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Comparative Evaluation of NCCLS M27-A and EUCAST Broth Microdilution Procedures for Antifungal Susceptibility Testing of Candida Species

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A two-laboratory study was performed to evaluate the correlation between the NCCLS M27-A and EUCAST microdilution procedures for antifungal testing of *Candida* spp. A panel of 109 bloodstream isolates was tested against amphotericin B, flucytosine, fluconazole, and itraconazole. Overall, the agreement was 92% and the intraclass correlation coefficient was 0.90 ($P < 0.05$).

The Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) has developed a proposed standard broth microdilution procedure for the determination of antifungal MICs of fermentative species of yeasts (5). This standard is based on the M27-A broth microdilution reference procedure of the National Committee for Clinical Laboratory Standards (NCCLS) (6) but incorporates some modifications in order to allow for automation of the susceptibility method and to permit the incubation period to be shortened from 48 to 24 h (4, 13). The modifications include the use of RPMI 1640 supplemented with 2% glucose as the test medium, an inoculum size of 0.5×10^5 to 2.5×10^5 CFU/ml, flat-bottomed trays, and spectrophotometric reading with a 50% inhibition endpoint for azole agents and flucytosine (5). A recent multicenter collaborative study has confirmed that the EUCAST procedure for antifungal susceptibility testing is a reproducible method, with an overall level of agreement between laboratories of 94% (M. Cuenca-Estrella, C. B. Moore, F. Barchiesi, J. Bille, E. Chryssanthou, D. W. Denning, J. P. Donnelly, F. Dromer, B. Dupont, J. H. Rex, M. D. Richardson, B. Sancak, P. E. Verweij, J. L. Rodríguez-Tudela and The AFST Subcommittee of European Committee on Antibiotic Susceptibility Testing, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. J569, 2001). In addition, a previous evaluation showed that MICs obtained by the EUCAST broth microdilution method are in close agreement with those obtained using the NCCLS reference procedure (4).

We performed a two-laboratory collaborative study to compare the susceptibility test results obtained by the NCCLS and EUCAST methodologies. We tested a panel of 109 bloodstream isolates of five different *Candida* spp. against amphotericin B, flucytosine, fluconazole, and itraconazole. The Centers for Disease Control and Prevention performed susceptibility testing as outlined in NCCLS document M27-A (6). The Spanish National Center for Microbiology determined MICs of antifungal agents using the AFST-EUCAST proposed standard procedure.

A total of 109 bloodstream isolates of *Candida* spp. (21 *Candida albicans* isolates, 19 *Candida tropicalis* isolates, 29 *Candida parapsilosis* isolates, 20 *Candida glabrata* isolates, and 20 *Candida krusei* isolates) were selected from a collection of strains obtained as part of a population-based active surveillance program in Baltimore, Md., and the state of Connecticut. Species identification was confirmed at the Centers for Disease Control and Prevention using standard methods, including API20C biochemical profiles, and morphological appearance on Dalmau cornmeal agar plates. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as quality control strains in each set of experiments (6).

The antifungal agents used in this study were the following: amphotericin B (Sigma Aldrich Química, Madrid, Spain, and Bristol Myers Squibb, Princeton N.J.), flucytosine (Sigma Aldrich Química, Madrid, Spain, and Hoffman La Roche, Nutley, N.J.), fluconazole (Pfizer, Madrid, Spain, and Pfizer Pharmaceuticals Group, Central Research Division, Groton, Conn.), and itraconazole (Janssen Pharmaceutica, Madrid, Spain, and Janssen Research Foundation, Beerse, Belgium).

NCCLS susceptibility testing procedure. Stock solutions were prepared in water (flucytosine and fluconazole) or dimethyl sulfoxide) (amphotericin B and itraconazole). Further dilutions of each antifungal agent were prepared with RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) which had been buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (Sigma), as outlined in document M27-A. The drug dilutions were dispensed into 96-well round-bottom microdilution plates that were sealed and stored frozen at −70°C until needed. The yeast inoculum was adjusted to a concentration of 0.5×10^3 to 2.5×10^3 CFU/ml (6), and an aliquot of 100 μl was

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TABLE 1. Antifungal susceptibilities of 109 isolates of *Candida* spp. as determined by two standardized broth microdilution procedures

Species (no. of isolates)	Method	MIC (μg/ml)							
		Amphotericin B		Flucytosine		Fluconazole		Itraconazole	
		Range	Geometric mean	Range	Geometric mean	Range	Geometric mean	Range	Geometric mean
C. albicans (21)	NCCLS	0.5–2	0.82	0.25–64	0.39	0.125–2	0.26	0.015–0.25	0.05
	EUCAST	0.125–0.5	0.42	0.25–16	0.33	0.125–4	0.25	0.015–0.06	0.03
C. tropicalis (19)	NCCLS	0.5–1	0.86	0.25–1	0.28	0.25–64	1.80	0.03–8	0.25
	EUCAST	0.125–0.5	0.39	0.25–0.5	0.25	0.125–64	0.34	0.015–4	0.04
C. parapsilosis (29)	NCCLS	0.125–1	0.46	<0.25–0.25	0.25	0.25–32	0.76	0.03–0.5	0.11
	EUCAST	0.125–0.5	0.32	<0.25–0.25	0.25	0.25–4	0.47	0.015–0.12	0.03
C. glabrata (20)	NCCLS	0.25–1	0.81	<0.25–0.25	0.25	1–64	9.80	0.125–2	0.61
	EUCAST	0.125–1	0.29	<0.25–0.25	0.25	1–64	4.70	0.06–0.5	0.17
C. krusei (20)	NCCLS	0.5–2	1.27	2–32	5.8	8–64	40.7	0.125–2	0.59
	EUCAST	0.25–1	0.59	2–8	3.5	8–64	26.9	0.06–0.5	0.19

added to each well of the microdilution plate. The plates were incubated at 35°C for 48 h.

EUCAST susceptibility testing procedure. Stock solutions were prepared in dimethyl sulfoxide, except flucytosine, which was dissolved in sterile distilled water (5). The assay medium was RPMI 1640 without sodium bicarbonate and with L-glutamine buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid and supplemented with 18 g of glucose per liter to reach a final concentration of 2% (RPMI–2% glucose; Oxoid S.A., Madrid, Spain). Culture medium was prepared as a double-strength solution and sterilized by filtration. Sterile plastic microtitration plates containing flat-bottomed wells were utilized (Corning Costar Europe, Badhoevedorp, The Netherlands). The plates contained 100 μl of twofold serial dilutions of the antifungal drugs per well. Two drug-free medium wells for sterility and growth controls were employed. The final inoculum suspension contained between 0.5×10^5 and 2.5×10^5 CFU/ml, and an aliquot of 100 μl was added to each well of the microdilution plate. The plates were incubated at 35°C for 48 h.

Endpoint determination: NCCLS procedure. Endpoints were determined by visual reading. The plates were agitated prior to reading to ensure that the contents were resuspended. The MICs of flucytosine, fluconazole, and itraconazole were determined according to a 0-to-4 scale (6). The MIC was defined as the lowest concentration of drug that produced a prominent decrease in turbidity compared with that of the drug-free control (score < 2). For amphotericin B, the MIC was defined as the lowest concentration of the drug that completely inhibited the growth of the strain (6).

Endpoint determination: EUCAST procedure. The optical density of each microplate well was measured after 24 h of incubation with a microplate spectrophotometer set at a wavelength of 530 nm (Dynatech, Cultek, Madrid Spain). For flucytosine and the azoles, the MIC endpoint was defined as the lowest drug concentration resulting in a reduction of growth of 50% or more compared with growth of the control. For amphotericin B, the endpoint was the lowest concentration that resulted in a reduction in growth of 90% or more compared with growth of the control.

Statistical analysis. The reproducibility of the results obtained by the two laboratories was evaluated by determining the percent agreement between MICs. Agreement was defined as discrepancies in MIC results of no more than two $\log_2$ dilutions. In addition, intraclass correlation coefficients (ICCs) were calculated. The ICC indicates the correlation between MICs, offering a measurement of statistical significance since it takes into account the number of cases and the absolute value of the MICs. The ICC is expressed based on a maximum value of 1, and a P value of <0.05 was considered significant. Both on-scale and off-scale results were included in the analysis. The off-scale MICs were converted to the next concentration up or down. All statistical analysis was done with the Statistical Package for the Social Sciences (SPSS, version 11.0) (SPSS S.L., Madrid, Spain).

Results and discussion. Table 1 summarizes the in vitro susceptibilities of the 109 isolates of *Candida* spp. to the four antifungal agents as determined by the two broth microdilution procedures. The data are presented as MIC ranges and geometric MICs obtained after the recommended incubation period for each procedure. A broad range of on-scale MICs was observed with each antifungal agent. In general, the MICs of each agent were typical for each species tested. The MICs for the two quality control isolates were within the accepted limits for both procedures on all occasions (3, 5).

Table 2 details the levels of agreement and ICCs between the two broth microdilution procedures for each *Candida* spp. and each antifungal agent when the MICs were determined after the recommended incubation period (NCCLS, 48 h; EUCAST, 24 h). Overall, levels of agreement were high, ranging from 80.7 to 100% by antifungal agent and 81.9 to 97.4% by organism. In general, the levels of agreement were similar for the different *Candida* spp. tested; however, lower levels of agreement were observed for *C. tropicalis* (Table 2). By anti-

TABLE 2. Agreement and ICC between NCCLS and EUCAST broth microdilution procedures

Species	% Agreement/ICC[b]			
	Amphotericin B	Flucytosine	Fluconazole	Itraconazole
C. albicans	94.5/0.92[a]	100/0.95[a]	93.9/0.93[a]	80.4/0.83[a]
C. tropicalis	93.1/0.91[a]	100/0.92[a]	88.5/0.86[a]	78.8/0.79[a]
C. parapsilosis	95.1/0.93[a]	100/0.94[a]	94.6/0.94[a]	80.7/0.82[a]
C. glabrata	95.3/0.94[a]	100/0.94[a]	93.8/0.93[a]	81.0/0.86[a]
C. krusei	94.5/0.94[a]	100/0.92[a]	92.7/0.92[a]	81.6/0.88[a]

[a] $P < 0.05$.
[b] The table shows coefficients obtained after the recommended incubation period (NCCLS, 48 h; EUCAST, 24 h).

fungal agent, the highest level of agreement between the two procedures was obtained with flucytosine and the lowest was obtained with itraconazole (80.7%). Table 2 also summarizes the ICCs and statistical significance for each antifungal agent and *Candida* species combination. Overall, the ICC was 0.90, a value that was statistically significant ($P < 0.05$).

The NCCLS M27-A reference procedure has proved to be a reliable and reproducible method for susceptibility testing of yeasts since its introduction in 1997 (6). However, despite the considerable effort that went into the development and evaluation of this procedure, the NCCLS method still has some unresolved limitations. These include the trailing growth phenomenon seen in tests with azole antifungal agents, the unreliable detection of resistance to amphotericin B, the subjective visual determination of MIC endpoints, and the need for an extended turnaround time to obtain the MIC (7, 8). Nevertheless, the NCCLS M27-A procedure is generally accepted as the reference method for antifungal susceptibility testing of yeasts and as the procedure with which any new methods in this field must be compared (12).

The EUCAST proposed standard procedure has been developed for the determination of antifungal drug MICs for fermentative species of yeasts by broth microdilution methodology (5). This method is based on the NCCLS procedure, and preliminary studies have demonstrated a high level of agreement between the results obtained with the two methods (4). The results of this two-laboratory collaborative study showed a high level of agreement between the NCCLS broth microdilution reference method and the EUCAST proposed standard procedure. The overall level of agreement was 92% when MICs obtained after recommended incubation periods (NCCLS, 48 h; EUCAST, 24 h) were compared. For most isolates included in the study, the difference in MICs between readings at 24 and 48 h was minimal. However, some strains showed lower rates of agreement, particularly for fluconazole and itraconazole MICs. Four clinical isolates (two of *C. tropicalis* and two of *C. glabrata*) were categorized as fluconazole susceptible using EUCAST methodology and as fluconazole resistant by the NCCLS procedure (11). In addition, 11 strains (five *C. tropicalis* strains, four *C. glabrata* strains, and two *C. krusei* strains) were categorized as itraconazole susceptible by the EUCAST procedure and itraconazole resistant or susceptible, depending on dose, by the NCCLS method. Some of these strains *(C. tropicalis* isolates) exhibited significant trailing growth, such that the MICs after 48 h were markedly higher than after 24 h. It should be noted that the recommended incubation time is 24 h for the EUCAST method and 48 h for the NCCLS procedure. It has previously been estimated that about 5% of *C. albicans* isolates display trailing growth when tested against fluconazole (2). More recently, Arthington-Skaggs et al. (1) found that 16 to 18% of *C. albicans* bloodstream isolates showed trailing in tests with fluconazole and itraconazole while 30 to 59% of *C. tropicalis* isolates displayed this phenomenon. However, both this in vitro study and two in vivo investigations that employed murine models of disseminated candidiasis have shown that isolates exhibiting this phenomenon should be categorized as susceptible rather than resistant (2, 10, 14). This concept has been supported by a clinical demonstration that oropharyngeal candidiasis due to strains exhibiting significant trailing growth responds to low doses of fluconazole, similar to those used to treat infections due to typical susceptible isolates (9). It is apparent that the trailing phenomenon can be an important potential source of inaccuracy in MIC determination after 48 h of incubation. A recent study that used the M27-A methodology suggests that MIC results determined by visual reading after 24 h or by spectrophotometric reading after 48 h may be more predictive of in vivo outcome for isolates that give unclear visual endpoints at 48 h due to trailing growth (1).

In conclusion, this study has demonstrated that antifungal susceptibility results obtained by using the AFST-EUCAST proposed standard reference procedure are in close agreement with those achieved by the NCCLS M27-A procedure. In addition, the proposed EUCAST standard has the advantage of reducing the incubation time needed to determine the MIC.

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