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Comparison of Results Obtained by Testing with Three Different Agar Media and by the NCCLS M27-A Method for In Vitro Testing of Fluconazole against *Candida* spp.

M. Carmen Rubio,[1]* Joaquina Gil,[1] Inmaculada Ramírez de Ocáriz,[1] Rafael Benito,[1] and Antonio Rezusta[2]

Microbiology, Hospital Clínico Universitario Lozano Blesa, Zaragoza,[1] and Facultad de Ciencias de la Salud y del Deporte, Huesca,[2] Spain

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Fluconazole susceptibilities of 150 *Candida* isolates were determined by a 25-μg fluconazole disk diffusion agar test and compared with the microdilution NCCLS M27-A method. The agar test used three different media and was read at 24 and 48 h. When only the susceptible and nonsusceptible categories were used, disk diffusion with Müeller-Hinton agar supplemented with 2% glucose and 0.5 μg of methylene blue (MHGM) per ml had a 95.37% correlation with the MIC method at 24 h, followed by RPMI 1640–2% of glucose agar (correlation, 94%) and Shadomy medium (SHDM) (correlation, 92.6%). The growth of microcolonies inside the inhibition zones was common (>63%) in the RPMI and SHDM media and minimal with MHGM (8.7%). At 48 h, MHGM and SHDM still had a >91% correlation with the MIC, while RPMI results had dropped to 75%. The best overall agreement was obtained with *C. dubliniensis* (100%).

Fungal infection with *Candida* species is still an important cause of morbidity and mortality in immunocompromised patients. With the widespread use of fluconazole for the treatment and prevention of oropharyngeal and/or esophageal candidiasis, particularly in AIDS patients, clinical resistance is becoming a serious problem (5, 7, 8, 12, 21).

What is needed is a rapid, easy, reproducible, and inexpensive in vitro method of obtaining susceptibility data which can guide the treatment of clinical yeast infections.

The M27-A and M27-A2 reference NCCLS methods for antifungal susceptibility testing (15, 17) are cumbersome and costly, and reading the endpoints of the azoles is difficult. Alternative methods such as the broth microdilution adaptation of the M-27A and M27-A2 methods or the E test are simplified tests but are not easily adapted to the screening of yeasts for fluconazole susceptibility, nor are they cost-effective enough to be performed routinely in most clinical microbiology laboratories (6, 18, 20, 24).

Recent studies (2, 4, 9, 11, 13, 14, 19; C. Durussel, A. M. Daoui-Hassani, and J. Bille, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. J120, 1998) have demonstrated that an agar disk diffusion method similar to that used for testing antibacterial agents (NCCLS M2-A6) (16) can reproducibly and accurately determine the susceptibility of yeasts to fluconazole and can easily be incorporated into a clinical laboratory as an effective means for fluconazole susceptibility screening.

The aim of this report is to study the agreement between the broth microdilution NCCLS method and a 25-μg fluconazole disk diffusion test performed using RPMI 1640–2% glucose agar (RPMIG), Müeller-Hinton agar supplemented with 2% glucose and 0.5 μg of methylene blue (MHGM) per ml, and Shadomy medium (SHDM).

A total of 150 isolates of *Candida* spp. were collected from the clinical microbiology laboratory of Hospital Clínico Universitario Lozano Blesa (Zaragoza, Spain) and submitted for fluconazole susceptibility testing by microdilution and agar diffusion. The *Candida* species tested were 63 *C. albicans* isolates, 25 *C. dubliniensis* isolates, 25 *C. glabrata* isolates, 22 *C. parapsilosis* isolates, 10 *C. krusei* isolates, and 5 *C. tropicalis* isolates.

Fluconazole powder was obtained from Pfizer (Madrid, Spain), and 25-μg fluconazole disks were obtained from Mast Diagnostics. RPMI 1640 broth with L-glutamine was purchased from GIBCO BRL (Barcelona, Spain) and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) organic buffer. Agar formulations which we used for the disk diffusion test were RPMI 1640 with L-glutamine supplemented with 1.5% agar and 2% glucose from Sigma Chemical Company, which was then buffered with MOPS (RPMIG). Müeller-Hinton agar was purchased from bioMerieux (Marcy l'Etoile, France) and solidified after the addition of 0.5 μg of methylene blue per ml from Sigma Chemical Company and 2% glucose (MHGM). Shadomy medium was acquired from Rosco (Taastrup, Denmark) (SHDM). Isolates were stored at −80°C and were then subcultured onto a Sabouraud dextrose agar plate and then subcultured again to select isolated colonies (bioMerieux). Stock inoculum suspensions were prepared as described in the NCCLS document, and the turbidity of the suspension was adjusted to a 0.5 McFarland standard (1×10^6 to 5×10^6 CFU/ml). An inoculum concentration of 0.5×10^3 to 2.5×10^3 CFU/ml was used for testing and confirmed by quantitative subculture. Final fluconazole concentrations ranged from 0.125 μg/ml to 64 μg/ml. Microdilution trays were incubated in air at 35°C and read after 48 h of incubation. Ten-centimeter-diameter plates containing agar at a depth of 4.0

* Corresponding author. Mailing address: Servicio de Microbiologia, Hospital Clínico Universitario Lozano Blesa, Domingo Miral s/n, Zaragoza 50009, Spain. Phone: 34-976-556400, ext. 4322. Fax: 34-976-761693. E-mail: mcrubio@posta.unizar.es.

TABLE 1. Comparison between results of 24- and 48-h disk tests on RPMIG, MHGM, and SHDM agar plates and the NCCLS broth microdilution method for each species group

Species	No. of isolates tested	Category by microdilution	No. of isolates per category	No. of isolates tested by disk diffusion at 24 h/no. tested at 48 h								
				RPMIG			MHGM			SHDM		
				S	S-DD	R	S	S-DD	R	S	S-DD	R
C. albicans	63	S	62	62/34	0/0	0/28	62/62	0/0	0/0	62/58	0/0	0/4
		S-DD	1	0/0	1/0	0/1	0/0	1/0	0/1	0/0	0/0	1/1
		R	0									
C. dubliniensis	25	S	25	25/25	0/0	0/0	25/25	0/0	0/0	25/25	0/0	0/0
		S-DD	0									
		R	0									
C. glabrata	25	S	8	3/1	3/0	2/7	4/4	3/0	1/4	1/1	0/0	7/7
		S-DD	15	1/1	5/4	9/10	3/4	3/3	9/8	1/1	4/3	10/11
		R	2	0/0	0/0	2/2	0/0	0/0	2/2	0/0	0/0	2/2
C. parapsilosis	22	S	21	21/20	0/1	0/0	21/21	0/0	0/0	21/21	0/0	0/0
		S-DD	0									
		R	1	0/0	0/0	1/1	0/0	0/0	1/1	0/0	0/0	1/1
C. krusei	10	S	0	2/0	1/1	3/5	0/0	0/0	6/6	2/0	1/1	3/5
		S-DD	6	0/0	1/0	3/4	0/0	1/0	3/4	0/0	1/0	3/4
		R	4									
C. tropicalis	5	S	4	4/4	0/0	0/0	4/4	0/0	0/0	4/3	0/0	0/1
		S-DD	0									
		R	1	1/0	0/0	0/1	0/0	1/1	0/0	1/0	0/0	0/1
Total	150	S	120	115/84	3/1	2/35	116/116	3/0	1/4	113/108	0/0	7/12
		S-DD	22	3/1	7/5	12/16	3/4	4/3	15/15	3/1	5/4	14/17
		R	8	1/0	1/0	6/8	0/0	2/1	6/7	1/0	1/0	6/8

[a] RPMIG, RPMI–2% glucose; MHGM, Müeller-Hinton–2% glucose and 5 μg of methylene blue per ml; SHDM, Shadomy; S, susceptible (≤8 μg/ml or ≥19 mm); S-DD, susceptible-dose dependent (16 to 32 μg/ml or 15 to 18 mm); R, resistant (≥64 μg/ml or ≤14 mm).

mm were used for the agar diffusion test. The agar surface was inoculated in three directions by using a swab moistened in an inoculum suspension that was also adjusted to a 0.5 McFarland standard. One 25-μg fluconazole disk was applied to each inoculated plate. The plates were incubated in air at 35°C and read at 24 and 48 h after incubation. The MIC reading was defined as the lowest concentration inhibiting at least 50% of the growth. For the agar-based test, inhibitory zone diameters were measured at the transitional point at which there was a sharp decline in the amount of growth (approximately 80% inhibition).

Interpretive breakpoints used for fluconazole microdilution and the disk test were based on the NCCLS document (15; National Committee for Clinical Laboratory Standards, minutes from a meeting of the Subcommittee on Antifungal Susceptibility Tests, Tampa, Fla., 5 January 2002). Fluconazole interpretive criteria were defined as follows. For the susceptible category, a concentration of ≤8 μg/ml correlated with a ≥19-mm zone diameter; for the susceptible-dose-dependent category, a concentration of 16 to 32 μg/ml correlated with a 15- to 18-mm zone diameter; and for the resistant category, a concentration of ≥64 μg/ml correlated with a ≤14-mm zone diameter.

Major errors were defined as results in which the reference method result was susceptible and the agar medium method result was resistant, while very major errors were defined as results in which the reference method result was resistant and the disk diffusion method result was interpreted as susceptible. Minor errors were defined as variations in results from resistant to susceptible dose dependent or from susceptible dose dependent to susceptible between the two methods (1).

For quality control, *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were used as control strains. Our control MICs were within the limits proposed in the M27-A document (15). Disk tests yielded zones that fell into the following ranges for each control strain: *C. albicans* ATCC 90028, 28 to 39 mm; and *C. parapsilosis* ATCC 22019, 22 to 33 mm (NCCLS, minutes from a meeting of the Subcommittee on Antifungal Susceptibility Tests, Tampa, Fla., 5 January 2002). Disk test and microdilution NCCLS method results fell into these ranges for each of five determinations with each control strain.

Of the 150 *Candida* species MIC categories, 120 strains were susceptible, 22 were susceptible-dose dependents, and 8 were resistant. The fluconazole disk diffusion test categories on RPMIG, MHGM, and SHDM were affected by incubation time. The agreement between MIC categories and 24- and 48-h disk test categories for each species group and for each medium is shown in Table 1. Agreement between 48-h disk testing on RPMIG and SHDM media and MIC categories were inferior to that of 24-h disk testing. The MIC and disk test categories correlated better when susceptible and nonsusceptible categories were used rather than when susceptible, sus-

ceptible-dose-dependent, and resistant categories were considered. In a comparison of the MIC and disk diffusion test results using only the susceptible and nonsusceptible categories, a major agreement on MHGM was found at both 24 and 48 h (95.3 and 94.6%), followed by RPMIG (94 and 75.3%) and SHDM (92.6 and 91.3%).

When compared to the M27-A reference method, no very major errors were found with MHGM at 24 and 48 h, while RPMIG and SHDM exhibited one very major error at 24 h with one isolate of *C. tropicalis*. MHGH agar medium produced only one major error at 24 h with *C. glabrata*, and the very same major error occurred at 48 h with four other strains. SHDM exhibited 7 major errors at 24 h and 12 major errors at 48 h and included seven strains of *C. glabrata*, four strains of *C. albicans* (4), and one strain of *C. tropicalis* (1).

Minor errors were found on MHGM (five strains at 24 and 48 h) and four strains at 24 h and one strain at 48 h on RPMIG and SHDM.

The growth of microcolonies was found inside the inhibition zone in 64.7% of the strains on RPMIG and 63.4% of those on SHDM. However, this problem was infrequent and minimal on MHGM (8.7%), in which zone margins were clear and definite in 91.3% of the tests, thereby facilitating measurement of the zone sizes and minimizing subjectivity.

Regression analysis correlating 24-h zones of inhibition around 25-μg fluconazole disks on MHGM, RPMIG, and SHDM with 48-h MICs determined by the NCCLS microdilution method showed the following equations and correlation coefficients: $y = 37.21 - 2.91x, r = -0.7; y = 40.92 - 3.23x, r = -0.6$; and $y = 44.83 - 4.04x, r = -0.7$, respectively.

The ability of distinguishing fluconazole-susceptible from -nonsusceptible *Candida* strains by agar diffusion has also been investigated by Sandven et al. (22, 23), Barry and Brown (2), Kirkpatrick et al. (9), May et al. (13), and Kronvall and Karlsson (10). In this study, of the 120 susceptible strains determined by microdilution, 116 (96.7%) were also susceptible by the diffusion test on MHGM, 115 (95.8%) were susceptible on RPMIG, and 113 (94.2%) were susceptible on SHDM. Twenty-six (86.6%) of the 30 MIC nonsusceptible strains were detected by the disk test on RPMIG and SHDM, and 27 (90%) were detected on MHGM. The agreement was lower when all three categories (susceptible, susceptible-dose dependent, and resistant) were used to analyze the data as has been observed by others (2, 11, 14, 23).

The disk diffusion nonsusceptible strains (susceptible-dose dependent and resistant) can be tested by the MIC method if it is necessary to determine if a higher dose of fluconazole might elicit a favorable clinical response (2, 3, 11, 22).

C. albicans, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis* were the species for which the best agreement was achieved when all interpretive criteria were used. Major discrepancies, however, were found for *C. glabrata* and *C. krusei*. When only susceptible and nonsusceptible categories were considered, the agreement was better for all strains tested.

For species other than *C. albicans*, it may be prudent to routinely use a full 48-h incubation; this is particularly important for tests of *C. krusei*, in which resistance is not always seen after the first 24 h (2).

We have determined that the disk diffusion test for fluconazole offers several advantages. (i) It can be easily implemented in routine clinical microbiology laboratories. (ii) The disk diffusion procedure showed very good correlation with the microdilution NCCLS M27A method. MHGM provided the best correlation with the MICs followed by RPMIG and SHDM. (iii) MHGM medium appears to be a useful medium for disk diffusion testing because the minimal and infrequent growth of microcolonies inside the inhibition zone facilitates the measurement of the zone sizes and minimizes subjectivity. The one major disadvantage is that it did not adequately separate fully resistant strains from those with dose-dependent susceptibility. However, these nonsusceptible strains can be evaluated by a more quantitative procedure in order to separate susceptible-dose-dependent strains from those that are resistant. Overall, the 25-μg fluconazole disk diffusion test offers a more stable, less subjective, and less cumbersome alternative to microdilution susceptibility testing for yeasts.

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