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Quality Control Limits for Broth Microdilution Susceptibility Tests of Ten Antifungal Agents

A. L. BARRY,[1]* M. A. PFALLER,[2] S. D. BROWN,[1] A. ESPINEL-INGROFF,[3] M. A. GHANNOUM,[4] C. KNAPP,[5] R. P. RENNIE,[6] J. H. REX,[7] AND M. G. RINALDI[8]

The Clinical Microbiology Institute, Wilsonville, Oregon[1]; University of Iowa College of Medicine, Iowa City, Iowa[2]; Medical College of Virginia Commonwealth University, Richmond, Virginia[3]; University Center Reference Laboratory for Medical Mycology, Cleveland, Ohio[4]; Trek Diagnostic Systems, Inc., Westlake, Ohio[5]; National Center for Mycology, Edmonton, Alberta, Canada[6]; and University of Texas Health Sciences Center, Houston,[7] and University of Texas Health Sciences Center, San Antonio,[8] Texas

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Broth microdilution susceptibility tests of *Candida* species have now been standardized by the National Committee for Clinical Laboratory Standards (NCCLS). An eight-laboratory collaborative study was carried out in order to document reproducibility of tests of *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 by the NCCLS method. Replicate broth microdilution tests were used to define control limits for 24- and 48-h MICs of amphotericin B, flucytosine, fluconazole, voriconazole, ketoconazole, itraconazole, caspofungin (MK 0991), ravuconazole (BMS 207147), posaconazole (SCH 56592), and LY 303366.

A standard reference method for testing the susceptibility of yeasts to antifungal agents has now been defined by the National Committee for Clinical Laboratory Standards (NCCLS) (3). Initially, the test was performed in 12-by-75-mm plastic tubes, but a more convenient broth microdilution version of that test (2) has been added to the current NCCLS document (3). Both methods are carried out in RPMI 1640 broth with glutamine and a phenol red pH indicator and with 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (0.165 mol/liter for pH 7.0). The inoculum is adjusted to achieve a final concentration of 0.5×10^3 to 2.5×10^3 CFU/ml. The MICs are determined after 48 h of incubation at 35°C. For tests of azoles and flucytosine, the MICs are defined as the lowest concentration with a prominent decrease (at least an 80% decrease) in turbidity but, for amphotericin B, the endpoint is complete inhibition of growth. Although the reference method requires a 48-h incubation period, there is some evidence that a 24-h incubation time might be more appropriate for tests of the azoles (8, 9).

Early studies with the NCCLS tube dilution method have been carried out to describe MIC limits for tests of five different antifungal agents (3, 6, 10) against two designated quality control strains (5): *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258. The current report describes the results of an eight-laboratory collaborative effort to select quality control limits for broth microdilution tests of 10 antifungal agents tested against the two control strains. Both 24- and 48-h test results were recorded since there are differences of opinion concerning the question of which incubation period is most clinically relevant.

The experimental design followed the guidelines that have been specified for antibacterial agents (4), but with appropriate modifications for testing yeasts. The NCCLS broth microdilution method (3) was strictly followed except that MICs were recorded at 24 h as well as the standard 48-h time point. Frozen microdilution panels were prepared by S. Killian (Trek Diagnostic Systems, Westlake, Ohio). The trays contained serial dilutions of the antifungal agents in three different lots of RPMI 1640 broths (Sigma lot 77H46141, Cellgrow lot 90022025, and Irvine Scientific lot 951370226B); all lots were supplemented with glutamine (Sigma lot 484H1013) and 0.165 mol of MOPS buffer (Sigma lot 56H57035) per liter. Each well contained 100 μl of a 2× concentration of the study agent. The range of concentrations of each drug was broad enough to ensure that the majority of MICs were on scale. After inoculation of the test panels with 100 μl of a standardized inoculum prepared in RPMI 1640 broth, the desired final concentration of drug was achieved. The participants were provided with frozen microdilution trays as well as samples of all three lots of broth for inoculum preparation. The microdilution trays were stored at −60°C or colder until needed. In addition to the two established control strains, a strain of *C. albicans* (ATCC 90028) was also studied, but the results are not shown here because MICs of most drugs were too variable to be of value for quality control purposes. Other strains of *C. albicans* are currently being considered for this use.

On 10 different working days, the participants prepared inocula of the control strains, adjusted to match the turbidity of a McFarland 0.5 standard, as determined with a spectrophotometer (7), and further diluted in each of the three lots of RPMI 1640 broths. Microdilution trays were allowed to thaw and then inoculated with a multichannel pipette, being certain to match the lot of broth in the test panel to the lot used to prepare the inoculum. Immediately after inoculation, randomly selected trays were mixed by gentle tapping, and then 10 μl was removed from the growth control well for determination of the actual inoculum density. Each participant performed at least one colony count on each of 10 test days. The eight participants reported 139 such determinations for each control strain. The inocula for *C. parapsilosis* ATCC 22019 ranged from 0.5×10^3 to 6.4×10^3 CFU/ml (mean, 1.8×10^3 CFU/ml), and for *C. krusei* ATCC 6258 the counts ranged from 0.2×10^3 to 2.5×10^3 CFU/ml (mean, 1.2×10^3 CFU/ml). Trays were incubated at 35°C, and MICs were recorded after 24 h and again after 48 h. MICs were defined as the highest

* Corresponding author. Mailing address: Clinical Microbiology Institute, 9725 SW Commerce Circle, Ste. A-1, Wilsonville, OR 97070. Phone: (503) 682-3232. Fax: (503) 682-2065. E-mail: cmi@hevanet.com.

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TABLE 1. Distribution of MICs reported by eight participating facilities each testing two control strains on 10 separate days

Antifungal agent and control strain[a]	Incubation time (h)	No. of times the following MICs (μg/ml) were reported ($n = 240$)[b]:													
		≤0.016	0.03	0.06	0.12	0.25	0.5	1.0	2.0	4.0	8.0	16	32	64	128
Amphotericin B															
C. parapsilosis	24				7	[1	120	109	3]						
	48					20	[1	88	123	8]					
C. krusei	24						[2	141	97]						
	48							[10	202	28]					
Flucytosine															
C. parapsilosis	24			[43[c]	152	43]	1	1							
	48			4[c]	[12	189	34]	1							
C. krusei	24							1	5	[36	119	79]			
	48									1	[74	162	3]		
Fluconazole[d]															
C. parapsilosis	24				1	2	[4	55	91	15]					
	48			1	2	1		[42	119	41]					
C. krusei	24										[5	95	76	4]	
	48											[4	108	98	0]
Voriconazole															
C. parapsilosis	24	[9	86	141	4]										
	48		[21	113	83	3]									
C. krusei	24		4	[17	76	139	4]								
	48				[0	110	125	5]							
Ketoconazole															
C. parapsilosis	24	6	[7	106	108	13]									
	48		2	[23	114	82	17]	2							
C. krusei	24		1	10	[32	74	123	0]							
	48					[25	143	70]	1						
Itraconazole															
C. parapsilosis	24	5		4	[34	129	66]	1							
	48			5	[28	149	57]	1							
C. krusei	24		1	9	[23	68	136	3]							
	48					[23	170	46]							
Caspofungin (MK0991)															
C. parapsilosis	24	6	1		1	[29	159	44]							
	48				1	5	[11	143	67	2]			9	2[c]	
C. krusei	24			3	[22	98	116	1]							
	48				4	[22	164	48]	2						
Ravuconazole (BMS 207147)															
C. parapsilosis	24	[9	93	121	7]	10									
	48	3	[8	114	92	22]	1								
C. krusei	24	6	10	[17	72	96	39]								
	48					[83	152	5]							
Posaconazole (SCH 56592)															
C. parapsilosis	24	7		[36	119	77]	1								
	48	1	1	[15	161	61]	1								
C. krusei	24			[12	51	107	70]								
	48			1	[3	93	140	3]							
LY 303366															
C. parapsilosis	24		4	3			2	[43	111	77	0]				
	48			1				[31	83	123	2]				
C. krusei	24	1	[8	130	75	23]	2	1							
	48	1	2	[59	108	44	14]	10	2						

[a] *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258.
[b] Proposed QC limits are indicated by brackets.
[c] MICs outside the range of flucytosine or caspofungin that were actually tested.
[d] Data from one laboratory were excluded because MICs of the control drug, amphotericin B, were outside of the proposed limits; another laboratory reported only 48-h readings. A third laboratory experienced growth failures with two lots of broth, especially after 24 h. Consequently, the total number of evaluable fluconazole MICs was less than the 240 expected test results.

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TABLE 2. Proposed MIC ranges of various antifungal agents for two quality control strains of *Candida* spp. when tested by the NCCLS microdilution procedure

Antifungal agent	MIC (μg/ml) ranges for microdilution tests with:			
	C. parapsilosis ATCC 22019 at:		*C. krusei* ATCC 6258 at:	
	24 h	48 h	24 h	48 h
Amphotericin B	0.25–2.0	0.5–4.0	0.5–2.0	1.0–4.0
Flucytocine	0.06–0.25	0.12–0.5	4.0–16	8.0–32
Fluconazole	0.5–4.0	1.0–4.0	8.0–64	16–128
Voriconazole	0.016–0.12	0.03–0.25	0.06–0.5	0.12–1.0
Ketoconazole	0.03–0.25	0.06–0.5	0.12–1.0	0.25–1.0
Itraconazole	0.12–0.5	0.12–0.5	0.12–1.0	0.25–1.0
Caspofungin (MK0991)	0.25–1.0	0.5–4.0	0.12–1.0	0.25–1.0
Ravuconazole (BMS 207147)	0.016–0.12	0.03–0.25	0.06–0.5	0.25–1.0
Posaconazole (SCH 56592)	0.06–0.25	0.06–0.25	0.06–0.5	0.12–1.0
LY303366	1.0–8.0	1.0–8.0	0.03–0.25	0.06–0.5

concentration that showed a sharp decline in the density of growth.

Because of technical problems with the first batch of fluconazole trays, new trays were prepared for retesting. The same protocol was used except that dilutions of amphotericin B were included for control purposes since MIC control limits for amphotericin B were defined by the first series of tests. Data from one of the eight participants in this second series were excluded because amphotericin B MICs were too high. Fluconazole MIC limits were based on data from the seven remaining laboratories even though the excluded fluconazole data were within the proposed MIC limits.

There were no major differences (>2 dilutions) between lots of RPMI 1640 broths, nor were there consistent discrepancies between laboratories. For that reason the data were pooled for analysis. The overall distribution of MICs are displayed in Table 1. There were 240 MICs for each drug-microorganism combination and for each incubation time. Fewer MICs were available for evaluating fluconazole in the second trial because data from one laboratory were excluded and because another participant reported only the 48-h MICs. Control limits were described as ranges which included one doubling concentration on either side of the mode (1). When two adjacent concentrations displayed similar frequencies, the mode was assumed to be somewhere between the even $\log_2$ concentrations that were tested, and a four-dilution range was proposed (1). For most drugs, the 48-h MICs were approximately one doubling concentration greater than those recorded after 24 h. Every effort was made to select MIC ranges that included at least 95% of the recorded values and that occasionally required a four-dilution range around a clearcut mode.

Considering the nature of the endpoints that were being determined and the technical problems that needed careful attention, it was pleasing to see the degree of precision that was achieved when standard control strains were tested by the NCCLS microdilution method. Reproducibility with control strains was similar to that normally seen with antibacterial agents (1). On the other hand, our inability to achieve satisfactory precision with a reference strain of *C. albicans* is rather disturbing. Satisfactory results with the control strains do not guarantee accuracy with the clinical isolates being tested. Experience in determining MIC values and careful attention to procedural details are critically important when performing antifungal susceptibility tests.

With the quality control limits that we now propose (Table 2), clinical or research laboratories will have a mechanism for assuring themselves that the antifungal microdilution test is being performed appropriately. The 24- and 48-h MIC limits that we propose (Table 2) have now been accepted by the NCCLS subcommittee on antifungal susceptibility tests and will appear in the next publication of that document.

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