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Quality Control Limits for Fluconazole Disk Susceptibility Tests on Mueller-Hinton Agar with Glucose and Methylene Blue

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An international collaborative study was performed in order to propose quality control limits for fluconazole disk diffusion susceptibility tests on Mueller-Hinton agar with 2% glucose and 0.5 μg of methylene blue per ml. The supplements may be added before autoclaving the agar, or Mueller-Hinton agar plates may be flooded with a glucose-methylene blue solution. Replicate tests on both types of agar plates generated data to propose zone size limits for tests of *Candida parapsilosis* ATCC 22019 (22 to 33 mm), *C. tropicalis* ATCC 750 (26 to 37 mm), and *C. albicans* ATCC 90028 (28 to 39 mm). *C. krusei* ATCC 6258 was not useful for this purpose.

Immunocompromised patients are often colonized by or infected with fungi, especially *Candida* spp. Consequently, they frequently receive antifungal agents such as fluconazole throughout their period of hospitalization. Prolonged exposure to an azole can select strains with diminished susceptibilities (4), and consequently, increasing doses may be necessary. For that reason, clinical laboratories may be asked to monitor the susceptibility of isolates from such patients in order to determine when strains with diminished susceptibility have been selected (3). For many laboratories, the disk diffusion procedure may be the most practical method for this purpose. A standardized method and well-defined quality control procedures are essential in order to determine when significant changes are being observed over an extended period of time.

In 1996, we proposed a disk diffusion procedure that was performed on RPMI 1640 broth with 2% glucose and 1.5% agar (1). Subsequent studies were carried out to find an agar medium that should be readily available in most clinical laboratories. Mueller-Hinton (MH) agar could be used for this purpose, but it had to be supplemented with 2% glucose in order to increase the number of clinical isolates that would grow adequately. Unfortunately, the added glucose resulted in a significant amount of growth within zones of inhibition and zone edges that were not well defined. Addition of a low concentration of methylene blue (0.5 μg/ml) made the zones of inhibition clearer and easier to measure precisely (2, 8).

Plates with MH–glucose-methylene blue (GMB) agar can be prepared in advance and stored until needed, but the shelf life of such plates has not been determined. Prepared agar plates are practical for surveys, when the number of tests can be predicted in advance and prolonged storage of the plates is not necessary. An alternative approach is preferred for clinical laboratories where susceptibility tests are only sporadically needed. Fresh MH agar plates are commonly available in clinical laboratories, and they can be supplemented by flooding the surface with a GMB solution.

Flooding procedure. A stock solution of methylene blue (5 mg/ml) was prepared and refrigerated at 2 to 8°C. To 100 ml of a 40% glucose solution, 200 μl of the stock methylene blue was added to give 10 μg of methylene blue per ml of 40% glucose (GMB solution). The GMB solution was dispensed into screw-cap tubes (3.5 ml for 150-mm-diameter plates or 1.5 ml for 100-mm-diameter plates), and that solution was then sterilized by autoclaving. The day before testing, refrigerated tubes with the GMB solution were allowed to warm to room temperature and at the same time MH agar plates were dried in a 35°C incubator with lids ajar until all of the surface moisture evaporated (usually 1 to 2 h). The dried agar surfaces were then flooded with the GMB solution, and that liquid was allowed to adsorb overnight at room temperature on a flat surface. Adsorption was completed within a few hours when the MH agar plates had been dried by prolonged storage, but freshly prepared agar plates required a longer time. Assuming that the supplements are completely adsorbed and evenly dispersed throughout the 70 ml of agar in each 150-mm-diameter plate, the final concentrations of glucose and methylene blue should be 2% and 0.5 μg/ml, respectively.

A preliminary study with 226 clinical isolates was performed to determine whether the two types of MH-GMB agar plates give similar results (unpublished data). Because some zones of inhibition were poorly defined and difficult to measure, zones on the two types of MH-GMB agar plates differed by as much as 10 mm, with the flooded plates averaging zones 2 to 3 mm larger than those on plates prepared in advance. Although these differences are relatively minor, we felt that we should evaluate both types of agar plates in order to propose quality control limits for fluconazole disk diffusion tests.

Quality control study. An international collaborative study was performed at the eight institutions we represent. Three

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TABLE 1. Results of replicate tests in eight laboratories using 25-μg fluconazole disks on two types of MH agar with GMB[a]

24-h zone diam (mm)	No. of times each zone diam was recorded[b]					
	C. parapsilosis ATCC 22019[c]		*C. tropicalis* ATCC 750[d]		*C. albicans* ATCC 90028[e]	
	Prepared (469 zones)	Flooded (450 zones)	Prepared (478 zones)	Flooded (468 zones)	Prepared (479 zones)	Flooded (472 zones)
19		1				
20		1				
21	10	2				
22	9	3		2		
23	10	7	3			
24	20	12	1	4		
25	35	32	8	7		
26	36	39	11	6	1	6
27	50	60	15	10	3	5
28	(74)	56	21	23	5	4
29	64	(70)	35	33	9	7
30	70	78	68	55	11	15
31	37	38	52	56	33	29
32	27	23	(69)	(69)	44	53
33	15	18	52	54	85	59
34	5	4	53	40	(68)	(85)
35	1	5	33	34	60	75
36	3	1	13	19	52	41
37	1		17	23	31	35
38	2		8	19	23	24
39			11	9	15	9
40			7	5	9	13
41			1		8	5
42					9	4
43					6	2
44						1

[a] MH agar was supplemented by adding GMB before autoclaving (prepared agar) or by flooding M H agar with a GMB solution on the day before testing (flooded agar).

[b] Horizontal lines designate the quality control limits for tests with each control strain (defined on the bottom line), and parentheses designate the median zone diameter for each data set. The quality control limits and percentage of zones that fell within the proposed control limits, as calculated for data from all eight laboratories or from seven laboratories, excluding data from the laboratory that reported unusually large zones were 22 to 33 mm for *C. parapsilosis* (96% of all eight laboratories; 97% of seven laboratories); 26 to 37 mm for *C. tropicalis* (91% of all eight laboratories; 96% of seven laboratories); and 28 to 39 mm for *C. albicans* (92% of all eight laboratories; 96% of seven laboratories).

[c] Of the 22 zone diameters of >33 mm, 11 were reported by laboratory no. 8.

[d] Of the 60 zone diameters of >37 mm, 48 were reported by laboratory no. 3.

[e] Of the 57 zone diameters of >39 mm, 41 were reported by laboratory no. 3.

lots of MH agar (Acumedia lot 0101-126, Difco lot 1005002, and Beckton Dickinson Microbiology Systems lot 1031005) were prepared as MH-GMB agar plates or poured for flooding with a GMB solution when needed. Each test plate received two 25-μg fluconazole disks (Bector Dickinson Microbiology Systems lot 906548 and Sanofi lot 9L010). The GMB solutions used for flooding were from a single source, as were the agar plates, disks, and control strains.

Disk diffusion tests were performed by preparing a saline suspension of freshly isolated colonies that was then adjusted to match the turbidity of a McFarland 0.5 standard. A sterile applicator swab was moistened in this cell suspension and then used to inoculate the surface of each 150-mm-diameter agar plate. After inoculation of the plates, two fluconazole disks were applied to the surface. The inverted plates were then incubated at 35°C for 20 to 24 h. Calipers were used to measure the diameter of each zone of inhibition at the point where there was a sharp decline in the density of growth.

On each of 10 separate days, each participant tested the four control strains on both types of MH-GMB agar plates. *Candida krusei* ATCC 6258 was not useful for monitoring tests of fluconazole because 55% of the tests had no zone of inhibition and others had zones of ≤18 mm. For each control strain, every participant was to record 60 zone diameters on prepared MH-GMB agar plates and 60 zone diameters on plates prepared by flooding MH agar plates (three lots of MH agar and two lots of fluconazole disks tested on 10 separate test days). Because of logistical problems, our target of 480 zone measurements for each strain was not achieved but 468 to 479 zones were recorded. Table 1 displays the overall distribution of zone diameters recorded for three of the four control strains when determined after 20 to 24 h of incubation. Zones were also measured after 48 h, but the first measurements were more reproducible and a 20- to 24-h incubation period is now recommended for disk tests.

The overall spread of zone diameters was skewed by one laboratory that reported unusually large zones for *C. tropicalis* ATCC 750 and *C. albicans* ATCC 90028 but not for the *C. parapsilosis* strain. Another participant reported exceptionally large zones for *C. parapsilosis* ATCC 22019 but not for the other control strains. Quality control limits were selected to best fit data from the seven participants that were in closer agreement (5). Control limits thus calculated were then applied to data from all eight facilities. Reasonable control limits

could be defined to include a 12-mm range of zone diameters. That is only slightly broader than the 8- to 10-mm range that is commonly applied to tests of bacteria. Because of the nature of the endpoints that are being observed, tests of antifungal agents cannot be expected to be as precise as those of antibacterial agents. Addition of methylene blue to the agar medium allowed zones of inhibition to be measured with a reasonable degree of precision when the standard control strains were being tested.

The two methods of preparing MH-GMB agar plates provided essentially identical results with the three control strains in Table 1; i.e., median zone sizes were the same or differed by only 1 mm. Consequently, we propose one set of quality control limits that can be used for either type of MH-GMB agar plate. The limits are noted in Table 1, footnote *b*, and include 91 to 96% of all of the zones reported or 96 to 97% of the zones remaining after exclusion of the facility reporting exceptionally large zones. The quality control limits have been approved by the NCCLS Subcommittee for Antifungal Susceptibility Testing and will be included in a document that will soon be published by that group. Tentative quality control limits proposed by Meis et al. (8) were about 4 mm larger. Other disk diffusion techniques have been described in the literature (6, 7, 9), but well-documented quality control parameters have not been defined for those methods.

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