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Collaborative Investigation of Variables in Susceptibility Testing of Yeasts

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A multicenter study was performed to evaluate the effect of medium, incubation time (24 and 48 h), and temperature (30 and 35°C) on intra- and interlaboratory variations in MICs of flucytosine, amphotericin B, and ketoconazole for yeasts. Testing was performed on coded isolates of *Candida* species (11 strains) and *Cryptococcus neoformans* (2 strains) by using a standard macrodilution protocol in 11 laboratories. Four chemically defined media buffered to pH 7.0 with morpholinepropanesulfonic acid were evaluated, including buffered yeast nitrogen base, synthetic amino acid medium-fungal, RPMI 1640 medium, and high-resolution antifungal assay medium. Intralaboratory variability was less than or equal to fourfold for 97% of the replicate sets of data. The highest level of interlaboratory agreement, irrespective of antifungal agent or incubation conditions, was observed with RPMI 1640 medium. Interlaboratory variability was less than or equal to fourfold for 93% of the determinations with ketoconazole and 100% with flucytosine tested in RPMI 1640 medium at 35°C for 24 h. Variability in amphotericin B results was less than or equal to fourfold for 81% of the determinations in RPMI 1640 medium at 35°C for 48 h. The rank order of MICs within each antifungal test group was similar among the various laboratories and was generally in agreement with the reference rank order regardless of the test medium that we used.

Two previous multicenter studies have documented unacceptable variations in broth dilution MICs obtained for different yeast isolates tested in different laboratories (3, 9). In other studies, variations in key technical steps such as inoculum preparation, medium composition, pH, length of incubation, and method of endpoint reading have been cited as reasons for variabilities of test results (1, 2, 6–8, 10, 11, 15, 17, 19–21, 28, 29). One of the test variables, inoculum preparation, was studied in a multicenter evaluation conducted by the National Committee for Clinical Laboratory Standards Subcommittee on Antifungal Susceptibility Testing (21). As a result of that study, the spectrophotometric method was recommended as the standardized method of choice for inoculum preparation for susceptibility testing of yeasts. It has been suggested that analysis and standardization of other test conditions might increase the reproducibility of the broth dilution method for fungal susceptibility testing (6–8, 17, 29).

The present collaborative study was performed to evaluate the effect of medium, incubation time (24 versus 48 h), and temperature (30 versus 35°C) on intra- and interlaboratory variations of MICs of currently licensed antifungal agents (amphotericin B, flucytosine [5-FC], and ketoconazole) for yeasts by a broth macrodilution method. The goals of this study were to (i) determine the ability of four chemically defined media to support the growth of clinically significant yeasts, (ii) examine the reproducibility of MIC results within laboratories, (iii) assess the ability of the various media and incubation conditions to define a rank order of susceptibilities relative to results obtained with an arbitrarily chosen reference method, and (iv) examine the extent of agreement among laboratories for each antifungal agent and set of test conditions. We elected to evaluate only chemically defined media because such media allow analysis of potential in vitro antagonism previously identified with an undefined medium, such as that of pyrimidine analogs with 5-FC (22, 23) and sterols with amphotericin B (13). Furthermore, factors in complex, undefined medium also have been postulated to antagonize the in vitro activity of azoles (14, 19, 20).

MATERIALS AND METHODS

Antifungal agents. Three antifungal agents were used in this study: amphotericin B (E. R. Squibb & Sons, Princeton, N.J.), 5-FC (Hoffmann-La Roche Laboratories, Nutley, N.J.), and ketoconazole (Janssen Pharmaceutica, Inc., Piscataway, N.J.). The antifungal agents were obtained from the manufacturers as standard powers, each from a single lot, and distributed to the participating laboratories. Upon receipt, concentrated stock solutions (amphotericin B, 5,000 μg/ml; 5-FC, 10,000 μg/ml; ketoconazole, 5,000 μg/ml) of

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TABLE 1. Study isolates and median MICs obtained in each test medium

Antifungal agent (organism)	ATCC[a] strain no.	Study strain no.	Median MIC (μg/ml) in each test medium[b]			
			BYNB	SAAMF	RPMI 1640	HR
Amphotericin B						
Candida albicans	64546	R-716	0.5	0.7	0.18	0.5
Candida albicans	64548	R-717	0.5	1.7	0.25	0.5
Candida lusitaniae	64125	R-727	1.0	0.35	0.35	0.7
Candida albicans	64552	R-718	1.0	2.8	1.4	1.0
5-FC						
Candida albicans	64551	R-719	0.5	0.7	0.5	0.35
Candida albicans	64544	R-720	11.3	8.0	4.0	4.0
Cryptococcus neoformans		87-29[c]	16	16	4.0	16
Candida albicans	64553	R-721	>64	>64	>64	>64
Candida albicans	64545	R-722	>64	>64	>64	>64
Cryptococcus neoformans		87-108[c]	>64	>64	>64	>64
Ketoconazole						
Candida albicans	64547	R-723	0.35	>2.0	2.0	2.0
Candida albicans	64546	R-728	0.09	>2.0	0.25	0.18
Candida albicans	64549	R-724	0.18	>2.0	0.5	0.25
Candida albicans	64550	R-725	1.4	0.5	1.0	2.0

[a] ATCC, American Type Culture Collection (Rockville, Md.).
[b] Median MICs for all tests performed on each isolate by all laboratories.
[c] Reference MICs were determined by the broth macrodilution method.

each antifungal agent were prepared in dimethyl sulfoxide and frozen at −60°C until they were used.

Test organisms. Four strains of *Candida* species were selected for use with each antifungal agent to obtain a wide range of susceptibility results, as determined previously by using an arbitrarily selected reference method (9–11, 16, 26, 27, 30). In addition, two clinical isolates of *Cryptococcus neoformans* from patients with meningitis were included for testing against 5-FC (Table 1). These isolates were selected for inclusion in the study based on widely different susceptibilities to 5-FC, as determined by broth macrodilution testing (15).

Media. Only chemically defined media that were previously used for antifungal testing were used in this study. Yeast nitrogen base medium (BYNB; Difco Laboratories, Detroit, Mich.) and RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) were obtained from their respective manufacturers. Synthetic amino acid medium-fungal (SAAMF) was obtained from Paul D. Hoeprich, University of California Davis Medical Center, Sacramento, and high-resolution antifungal assay medium (HR) was obtained from Peter F. Troke, Pfizer Central Research, Kent, United Kingdom. A single lot of each medium was distributed among the participating laboratories, along with detailed preparation instructions. All media were buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma).

Although this study focused on the performance of buffered synthetic media, comparison of the ability to support the growth of *Candida* species and *C. neoformans* was made with two nonsynthetic media, yeast extract peptone dextrose medium (Difco) and Sabouraud dextrose medium (Difco), in one of the participating laboratories. Sidearm flasks containing 50 ml of each test medium were inoculated with 10^6 CFU of *Candida albicans* (University of Iowa strain 320-40), *Candida tropicalis* (University of Iowa strain 153-30), or *C. neoformans* (strain 87-29) per ml. Duplicate sets of flasks were incubated at 30 and 35°C. The doubling time (in minutes) of each isolate for each medium and incubation temperature was determined by measuring the optical density (530 nm) of the organism suspension at 30-min intervals for a total of 6 to 8 h (12).

Susceptibility testing procedure. Detailed instructions for performing twofold dilutions, inoculum preparation, and endpoint determination were provided for each laboratory. Broth macrodilution testing was performed with twofold drug dilutions in all test media. Stock solutions of antifungal agents were diluted by previously described methods recommended for minimizing systematic pipetting errors (18). Final drug concentrations were 0.06 to 4 μg/ml for amphotericin B, 0.12 to 64 μg/ml for 5-FC, and 0.004 to 2 μg/ml for ketoconazole.

Yeast inocula were prepared as described previously (21). Briefly, yeasts were grown on Sabouraud dextrose agar for 48 h at 30°C, and the inoculum suspension was prepared by picking five colonies of at least 1 mm in diameter and suspending the material in 5 ml of sterile 0.85% saline. The turbidity of the cell suspension measured at an optical density of 530 nm was adjusted to match that of a commercial barium sulfate turbidity standard (0.5 McFarland; Difco) by using sterile saline as needed. The saline suspension then was diluted 1:100 with the desired test medium. By this procedure, inocula for all isolates were $(3.2 \pm 1.8) \times 10^4$ yeasts per ml, as judged by enumeration of CFU obtained by subculture on Sabouraud dextrose agar by each collaborating laboratory.

Yeast inocula (0.9 ml) were added to polystyrene plastic tubes (12 by 75 mm; Falcon 2054, Becton Dickinson, Lincoln Park, N.J.) containing one of the antifungal agents (0.1 ml) by using sterile, individual 1-ml pipettes. The contents of the tubes were mixed by inversion. Replicate tests were incubated in air at 30 and 35°C, and the loosely capped tubes were inspected 24 and 48 h later. Drug-free and yeast-free controls were included.

To assess drug activity more precisely, growth in each tube was scored and recorded as follows: 0, optically clear; 1+, slightly hazy; 2+, prominent reduction in turbidity compared with that of the drug-free control; 3+, slight reduction in turbidity compared with that of the drug-free control; 4+, no reduction in turbidity compared with that of

TABLE 2. Requirements for agreement with reference rank order

Antifungal test group	Relative MICs for each test isolate[a]
Amphotericin B	R-716 ≦ R-717 ≦ R-727 ≦ R-718
5-FC	R-719 ≦ R-720 ≦ 87-29 ≦ R-721 ≦ R-722 = 87-108
Ketoconazole	R-723 ≦ R-728 ≦ R-724 ≦ R-725

[a] The data set was unrankable if three of the four isolates in the amphotericin B or ketoconazole group or four of the six isolates in the 5-FC group had identical MICs.

the drug-free control. Optimal agreement resulted when the endpoint for amphotericin B and 5-FC was defined as the lowest concentration in which the growth score was 0 (optically clear) and when the endpoint for ketoconazole was defined as the lowest concentration in which the growth score was no more than 1+. These criteria were used to define the MIC.

Study design and analysis of results. Eleven laboratories (referred to as laboratories A through K) participated in the study. All trials by participating laboratories were blinded by using coded test isolates. Laboratories A, B, and C performed all aspects of the study and evaluated the effects of the various media and incubation conditions on the susceptibilities of all three groups of test organisms to their appropriate antifungal agents. The remaining laboratories (D through K) evaluated the same media and incubation conditions but were assigned only one of the three antifungal agents and the corresponding group of test organisms, as follows: amphotericin B group, laboratories D and E; 5-FC group, laboratories F, G, and H; and ketoconazole group, laboratories I, J, and K. Each laboratory received triplicate subcultures of the assigned test isolates, each of which was identified only by a coded number. The accompanying instructions indicated which isolates were to be tested in each of the four test media and two incubation temperatures on a given day against the assigned antifungal agent. Intralaboratory variation was assessed by testing each isolate under all conditions on each of 3 separate days. Results were recorded on data sheets supplied to each laboratory and were submitted to a coordinating laboratory, where they were decoded for analysis.

The median value from each laboratory for each isolate and each set of test conditions was determined from the replicate MICs. These median values were used to examine agreement among laboratories and to determine the rank order of isolates within each antifungal test group for each laboratory and set of test conditions. For most isolates, analysis was restricted to MICs for which endpoints were obtained. However, because two isolates in the 5-FC group (R-722 and 87-108; Table 1) were selected to be off-scale resistant (>64 μg/ml), MICs of >64 μg/ml obtained with these isolates were included and assigned an endpoint value of 128 μg/ml. Isolates in each antifungal susceptibility test group (four isolates each for amphotericin B and ketoconazole and six isolates for 5-FC; Tables 1 and 2) tested under a single set of medium and incubation conditions were ranked according to their relative susceptibilities (low to high) to the test agent, and the resulting rank order was compared with that established by the reference method in previous reports (9, 16, 26, 27, 30). For candidal isolates, the reference rank order also was supported by previously published in vivo correlations (16, 26, 27, 30). The data set was classified as unrankable if three of the four isolates in the amphotericin B or ketoconazole group or four of the six isolates in the 5-FC group had identical MICs. For the amphotericin B group, agreement with the reference rank order required that the MIC for isolate R-716 be the lowest and that the MIC for R-718 be the highest for the four test strains (Table 2). The MIC for isolate R-717 was required to be greater than or equal to that for R-716 and less than or equal to that for R-727. For 5-FC, the MIC for isolate R-719 was required to be the lowest and the MICs for isolates R-722 and 87-108 were required to be the highest of those for the six test strains (Table 2). The MIC for isolate R-720 was required to be greater than or equal to that for R-719 and less than or equal to the MIC for strain 87-29. Similarly, the MIC for isolate 87-29 was required to be less than or equal to that for R-721. For the ketoconazole group, the MIC for isolate R-723 was required to be the lowest and that for R-725 was required to be the highest for the four test isolates (Table 2). The MIC for isolate R-728 was required to be greater than or equal to that for R-723 and less than or equal to that for R-724. Any deviation from these guidelines resulted in classification of the data set as not being in agreement with the reference rank order.

TABLE 3. Ability of test media to support the growth of *Candida* species and *C. neoformans*

Test organism	Incubation temp (°C)	Doubling time (min) in each medium[a]					
		BYNB	SAAMF	RPMI 1640	HR	Sab	YPD
Candida albicans	30	96	129	129	104	NT	79
	35	105	86	97	78	NT	57
Candida tropicalis	30	115	101	130	106	NT	67
	35	69	71	75	59	NT	38
Cryptococcus neoformans	30	164	207	202	159	167	176
	35	189	163	165	142	141	118

[a] Abbreviations: BYNB, buffered yeast nitrogen base; SAAMF, synthetic amino acid medium-fungal; RPMI, RPMI 1640 medium; HR, high-resolution antifungal assay medium; Sab, Sabouraud dextrose medium; YPD, yeast extract peptone dextrose medium; NT, not tested.

The data were analyzed by analysis of variance applied to the arcsine transformation of the raw proportions. Differences in agreement of susceptibility testing results under the various testing conditions within each laboratory and among the different laboratories were assessed by the Tukey Studentized range test. Alpha values of less than 0.05 were considered significant (4, 25).

RESULTS

Growth studies. Overall, the growth rates of the test isolates were similar in each of the defined media and slower than those observed in the undefined media (Sabouraud dextrose agar and yeast extract peptone dextrose medium) (Table 3). *C. neoformans* grew more slowly than *C. albicans* or *C. tropicalis* did in each medium. The doubling times of *Candida* species and *C. neoformans* were more rapid at an incubation temperature of 35°C than they were at 30°C in each medium except BYNB. Of the defined media, the most rapid growth at 35°C was observed with HR.

Variability of replicates within laboratories. Differences among replicates tested under each of the various medium and incubation conditions within the same laboratory were used to estimate intralaboratory variability when endpoints for at least two of the three replicate determinations could be defined. Overall, 897 (70%) of 1,280 replicate sets were

TABLE 4. Reproducibility of MIC results within each participating laboratory[a]

Laboratory	% of replicate tests with endpoint readings within fourfold for each antifungal agent		
	Amphotericin B	5-FC	Ketoconazole
A	100	98	96
B	99	99	99
C	100	93	100
D	100		
E	100		
F		91	
G		98	
H		99	
I			97
J			90
K			93
Overall	99.9	96	96

[a] Values are for 897 replicate sets of MICs with at least two endpoint determinations (total of 2,509 datum points). The overall reproducibility for all laboratories and all test conditions was 97%.

TABLE 6. Interlaboratory agreement of MIC results stratified by antifungal agent, incubation conditions, and test medium

Antifungal agent (no. of participating laboratories)	Incubation temp (°C)/ time (h)	% of endpoint readings that varied fourfold or less in each medium			
		BYNB	SAAMF	RPMI 1640	HR
Amphotericin B (5)	30/24	79	60	85	85
	30/48	67	69	73	85
	35/24	75	60	69	79
	35/48	76	67	81	79
5-FC (6)	30/24	96	90	88	93
	30/48	91	92	89	93
	35/24	93	97	100	93
	35/48	96	92	92	92
Ketoconazole (6)	30/24	53	61	93	94
	30/48	88	71	83	75
	35/24	69	65	93	81
	35/48	91	89	93	58

evaluable and contained 2,509 datum points, of which 2,437 (97%) were within fourfold of other values in their set (Table 4). For amphotericin B, 235 (73%) of 320 sets were evaluable and contained 673 datum points. Of these, 99.9% were within fourfold of other values in their set. For 5-FC, 436 (76%) of 576 sets were evaluable and contained 1,235 datum points. Of these, 96% were within fourfold of the other values in their set. For ketoconazole, 226 (59%) of 384 sets were evaluable and contained 601 datum points, of which 96% were within fourfold of other values in their set. In greater than 90% of the replicate sets with either 5-FC or ketoconazole, the failure to obtain an endpoint was due to failure to detect drug activity. In contrast, apparent resistance to amphotericin B was responsible for only 55% of the offscale results.

Length of incubation. A comparison of MIC results obtained after 24 h of incubation versus those obtained after 48 h of incubation at 30 and 35°C in the four different media is given in Table 5. For both amphotericin B and 5-FC, fewer than 12% of results varied more than fourfold between 24 and 48 h with any medium at either temperature. However, with ketoconazole, fourfold variability was much more frequent. The best agreement was found with SAAMF at 30°C and BYNB and RPMI 1640 medium at 35°C, with which fourfold agreement was 100, 73, and 71%, respectively. In all cases the differences were due to an increase in MICs following 48 h of incubation.

Variability among laboratories. The levels of interlaboratory agreement of MIC results stratified by antifungal agent, incubation conditions, and test medium are given in Table 6.

TABLE 5. Agreement in MICs between 24 and 48 h of incubation

Medium	% of MICs that varied fourfold or less					
	Amphotericin B		5-FC		Ketoconazole	
	30°C	35°C	30°C	35°C	30°C	35°C
BYNB	94	93	91	92	55	73
SAAMF	92	100	96	96	100	44
RPMI 1640	100	100	93	92	40	71
HR	100	100	88	88	58	67

For amphotericin B, the highest level of agreement was observed with RPMI 1640 medium at 30°C for 24 h of incubation (85%) or HR at 30°C for 24 or 48 h of incubation (85%) followed closely by RPMI 1640 medium at 35°C for 48 h of incubation (81%). Excellent agreement among laboratories was observed with 5-FC, regardless of the medium or incubation conditions used; however, the highest level of agreement was observed with RPMI 1640 medium at 35°C for 24 h of incubation (100%). For ketoconazole, the highest level of agreement was observed with HR at 30°C for 24 h (94%), followed closely by RPMI 1640 medium at 35°C for 24 or 48 h of incubation (93%) or 30°C for 24 h of incubation (93%). Of the three antifungal agents tested, the agreement among laboratories was significantly higher with 5-FC than it was with either amphotericin B or ketoconazole ($P < 0.05$). Overall, the highest level of agreement among laboratories, regardless of antifungal agent, incubation time, or temperature, was observed with RPMI 1640 medium (87%) followed by HR (84%), BYNB (81%), and SAAMF (76%). Of the four media evaluated for interlaboratory agreement, only the comparison between RPMI 1640 medium and SAAMF was significantly different ($P < 0.05$).

Relative susceptibilities of isolates. To assess the degree to which laboratories identified a similar relative susceptibility pattern among isolates, we compared the rank order of susceptibility within each group of antifungal agents, as determined by each laboratory under the various testing conditions, with the reference rank order. The rank order of susceptibility within each test group, as determined by the median MIC for all tests in all laboratories, was similar for all medium-antifungal combinations with the exception of SAAMF-amphotericin B and SAAMF-ketoconazole (Table 1). The isolate that was most susceptible to ketoconazole (R-723) by the reference method was misclassified in all of the test media by all laboratories and was excluded from all subsequent analyses. Overall, 272 sets (1,280 datum points) of antifungal test group data were reported, and in 218 sets (80%) a rank order of MICs could be determined. The rank order was in agreement with the reference rank (Table 2) in 160 (73%) of the 218 ranked sets of data.

The agreement of susceptibility test results with the reference rank order for each antifungal agent under the various test conditions is shown in Table 7. The ranking of isolates

TABLE 7. Agreement of susceptibility test results with the reference rank order

Antifungal agent	Medium	% of data sets with reference rank order					
		30°C		35°C		Both temp	
		24 h	48 h	24 h	48 h	24 h	48 h
Amphotericin B	BYNB	20	20	80[a]	80[a]	50	50
	SAAMF	100	40	80	40	90	40
	RPMI 1640	80	100	80	80	80	80
	HR	60	60	60	100	60	60
	All	65	55	75	75	70	65
5-FC	BYNB	83	17	100	33	92	25
	SAAMF	100	33	100	33	100	33
	RPMI 1640	100	67	100	33	100	50
	HR	100	50	83	50	92	50
	All	96[b]	42	96[b]	38	96[b]	40
Ketoconazole[c]	BYNB	83	50	67	50	75	50
	SAAMF	33	0	33	0	33	0
	RPMI 1640	67	17	67	17	67	17
	HR	67	33	67	17	67	25
	All	63	25	58[b]	21	60[b]	23
Overall	BYNB	65	29	88	53	76	41
	SAAMF	76	24	71	24	74	24
	RPMI 1640	82	59	82	41	82[d]	50
	HR	76	47	71	53	74	50
	All	75[b]	40	78[b]	43	76[b]	41

[a] Significantly higher than that obtained at 30°C ($P < 0.05$).
[b] Significantly higher than that obtained at 48 h ($P < 0.05$).
[c] Excluding isolate R-723.
[d] Not significantly different from that obtained with BYNB, SAAMF, or HR ($P > 0.05$).

was similar to the reference rank order for each antifungal agent with the majority of incubation conditions and media that were used. The incubation temperature had little effect on the relative susceptibilities of the test isolates to the three antifungal agents, with the exception of amphotericin B when tested in BYNB, for which a significantly higher proportion of the data sets were in agreement with the reference rank order at 35 than that at 30°C ($P < 0.05$). The duration of incubation had little effect on the relative susceptibilities of the isolates to amphotericin B, regardless of the medium that was used; however, a significant effect was observed for 5-FC and ketoconazole in all media. The reference rank order was observed in 96% of all data sets at 24 h and in only 40% at 48 h for 5-FC ($P < 0.05$) and in 60% (24 h) versus 23% (48 h) of all data sets for ketoconazole ($P < 0.05$). In each case deviation from the reference rank order was due to a shift to a higher MIC following prolonged (48 h) incubation. Overall, 68 sets of data (320 datum points) were submitted for testing with each medium, and of these, the reference rank order was most commonly observed with RPMI 1640 medium (82% of all sets at 24 h of incubation); however, this was not significantly different from that observed with the other test media ($P > 0.05$).

DISCUSSION

An essential requirement of a medium selected for inclusion in an antimicrobial susceptibility testing method is that it support adequate growth of test organisms. Each of the four chemically defined media evaluated in this study supported adequate growth of *Candida* species, although growth was slower than that in undefined medium (yeast extract peptone dextrose medium; Table 3). Although *C. neoformans* grew more slowly than *Candida* species did in all of the defined media, five of the six laboratories in the 5-FC test group were able to distinguish accurately between the two strains in the test panel with regard to their susceptibility to 5-FC (Table 1). Additional testing with a larger panel of *C. neoformans* isolates will be needed to assess adequately the suitability of these media for susceptibility testing of this relatively fastidious organism.

Better growth was observed at 35 than at 30°C in all of the test media except BYNB (Table 3). This was in agreement with the previous observations of Radetsky et al. (24), who observed superior growth of *Candida* species in both RPMI 1640 medium and Casamino Acids medium (Difco) at 35 versus 30°C, and suggests that if other factors are equal, 35°C may be the preferred incubation temperature for antifungal susceptibility testing.

The intralaboratory reproducibility of MICs of each antifungal agent was >95%, regardless of media formulation or incubation conditions (Table 4). These findings are in agreement with those of recent studies (3, 5, 9) and support the suggestion by Galgiani et al. (9) that little of the variation in results among laboratories can be attributed to intralaboratory error.

The duration of incubation has been shown to be an important variable in antifungal susceptibility testing (1, 5, 11, 20, 29, 30). Although we observed very little effect of incubation time on the MICs of amphotericin B, fourfold or greater increases in MICs from observations at 24 to 48 h were more frequent with 5-FC and were common with ketoconazole (Table 5). Furthermore, we also found that the duration of incubation had significant effects on the relative ranking of susceptibilities of the test isolates to 5-FC and ketoconazole in each of the four test media (Table 7). A significantly higher level of agreement with the reference rank order of susceptibilities was observed following 24 h, as opposed to 48 h, of incubation for both 5-FC and ketoconazole. By comparison, the rank order of amphotericin B susceptibilities was unaffected by duration of incubation and was generally in agreement with the reference rank order. In agreement with our data, Doern et al. (5) reported significantly higher MICs for 5-FC, ketoconazole, and miconazole, but not amphotericin B, following incubation for 48 versus 24 h. Those investigators suggested that 48 h of incubation is preferable to 24 h for clinical use in order to avoid reporting falsely susceptible results for 5-FC and the azoles. On the basis of our findings, we suggest that the converse is true and that 24 h may be the optimal preferred incubation time for these two antifungal agents. Although this conclusion is further supported by currently available relationships of the reference rank order to in vivo correlation (16, 26, 27, 30), additional clinical data will be needed to resolve this question.

The level of interlaboratory agreement of MIC results was improved considerably in the present study over that observed in the earlier studies of Calhoun et al. (3) and Galgiani et al. (9). This was particularly evident for 5-FC (Table 6). Whereas Calhoun et al. (3) and Galgiani et al. (9) observed MIC variations of several thousand-fold for 5-FC among laboratories, we found that agreement among laboratories was significantly higher for 5-FC than for either amphotericin B or ketoconazole and that 88 to 100% of 5-FC MICs were within two doubling dilutions of each other, depending on the medium and incubation conditions that were used. Among laboratories, the level of agreement observed with amphotericin B and ketoconazole was more dependent on

the choice of medium; the highest agreement was observed with RPMI 1640 medium and HR (Table 6). As in previous studies, susceptibility testing with ketoconazole was less reproducible; this was due, in part, to the well-recognized problem of incomplete inhibition of growth (trailing endpoints) over a wide range of concentrations (19, 20).

Overall, the highest level of interlaboratory agreement, irrespective of antifungal agent or incubation conditions, was observed with RPMI 1640 medium; however, this was not significantly different from that observed with either BYNB or HR. The use of RPMI 1640 medium for antifungal susceptibility testing was previously recommended by Radetsky et al. (24), who noted that it was readily available, economical, quality controlled, and chemically defined and supported the growth of a wide range of *Candida* species. Similarly, we found that RPMI 1640 medium could be used for susceptibility testing of amphotericin B, 5-FC, and ketoconazole and that the incubation conditions providing the best growth, most accurate rank order, and best agreement among laboratories was 35°C at 24 h for 5-FC and ketoconazole and 35°C at 48 h for amphotericin B.

In agreement with the earlier studies of Calhoun et al. (3) and Galgiani et al. (9), we found that the rank order of MICs within each antifungal agent test group was similar among the various laboratories and was generally in agreement with the reference rank order, regardless of the test medium that was used (Table 7). A notable exception to the otherwise excellent agreement of test results with the reference rank order was the misclassification of isolate R-723 (Table 1). This isolate, which was the most susceptible to ketoconazole by the reference method, was misclassified as either the most resistant or second most resistant by every laboratory, regardless of media or incubation conditions. Of interest is the fact that this isolate was also misclassified as resistant in a previous study (9). The reasons for these disagreements are unclear and merit further study.

Our data, which were obtained by a single test method in all 11 laboratories, compared favorably with those obtained in the study of Galgiani et al. (9), in which a total of eight different nonstandardized methods were used by three laboratories. Using the same panel of test isolates, Galgiani et al. (9) defined a rank order of MICs in only 46% of the sets of data, whereas in the present study we defined a rank order in 80% of the sets and results were in agreement with the reference rank order for 73% of the sets. The only significant effect of incubation conditions on the relative susceptibilities of the test isolates was the negative effect of prolonged incubation (48 h) on the rank order of isolates in the 5-FC and ketoconazole test groups (Table 7). The choice of test medium did not significantly influence the relative susceptibilities of the isolates to the three antifungal agents; however, the reference rank order was most commonly observed with RPMI 1640 medium.

In conclusion, the results of this multicenter investigation indicate that broth macrodilution antifungal susceptibility testing, when performed according to the protocol used in this study, provides excellent intralaboratory reproducibility, improved interlaboratory agreement, and uniform ranking of test isolates according to their relative MICs of amphotericin B, 5-FC, and ketoconazole. Furthermore, 35°C appears to be a more optimal incubation temperature, and incubation periods longer than 24 h may result in less discriminating test results for both ketoconazole and 5-FC. Our results do not indicate a clear-cut choice of medium for antifungal susceptibility testing but, rather, suggest that the use of RPMI 1640 medium, HR, or BYNB may produce satisfactory results. Nevertheless, based on the overall agreement among laboratories and agreement with the reference rank order, it appeared that RPMI 1640 medium performed slightly better than either HR or BYNB did. Given these results, it would seem reasonable to use RPMI 1640 medium as the test medium, 35°C as the incubation temperature, and 24 h (5-FC and azoles) or 48 h (amphotericin B) as the duration of incubation for subsequent studies designed to examine additional variables such as inoculum size and performance of this broth macrodilution method with a larger number of yeast isolates.

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