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Quality Control Guidelines for National Committee for Clinical Laboratory Standards Recommended Broth Macrodilution Testing of Amphotericin B, Fluconazole, and Flucytosine

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Amphotericin B, fluconazole, and flucytosine (5FC) were tested in a multilaboratory study to establish quality control (QC) guidelines for yeast antifungal susceptibility testing. Ten candidate QC strains were tested in accordance with National Committee for Clinical Laboratory Standards M27-P guidelines against the three antifungal agents in each of six laboratories. Each laboratory was assigned a unique lot of RPMI 1640 broth medium as well as a lot of RPMI 1640 common to all of the laboratories. The candidate QC strains were tested 20 times each against the three antifungal agents in both unique and common lots of RPMI 1640. A minimum of 220 MICs per drug per organism were generated during the study. Overall, 95% of the MICs of amphotericin B, fluconazole, and 5FC fell within the desired 3 $\log_2$-dilution range (mode ± 1 $\log_2$ dilution). Excellent performance with all three drugs was observed for *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258. With these strains, on-scale 3 $\log_2$-dilution ranges encompassing 96 to 99% of the MICs of all three drugs were established. These two strains are recommended for QC testing of amphotericin B, fluconazole, and 5FC. Reference ranges were also established for an additional four strains for use in method development and for training. Four strains failed to perform adequately for recommendation as either QC or reference strains.

Over the past decade, a great deal of effort has gone into the development of a standardized method for broth dilution susceptibility testing of antifungal agents (2–6, 8–14). Most recently, the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Tests has evaluated 10 candidate quality control (QC) isolates and has proposed tentative QC limits for amphotericin B, flucytosine (5FC), and fluconazole (8). These strains and QC limits are considered tentative because they were developed with only a single lot of RPMI 1640 broth medium. On the basis of this work, both unique and common lots of RPMI 1640 can be used to produce final QC criteria for amphotericin B, 5FC, and fluconazole by using the guidelines of the NCCLS M23-A document (7).

In the present multilaboratory study, amphotericin B, 5FC, and fluconazole were studied by using six unique lots and one common lot of RPMI 1640 as recommended by NCCLS M23-A (7). Replicate testing of the 10 candidate QC strains was performed in each laboratory by using the NCCLS M27-P broth macrodilution reference method (6). Performance limits for each QC strain and antifungal agent were established by using the statistical recommendations of Barry et al. (1). The goal of this study was to identify one or more QC strains to be used for QC and to establish reference MIC performance limits for additional strains and antifungal agents for use in method development and for training.

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MATERIALS AND METHODS

Antifungal agents. Amphotericin B, 5FC, and fluconazole were obtained from their respective manufacturers. Stock solutions of each antifungal agent were prepared in each laboratory and were stored frozen at −20°C or lower until used in the study. The twofold dilution ranges for the antifungal agents were as follows: amphotericin B, 0.03 to 16 μg/ml; 5FC, 0.12 to 64 μg/ml; fluconazole, 0.25 to 128 μg/ml.

Yeast isolates. The 10 candidate QC strains tested previously (8) were all included in this study. The isolates included *Candida albicans* ATCC 90028, ATCC 24433, and ATCC 76615; *C. parapsilosis* ATCC 90018 and ATCC 22019; *C. tropicalis* ATCC 750; *C. krusei* ATCC 6258; *Saccharomyces cerevisiae* ATCC

TABLE 1. Distribution of broth dilution MICs of amphotericin B for 10 candidate QC isolates at six laboratories[a]

Organism	No. of occurrences[b] at MIC (μg/ml) of:					
	0.06	0.12	0.25	0.5	1.0	2.0
C. albicans ATCC 90028		2	16	[99	105	0]
C. albicans ATCC 24433			[21	110	90]	1
C. albicans ATCC 76615		1	[11	106	103]	
C. parapsilosis ATCC 90018			8	[98	114	2]
C. parapsilosis ATCC 22019		2	[11	124	85]	
C. tropicalis ATCC 750		1	13	[95	113	0]
C. krusei ATCC 6258			1	[32	184	5]
S. cerevisiae ATCC 9763	1	38	[40	108	34]	
T. glabrata ATCC 90030		1	[15	147	59]	
C. neoformans ATCC 90112	4	21	[52	128	16]	

[a] All isolates were tested in six unique lots and one common lot of RPMI 1640 broth by NCCLS M27-P methods (6).

[b] Brackets enclose the proposed QC or reference ranges. Blank cells indicate no occurrences.

TABLE 2. Distribution of broth dilution MICs of fluconazole for 10 candidate QC isolates at six laboratories[a]

Organism	No. of occurrences[b] at MIC (μg/ml) of:											
	<0.25	0.25	0.5	1.0	2.0	4.0	8.0	16	32	64	128	>128
C. albicans ATCC 90028		[92	97	27]	5	1						
C. albicans ATCC 24433		[81	101	31]	6	3						
C. albicans ATCC 76615	[0	165	44]	7	3	2		1				
C. parapsilosis ATCC 90018		[1	149	68]	4							
C. parapsilosis ATCC 22019				1	[82	105	33]		1			
C. tropicalis ATCC 750				[47	130	35]	7	2	1			
C. krusei ATCC 6258							2	[5	198	17]		
S. cerevisiae ATCC 9763					3	[70	129	16]	3			
T. glabrata ATCC 90030		1		3	13	6	[58	78	38]	5		20
C. neoformans ATCC 90112			3	[66	106	35]	11					

[a] All isolates were tested with six unique lots and one common lot of RPMI 1640 broth by NCCLS M27-P methods (6).
[b] Brackets enclose the proposed QC or reference ranges. Blank cells indicate no occurrences.

9763; *Torulopsis glabrata* ATCC 90030; and *Cryptococcus neoformans* ATCC 90112.

Study design and susceptibility testing methods. The study design followed the recommendations of NCCLS document M23-A (7) for determination of QC parameters. Current RPMI 1640 base lots were obtained from American Biorganics, Inc. (Niagara Falls, N.Y.; lots B3040 and K3002), JRH Biosciences (Lenexa, Kans.; lot 2N3901), HyClone Laboratories, Inc. (Logan, Utah; lot AAK0146A), Irvine Scientific (Santa Ana, Calif.; lot 951221120), Gibco-Life Technologies, Inc. (Grand Island, N.Y.; lot 67N8041), and ICN (Costa Mesa, Calif.; lot 10601002).

The media were prepared as recommended by the manufacturer and included glutamine but not bicarbonate and were buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer. Six separate laboratories participated in these evaluations. Broth macrodilution susceptibility tests were performed in accordance with NCCLS M27-P standards (6).

Each of the six participating laboratories was assigned a specific lot of RPMI 1640 medium, and five laboratories also tested American Biorganics lot B3040, which was used as the common study lot. QC trials generated a minimum of 20 MICs per drug-organism pair at each participating facility with their unique RPMI 1640 broth base lots. An additional 20 MICs per drug-organism pair were generated in the five laboratories assigned the common lot of RPMI 1640. Thus, a minimum of 220 MICs per drug-organism pair were generated during the study. The statistical methods of Barry et al. (1) were used to define the MIC QC and reference ranges. With these methods, the proposed range includes the modal MIC $\pm$ 1 $\log_2$ dilution (1, 7). In general, the proposed range should include $\geq$95% of the MICs.

RESULTS AND DISCUSSION

Tables 1 to 3 summarize the MICs of amphotericin B, fluconazole, and 5FC, respectively, for the 10 candidate QC strains. The mode $\pm$ 1 $\log_2$ dilution encompassed $\geq$90% (91.9 to 99.5%) of the MICs of amphotericin B for 8 of the 10 QC strains (Table 1). For *S. cerevisiae* ATCC 9763 and *C. neoformans* ATCC 90112, a 4 $\log_2$-dilution range was required to encompass >90% of the MICs.

The mode $\pm$ 1 $\log_2$ dilution encompassed $\geq$93% (93.7 to 99.1%) of the fluconazole, MICs for 9 of the 10 QC strains (Table 2). The MIC distribution for the *T. glabrata* ATCC 90030 isolate was skewed by 20 off-scale (>128 μg/ml) results, 19 of which were reported by a single laboratory. When the results from that laboratory were omitted, 156 (85.7%) of the 182 MIC results for that isolate were included in the 3-dilution range defined by the mode $\pm$ 1 $\log_2$ dilution (8.0 to 32 μg/ml).

The mode $\pm$ 1 $\log_2$ dilution encompassed $\geq$90% (91.9 to 99.5%) of the 5FC MICs for 8 of the 10 QC strains (Table 3). Expansion to a 4-dilution range (0.5 to 4.0 μg/ml) would be required to encompass >90% (96.4%) of the MICs for isolate *C. neoformans* ATCC 90112. The MIC distribution for *C. albicans* ATCC 76615 was bimodal, with peaks at 8.0 and >64 μg/ml. Fifty-seven percent of the off-scale (>64 μg/ml) values were contributed by a single laboratory.

Overall, 95% of the amphotericin B, fluconazole, and 5FC MICs fell within the desired 3 $\log_2$-dilution range (mode $\pm$ 1 $\log_2$ dilution) for the 10 candidate QC strains tested with multiple lots of RPMI 1640 medium. Excellent performance of all three drugs was observed for *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258. With these strains, on-scale 3 $\log_2$-dilution ranges encompassing 96.8 to 99.5% of the MICs of all three drugs were established (Table 4). On the basis of this performance, these two strains and the accompanying MIC limits are recommended for use in quality control testing of amphotericin B, fluconazole, and 5FC (Table 4).

TABLE 3. Distribution of broth dilution MICs of 5FC for 10 candidate QC isolates at six laboratories[a]

Organism	No. of occurrences[b] at MIC (μg/ml) of:											
	<0.12	0.12	0.25	0.5	1.0	2.0	4.0	8.0	16	32	64	>64
C. albicans ATCC 90028		2	7	[93	120	8]	2					
C. albicans ATCC 24433			4	13	[47	111	46]	1				
C. albicans ATCC 76615			1			5	[57	94	24]	4	2	35
C. parapsilosis ATCC 90018	[0	206	15]	1								
C. parapsilosis ATCC 22019		[24	156	39]	1	1		1				
C. tropicalis ATCC 750	[0	212	8]	1								
C. krusei ATCC 6258		3					[1	122	92]	4		
S. cerevisiae ATCC 9763	[0	212	2]	1	1	1	1		2			
T. glabrata ATCC 90030	[0	215	4]	1			1					
C. neoformans ATCC 90112		1	1	17	[66	73	56]	6				

[a] All isolates were tested with six unique lots and one common lot of RPMI 1640 broth by NCCLS M27-P methods (6).
[b] Brackets enclose the proposed QC or reference ranges. Blank cells indicate no occurrences.

TABLE 4. Recommended MIC limits of three antifungal agents for two QC and four reference strains when tested by NCCLS M27-P methods

Organism	Purpose	Antifungal agent	MIC range (μg/ml)	% of MICs within range
C. parapsilosis ATCC 22019	QC	Amphotericin B	0.25–1.0	99.1
		Fluconazole	2.0–8.0	99.1
		5FC	0.12–0.5	98.6
C. krusei ATCC 6258	QC	Amphotericin B	0.5–2.0	99.5
		Fluconazole	16–64	99.1
		5FC	4.0–16	96.8
C. albicans ATCC 90028	Reference	Amphotericin B	0.5–2.0	91.9
		Fluconazole	0.25–1.0	97.3
		5FC	0.5–2.0	95.0
C. albicans ATCC 24433	Reference	Amphotericin B	0.25–1.0	99.5
		Fluconazole	0.25–1.0	95.9
		5FC	1.0–4.0	91.9
C. parapsilosis ATCC 90018	Reference	Amphotericin B	0.5–2.0	96.4
		Fluconazole	0.25–1.0	98.2
		5FC	≤0.12–0.25	99.5
C. tropicalis ATCC 750	Reference	Amphotericin B	0.5–2.0	93.7
		Fluconazole	1.0–4.0	95.5
		5FC	≤0.12–0.25	99.5

An additional four isolates, *C. albicans* ATCC 90028 and ATCC 24433, *C. parapsilosis* ATCC 90018, and *C. tropicalis* ATCC 750, performed well enough to be recommended as reference strains for use in method development and for training. With these strains, 91.9 to 99.5% of the amphotericin B MICs, 95.5 to 98.2% of the fluconazole MICs, and 91.9 to 99.5% of the 5FC MICs fell within the desired 3 $\log_2$-dilution range (mode ± 1 $\log_2$ dilution) (Table 4). These strains are not recommended as QC strains either because the mode ± 1 $\log_2$ dilution encompassed <95% of the MICs of one or more drugs or because the MICs of one or more of the antifungal agents tended to cluster towards the extreme low end of the dilution series. Nevertheless, all of these strains have performance characteristics that make them useful as reference strains for laboratories striving to establish broth macrodilution antifungal susceptibility testing.

C. albicans ATCC 76615, *S. cerevisiae* ATCC 9763, *T. glabrata* ATCC 90030, and *C. neoformans* ATCC 90112 failed to perform adequately for recommendation as either QC or reference strains. Expansion to a 4-dilution range would encompass ≥95% of the MICs of each of the three drugs for *C. neoformans* ATCC 90112, and perhaps this strain could be used as a reference. Each of the remaining three strains performed unacceptably with one or more of the study drugs (<90% agreement among laboratories; Table 5).

A comparison of the MIC QC and reference ranges obtained in the present study with those reported previously as tentative QC ranges (8) revealed only minor differences (data not shown). The amphotericin B MIC ranges for *C. parapsilosis* ATCC 22019 and *C. albicans* ATCC 24433 are 1 $\log_2$ dilution lower in the present study than those reported previously (0.25 to 1.0 versus 0.5 to 2.0 μg/ml).

As stated previously (8), in developing QC strains and MIC limits for antifungal testing we did not attempt to qualify strains that are resistant to each of the antifungal agents nor did we attempt to represent specific *Candida* or *Cryptococcus* species. We selected QC strains in accordance with NCCLS M23-A (7) and recommend those with reliable performance characteristics and with MIC endpoints that are on scale for all three of the antifungal agents tested. These QC strains and MIC limits will provide laboratories with the means of qualifying lots of RPMI 1640 medium for testing and will allow routine monitoring of the performance of the antifungal susceptibility testing method. The QC strains plus the reference strains (Table 4) will also be useful in assisting laboratories to develop alternatives to the NCCLS M27-P reference macrodilution method (9–11, 14). In addition, strains resistant to the major classes of antifungal agents described in this report are available. The incorporation of such strains into future studies is essential to control the ability of the procedure to detect resistance.

In conclusion, we established QC performance guidelines for two QC strains and amphotericin B, fluconazole, and 5FC

TABLE 5. Overall agreement among six laboratories when testing three antifungal agents by NCCLS-recommended broth macrodilution methods[a]

Organism	% of MICs within 3 $\log_2$-dilution range[b]		
	Amphotericin B	Fluconazole	5FC
C. albicans ATCC 90028	91.9	97.3	95.0
C. albicans ATCC 24433	99.5	95.9	91.9
C. albicans ATCC 76615	99.5	94.1	78.8
C. parapsilosis ATCC 90018	96.4	98.2	99.5
C. parapsilosis ATCC 22019	99.1	99.1	98.6
C. tropicalis ATCC 750	93.7	95.5	99.5
C. krusei ATCC 6258	99.5	99.1	96.8
S. cerevisiae ATCC 9763	70.0	97.3	97.3
T. glabrata ATCC 90030	99.5	78.4	99.1
C. neoformans ATCC 90112	88.7	93.7	88.6

[a] Each isolate was tested 20 times in unique and common lots of RPMI 1640 broth in each laboratory.

[b] Mode ± 1 $\log_2$ dilution.

by using the NCCLS M27-P method. We also identified four reference strains that may be useful in further method development and training of individuals to perform antifungal susceptibility testing. The approach used in the present study to establish the QC parameters for testing of amphotericin B, fluconazole, and 5FC should also be applied to new antifungal agents as they become available. The QC and reference strains identified in this study should facilitate these efforts.

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