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Collaborative Evaluation in Seven Laboratories of a Standardized Micromethod for Yeast Susceptibility Testing

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The new micromethod for yeast susceptibility testing, MYCOTOTAL, was evaluated with 10 reference strains in seven laboratories. Ready-to-use microtitration plates and the same synthetic medium were used with two dilutions of imidazoles, flucytosine, and amphotericin B, permitting the categorization of each strain as susceptible, intermediate, or resistant. The results were compared with the MIC for each reference strain, and the repeatability and reproducibility were evaluated. The yeasts tested presenting different patterns of susceptibilities in reference MICs included six strains of *Candida albicans*, two strains of *Candida tropicalis*, one strain of *Candida parapsilosis*, and one strain of *Torulopsis glabrata*. For 4,200 antifungal agent-yeast results, the repeatability was 99.3% and the reproducibility was 96.3%. The correlation between the reference MICs and the category results was 91.5% for seven laboratories (and 92.7% for six laboratories excluding the laboratory which did not follow exactly the same protocol). We observed only 7.9% minor discrepancies, 0.5% (0.29% for six laboratories) major discrepancies, and 0.1% uninterpretable results. The percentages of concording results were similar for each strain and each antifungal agent tested. The overall results indicated that MYCOTOTAL was a reliable and reproducible method, well correlated with reference MICs. This ready-to-use micromethod with the same medium for all antifungal agents would be an important step in the necessary standardization of yeast susceptibility testing.

It is widely accepted that infections caused by fungi have increased in terms of annual morbidity and mortality, mainly owing to the increased use of more effective cytotoxic and antibacterial agents. A variety of antifungal agents are now available for use in parental therapy of fungal infections, including the polyene antibiotic amphotericin B, the synthetic antimetabolite flucytosine (5-FC), and the synthetic imidazoles-triazoles (2, 9, 21, 29, 31, 39, 46). Unfortunately, resistance of yeasts to antifungal agents has become evident (10, 13, 17, 18, 20, 23, 37). For all these reasons, the clinical laboratory now is assuming a greater role in the selection and monitoring of antifungal chemotherapy.

Despite many efforts, in vitro antifungal susceptibility testing remains problematic (8, 16, 25, 26, 32, 39, 42, 47). In vitro test procedures with antifungal agents are similar in design to those with antibacterial agents, namely, serial dilutions and agar diffusion tests (39). Numerous factors may influence the results including the organism being tested, the inoculum size, conditions of incubation, test format, and composition of test medium. Several problems depend on the antifungal agent to be tested: amphotericin B is light sensitive and water insoluble, 5-FC must be tested in synthetic media free of antagonistic substances, and imidazoles are highly subject to considerable variations in MICs owing to inoculum and method of testing. In addition, azole antifungal in vitro testing results are poorly correlated with in vivo drug effects, especially for ketoconazole and fluconazole (2, 21, 22, 29, 30, 34, 35, 38, 40, 43). Recently, an improved method has been proposed based on azole-antibiotic interactions (28). Unfortunately, the method did not work routinely when tested with a larger number of *Candida* strains and particularly with non-*Candida albicans* strains (36). Results of a survey of antifungal susceptibility tests in the United States indicated a poor interlaboratory precision of broth dilution testing of 5-FC and amphotericin B (5). For amphotericin B, values varied 8- to 32-fold, and for 5-FC they varied 32- to >512-fold. Disparate results have also been reported by similar working groups in other countries (41); thus, improved standardization of fungal susceptibility tests is necessary before the results can be applied to a clinical situation.

Recently, ready-to-use micromethods have been described for yeast susceptibility testing with the same synthetic medium used for all antifungal agents (R. Guinet, IXth ISHAM Congress, Atlanta, Ga. 1985 [11]). Comparing MIC determinations with classical media, the method was reproducible, repeatable, sensitive, and in concordance with in vivo results for amphotericin B, 5-FC, and the imidazoles. The susceptibility of 1,850 yeast strains of medical importance was determined either with MICs or with two concentrations of each antifungal agent, assessing the value of the method in routine susceptibility testing (12). As this method is now commercially available (MYCOTOTAL; Behring Diagnostic, Rueil-Malmaison, France), the aim of this collaborative study was to evaluate the repeatability and reproducibility of this micromethod in seven different laboratories with 10 identical reference strains. For 4,200 antifungal agent-yeast results, the repeatability was 99.3%, the repro-

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TABLE 1. MICs for reference strains[a]

Strain	MIC (μg/ml)						
	Amphotericin B	5-FC	Miconazole	Econazole	Ketoconazole	Tioconazole	Clotrimazole
C. albicans							
A311[b]	0.78	>100	<0.10	<0.10	<0.10	<0.10	<0.10
B792[b]	0.39	>100	<0.10	<0.10	<0.10	<0.10	<0.10
DAR[c]	0.39	>100	12.5	6.25	6.25	3.12	0.39
GSR[c]	0.39	25	<0.10	<0.10	<0.10	<0.10	<0.10
ATCC 24433	0.20	>100	<0.10	<0.10	<0.10	<0.10	<0.10
ATCC 2091	0.78	3.12	<0.10	0.20	<0.10	<0.10	0.20
C. tropicalis							
CBS 94	0.78	0.78	0.20	0.20	<0.10	<0.10	0.20
CIM 104	1.56	>100	0.78	0.78	0.78	0.39	0.78
C. parapsilosis CBS 604	0.39	>100	1.56	6.25	0.20	0.78	0.39
T. glabrata CBS 138	0.39	0.78	0.78	0.78	0.78	0.20	0.78

[a] CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; CIM, Centre d'Immunochemie Microbienne, Institut Pasteur, Lyon, France.
[b] From National Institutes of Health, Bethesda, Md.
[c] From F. Riley (37).

ducibility was 96.3%, and the correlation between the reference MIC and the categorized results was 91.5% for all seven laboratories.

MATERIALS AND METHODS

The yeast strains were chosen with regard to both their importance in human pathology and differences in their susceptibility patterns. The strains tested included eight reference strains and two wild strains isolated from patients with chronic mucocutaneous candidiasis (kindly provided by F. Riley [37]). These strains were transmitted freeze-dried and with a code number to the seven laboratories. The origin of the strains and the reference MIC determined by the standardized micromethod are summarized in Table 1.

The reference MIC for each strain was determined earlier as previously described (R. Guinet, IXth ISHAM Congress, Atlanta, Ga., 1985 [11]). Briefly, microdilution plates were prepared with 11 dilutions of each antifungal agent ranging from 100 to 0.1 μg/ml, and one well for a control received the solvent. The yeast suspension was prepared with fresh cultures in the MYCOTOTAL synthetic medium supplemented with the vitamin solution (Behring Diagnostic), maintained liquid at 45°C in a water bath, and adjusted under the light microscope to contain 10^5 to 10^6 CFU/ml. The composition of the chemically defined MYCOTOTAL medium (pH 7) was as follows: asparagine, 2 g; ammonium sulfate, 3 g; D-glucose, 30 g; L-histidine monochloride, 10 mg; DL-methionine, 20 mg; DL-tryptophan, 20 mg; ferric chloride, 1 mg; manganese sulfate, 1 mg; zinc sulfate, 1 mg; biotin, 20 μg; calcium pantothenate, 2,000 μg; folic acid, 2 μg; inositol, 10,000 μg; *p*-aminobenzoïc acid, 200 μg; pyridoxine hydrochloride, 400 μg; riboflavin, 200 μg; thiamine hydrochloride, 400 μg; Berthelot solution of oligoelements, 10 drops; Bacto-Agar (Difco Laboratories, Detroit, Mich.), 15 g; distilled water, 1 liter. The MIC corresponded to the lowest concentration of the antifungal agent totally inhibiting yeast growth after 48 h of incubation at 28°C.

In this study, for each susceptibility test the yeasts were suspended (10^5 to 10^6 CFU/ml) in the MYCOTOTAL synthetic medium supplemented with the vitamin solution and maintained liquid at 45°C in a water bath as recommended by the manufacturer. Then a microdilution strip (two rows of eight wells each) containing the ready-to-use antifungal agents was inoculated with 100 μl in each well. After solidification, the microdilution strip was incubated at 28°C for 48 h with humidity. The growth in each well was read with the naked eye, and inhibition corresponded to a complete absence of growth. Each antifungal agent was tested at two concentrations, permitting the results to be categorized as susceptible, intermediate, resistant, or uninterpretable (Table 2).

Each reference strain was tested in each laboratory in triplicate the same day for repeatability, and this was done three times at different days for reproducibility. Thus, a total of nine determinations was realized for each strain in each of the seven laboratories involved in the collaborative evaluation. Each determination was compared with the reference MIC. Major discrepancies were defined as results that were sensitive by one method and resistant by the other, and minor discrepancies were defined as variations from resistant to intermediate or intermediate to sensitive between the two methods.

RESULTS

For 4,200 antifungal agent-yeast combinations, the repeatability was 99.3%, with four laboratories showing a perfect 100% score (Table 3). The reproducibility ranged from 92 to 99.5%, with a 96.3% average for the seven laboratories. When calculated for six laboratories, since laboratory 3 in Table 3 did not strictly follow the protocol, the reproducibility was 96.6%. The agreement of the results with the reference MICs was 91.5% (92.7% for six laboratories), with only 0.5% major discrepancies (0.29% for six laboratories)

TABLE 2. Interpretation of susceptibility testing with two concentrations

Antifungal agent	Concn (μg/ml)		Result[a]		Intrepretation
	High	Low	High	Low	
Control	0	0	+	+	Correct
Amphotericin B	4	1	−	−	Susceptible
5-FC	32	1	+	+	Resistant
Miconazole	8	1	−	+	Intermediate
Econazole	8	1	−	+	Intermediate
Ketoconazole	8	1	−	−	Susceptible
Tioconazole	8	1	−	−	Susceptible
Clotrimazole	8	1	+	−	Uninterpretable

[a] +, Growth; −, inhibition of growth.

TABLE 3. Repeatability, reproducibility, and concordance for 4,200 yeast-antifungal agent results in seven laboratories

Laboratory	%					
	Repeatability	Reproducibility	Concordance	Minor discrepancies	Major discrepancies	Uninterpretable
1	100	97.8	89.2	10.3	0.5	0
2	100	99.5	92.4	7.6	0	0
3	100	94.2	81	16.7	2.3	0
4	100	97.6	97.2	2.8	0	0
5	99.4	97.5	96	2.85	0.5	0.65
6	96.3	92	88.2	11.1	0.7	0
7	99.8	95.1	93.2	6.8	0	0
Total for seven laboratories	99.3	96.3	91.5	7.9	0.5	0.1
Total for six laboratories[a]	99.2	96.6	92.7	6.9	0.29	0.11

[a] There were 3,780 yeast-antifungal agent results for the six laboratories; laboratory 3 did not strictly follow the protocol.

and three uninterpretable results appearing on the same microplate (Table 3).

The concordance between MYCOTOTAL and the MICs for each strain ranged from 76.4 to 97% (Table 4), with no major discrepancies for four strains, 1.9% major discrepancies for *Candida tropicalis* CBS 94, and 22.9% minor discrepancies for *C. albicans* DAR (from Riley [37]). The concordance between MYCOTOTAL and MICs for each antifungal agent ranged from 86% for econazole and amphotericin B to 97% for ketoconazole (Table 5). Major discrepancies did not exceed 0.8%, and the most important minor discrepancies were observed for econazole, 5-FC, and amphotericin B (Table 5). Most of the discrepancies observed were for strains found resistant by MYCOTOTAL and susceptible by MIC test, since of 20 major discrepancies, 19 were strains found resistant with MYCOTOTAL and susceptible in MIC tests. For minor discrepancies, 80.5% were strains found intermediate or resistant by MYCOTOTAL and susceptible or intermediate, respectively, by MIC tests. Finally, the detailed analyses of minor discrepancies for each strain and each antifungal agent are presented in Table 6 and show that only five antifungal agent-yeast pairs had discrepancies over 5%, namely, *C. tropicalis* CBS 94 with econazole (5.1%), *C. tropicalis* CIM 104 with miconazole (6.9%) and econazole (6.9%) and *C. albicans* DAR with amphotericin B (9%) and econazole (11.7%).

TABLE 4. Concordance between MYCOTOTAL and MICs for each strain

Strain	%			
	Concordance	Minor discrepancies	Major discrepancies	Uninterpretable
C. albicans				
A311	96.2	3.8	0	0
B792	96.7	3.1	0	0.2
DAR	76.4	22.9	0.7	0
GSR	95	5	0	0
ATCC 24433	97	2.8	0.2	0
ATCC 2091	96	3.5	0.5	0
C. tropicalis				
CBS 94	87.2	10.9	1.9	0
CIM 104	86	14	0	0
C. parapsilosis CBS 604	92.6	6.2	0.6	0.6
T. glabrata GBS 138	92	7.3	0.7	0

DISCUSSION

This report represents the first multicenter study on the standardization of sensitivity testing of yeasts against 5-FC, amphotericin B, and imidazoles with ready-to-use microdilution plates containing antifungal agents and the same agar medium. The protocol for the evaluation was distributed, along with the 10 reference yeasts and the MYCOTOTAL kits for susceptibility testing, to seven laboratories. As in a previous study (5), participants were asked to test each strain-drug combination in triplicate and on 3 different days, but in contrast to Calhoun and Galgiani (5), we took into account all 4,200 results obtained. Despite this difference, the MYCOTOTAL micromethod was found to be very reliable since the repeatability was very close to 100% and the reproducibility was excellent, ranging from 92 to 99.5%. Thus, these results indicated that the method is well standardized and confirmed that it does not depend on inoculum variations, even with imidazoles (12).

The design of MYCOTOTAL was based on principles widely used in antimicrobial susceptibility testing (44), such as the determination of a category result (i.e., susceptible, intermediate, or resistant) with two critical concentrations of each antifungal agent being tested for each isolate. These two critical concentrations were chosen according to serum levels (1, 3, 4, 7, 8, 14, 15, 19, 21) achieved with normal and maximum dosages of systemic drugs (e.g., amphotericin B, 5-FC, miconazole, and ketoconazole), and under these conditions, it was possible to determine the category of the results. However, in some circumstances, the use of only two concentrations to determine the category of the result

TABLE 5. Concordance between MYCOTOTAL and MICs for each antifungal agent

Antifungal agent	%			
	Concordance	Minor discrepancies	Major discrepancies	Uninterpretable
Amphotericin B	86.2	13	0.8	0
5-FC	88.4	11.59	0.01	0
Miconazole	92.2	7.1	0.7	0
Econazole	86	13.3	0.7	0
Ketoconazole	97	3	0	0
Tioconazole	95	4.3	0.5	0.2
Clotrimazole	96.1	2.9	0.5	0.5

TABLE 6. Minor discrepancies for each strain and each antifungal agent (total, 100%)

Antifungal agent	% Discrepancy									
	C. tropicalis CBS 94	*C. albicans* A311	*C. tropicalis* CIM 104	*C. albicans* B792	*C. parapsilosis* CBS 604	*C. albicans* DAR	*C. albicans* GSR	*C. albicans* ATCC 24433	*T. glabrata* CBS 138	*C. albicans* ATCC 2091
Amphotericin B	3	0.3	2.1	0.3	0.6	9	2.7	2.1	1.2	2.1
5-FC		4.5	1.2	3.6	3	1.2	2.1	1.5	1.2	2.4
Miconazole	3.9		6.9		1.2		0.6		0.3	
Econazole	5.1		6.9			11.7			0.3	
Ketoconazole	1.8						0.9		3	
Tioconazole					2.7	4.2			0.9	
Clotrimazole						2.7			2.4	

might be insufficient and the clinical susceptibility also must be interpreted in terms of site of infection since some drugs diffuse poorly in the central nervous system and others can concentrate in urine. Furthermore, for nonsystemic antifungal agents, the category result is very pessimistic since higher local concentrations of antifungal agents are usually obtained. Nevertheless, all the category results were compared with the MICs for the reference strains, giving an overall agreement of 91.5% for seven laboratories, which should be regarded as a highly acceptable performance for a ready-to-use micromethod. Indeed, it should be remembered that the finite limit of complete agreement when any reference method is compared with itself has been estimated to be 92% (45). Furthermore, the highest levels of minor discrepancies were obtained with strain-antifungal agent combinations (Table 6) corresponding to MICs very near the critical concentrations used in MYCOTOTAL (Table 1), and this could correspond to differences no greater than one dilution in the MIC determination. Again, this confirms the good reproducibility of the micromethod since for *C. tropicalis* CIM 104 and *Torulopsis glabrata* CBS 138, the MICs of five antifungal agents were very near the critical concentrations and the agreements were still 86 and 92%.

The difficulties of determining reliable susceptibility data in traditional in vitro tests have been extensively reported (8, 9, 24, 25, 32, 39, 42, 47), and only limited success in interlaboratory and intralaboratory standardization has been achieved (5, 8, 26, 33, 41). The main reasons for variations in data include subjective difficulties in interpretation of cultures in which fungal growth is partially inhibited and objective variations in MIC with composition and pH of culture media, size of fungal inoculum, and the time at which the tests are read (6, 8, 16, 24–26, 39, 42, 47). The collaborative study reported here confirms that the MYCOTOTAL micromethod is very easy to handle and to read with clear endpoints of growth. Furthermore, MYCOTOTAL is very reproducible, insensitive to inoculum effect, and independent of the incubation time since no difference in susceptibility was observed after 2 or 3 days of incubation (data not shown). The method was also proved to be valuable for susceptibility testing of *Cryptococcus neoformans* and *Aspergillus* sp. (data not shown). This collaborative study demonstrates that the introduction of improved methodology for susceptibility testing of fungi can give consistent results allowing interlaboratory comparisons at a level never reached in the past. Furthermore, some evidence of correlations between susceptibility results with MYCOTOTAL and drug effects in vivo was previously shown (R. Guinet, IXth ISHAM Congress, Atlanta, Ga., 1985), and results obtained with strains isolated from patients with mucocutaneous candidiasis were in agreement either with minimum effective doses in vaginal and systemic experimental candidiasis for ketoconazole (34, 37) and ICI 153,066 or with the relative inhibition factors determined by Odds et al. (27, 31). In addition, the recent study of Guinet (12) concerning the comparative susceptibility of 1,850 yeast strains belonging to eight species by the standardized micromethods showed good concordance with the in vivo efficacy of antifungal agents. Variations in the susceptibility pattern were observed with yeast species and among *C. albicans* and *T. glabrata* strains, although none were resistant to amphotericin B and only 6% were resistant to 5-FC. *C. albicans* was highly susceptible to imidazoles (0.8 to 2.5% resistant strains), whereas *T. glabrata* showed much higher resistance rates (18% for tioconazole and 70% for ketoconazole). Such results correlate well with the effects of the drugs in vivo (14, 30, 40), and the relative resistance of *T. glabrata* infection to treatment with imidazoles is now widely recognized (9, 10, 23, 38).

In conclusion, the collaborative evaluation reported here indicates that the micromethod MYCOTOTAL constitutes a major improvement in yeast susceptibility testing. This standardized micromethod can be used in routine work even by unexperienced personnel since it is ready to use, insensitive to inoculum effect, easy to handle, and utilizes the same synthetic medium for all antifungal agents. We believe that MYCOTOTAL will be of considerable help in clarifying the confusing area of antifungal susceptibility testing. Despite these excellent results, the manufacturer must follow the evolution of antifungal drugs, and the most recent triazoles (fluconazole and itraconazole) should be introduced in the susceptibility test (2, 22, 29). The introduction of a liquid dilution system could also improve the method, providing automatization and fungicidal testing as well as inhibitory testing. Finally, the actual indications of the MYCOTOTAL susceptibility test can be used for all yeast isolates from deep mycoses and for all non-*C. albicans* isolates from superficial mycoses, but for *C. albicans* isolates from nonrecurrent superficial mycoses, the test is not useful owing to the great susceptibility in vitro (12) and in vivo to various imidazoles.

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