clin. Microbiol. **29**:1089–1094.
3. **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.
4. **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.
5. **Galgiani, J. N., J. Reiser, C. Brass, A. Espinel-Ingroff, M. A. Gordon, and T. M. Kerkering.** 1987. Comparison of relative susceptibilities of *Candida* species to three antifungal agents as determined by unstandardized methods. Antimicrob. Agents Chemother. **31**:1343–1347.
6. **Galgiani, J. N., M. G. Rinaldi, A. M. Polak, and M. A. Pfaller.** 1992. Standardization of antifungal susceptibility testing. J. Med. Vet. Mycol. **30**(Suppl. 1):213–224.
7. **National Committee for Clinical Laboratory Standards.** 1992. Reference method for broth dilution antifungal susceptibility testing for yeasts. Proposed standard. Document M27-P. National Committee for Clinical Laboratory Standards, Villanova, Pa.
8. **Odds, F. C.** 1985. Laboratory tests for the activity of imidazole and triazole antifungal agents in vitro. Semin. Dermatol. **4**:260–279.
9. **Pfaller, M. A., L. Burmeister, M. S. Bartlett, and M. G. Rinaldi.** 1988. Multicenter evaluation of four methods of yeast inoculum preparation. J. Clin. Microbiol. **26**:1437–1441.
10. **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.
11. **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.
12. **Pfaller, M. A., and M. G. Rinaldi.** 1992. In vitro testing of susceptibility of fluconazole, p. 10–22. *In* W. B. Powderly and J. W. Van't Wout (ed.), The antifungal agents, vol. 1. Fluconazole. Marius Press, Lancashire, United Kingdom.

13. **Pfaller, M. A., and M. G. Rinaldi.** 1993. Antifungal susceptibility testing: current state of technology, limitations, and standardization. Infect. Dis. Clin. N. Am. **7:**435–444.
14. **Pfaller, M. A., M. G. Rinaldi, J. N. Galgiani, M. S. Bartlett, B. A. Body, A. Espinel-Ingroff, R. A. Fromtling, G. S. Hall, C. E. Hughes, F. C. Odds, and A. M. Sugar.** 1990. Collaborative investigation of variables in susceptibility testing of yeasts. Antimicrob. Agents Chemother. **34:**1648–1654.
15. **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.
16. **Warren, N. G., and H. J. Shadomy.** 1991. Yeasts of medical importance, p. 617–629. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.