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Interlaboratory Comparison of Results of Susceptibility Testing with Caspofungin against *Candida* and *Aspergillus* Species

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Seventeen laboratories participated in a study of interlaboratory reproducibility with caspofungin microdilution susceptibility testing against panels comprising 30 isolates of *Candida* spp. and 20 isolates of *Aspergillus* spp. The laboratories used materials supplied from a single source to determine the influence of growth medium (RPMI 1640 with or without glucose additions and antibiotic medium 3 [AM3]), the same incubation times (24 h and 48 h), and the same end point definition (partial or complete inhibition of growth) for the MIC of caspofungin. All tests were run in duplicate, and end points were determined both spectrophotometrically and visually. The results from almost all of the laboratories for quality control and reference *Candida* and *Aspergillus* isolates tested with fluconazole and itraconazole matched the NCCLS published values. However, considerable interlaboratory variability was seen in the results of the caspofungin tests. For *Candida* spp. the most consistent MIC data were generated with visual "prominent growth reduction" (MIC_2) end points measured at 24 h in RPMI 1640, where 73.3% of results for the 30 isolates tested fell within a mode ± one dilution range across all 17 laboratories. MIC_2 at 24 h in RPMI 1640 or AM3 also gave the best interlaboratory separation of *Candida* isolates of known high and low susceptibility to caspofungin. Reproducibility of MIC data was problematic for caspofungin tests with *Aspergillus* spp. under all conditions, but the minimal effective concentration end point, defined as the lowest caspofungin concentration yielding conspicuously aberrant hyphal growth, gave excellent reproducibility for data from 14 of the 17 participating laboratories.

Caspofungin acetate (Cancidas, formerly MK-0991 and L-743872; Merck & Co., Inc.) is an echinocandin-class antifungal agent, recently approved in the United States and Europe for the treatment of invasive aspergillosis that is refractory to other antifungal treatments, esophageal *Candida* infections and invasive *Candida* infections (11). Caspofungin inhibits synthesis of β-1,3-D-glucan in fungal cell walls, a property that results in fungicidal effects against *Candida* species, in which this polysaccharide is vital to cell wall integrity (8).

Susceptibility tests of caspofungin against *Candida* species have been done according to the National Committee for Clinical Laboratory Standards (NCCLS) method M27-A (18) or its

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recent second edition M27-A2 (19), the reference protocol for testing azoles, flucytosine, and polyene antifungal agents against yeasts. Quality control (QC) limits have been established for caspofungin in M27-A tests with reference isolates of *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 (3). A European reference method (EUCAST) for antifungal susceptibility testing with *Candida* spp. gives results that closely match those obtained by method M27 for azole antifungals (6). The EUCAST method is very similar to M27-A2 but uses a higher yeast inoculum, a shorter incubation time, flat-bottom microdilution plates, and spectrophotometric end point determinations (6).

Published caspofungin MICs for *Candida* species suggest interlaboratory variation in the ranges of caspofungin MICs determined by NCCLS method M27-A. For example, whereas some authors found caspofungin MIC ranges against isolates of *C. albicans* from 0.03 to 1 μg/ml (12, 14, 16, 21, 23, 26), other researchers have reported higher ranges of 0.25 to 4 μg/ml (5, 9, 15). For tests with *C. tropicalis*, the highest caspofungin MIC from some laboratories (14, 16) was at or below the lowest MIC reported from others (5, 9, 15, 21). Differences of this order may represent intrinsic differences in the susceptibility of the panels of isolates tested, or they may indicate interlaboratory disparities in susceptibility tests with caspofungin.

For *Aspergillus* species, agreement in published MICs is unimpressive. For filamentous fungi, the NCCLS reference method is M38-A, approved for tests with azoles, flucytosine, and polyenes (17). For 26 *A. fumigatus* isolates, Arikan et al. reported geometric mean MICs of caspofungin of 0.43 μg/ml after 24 h of incubation and of >16 μg/ml after 72 h of incubation (1). By comparison, 72-h geometric mean MICs versus *A. fumigatus* were determined to be 2.15 μg/ml by Espinel-Ingroff (9) by using method M38-A and ≤0.09 μg/ml by Del Poeta et al. (7), who used a tube dilution method. Pfaller et al. (22) reported 72-h caspofungin MICs of 0.06 and 0.12 μg/ml for 12 isolates of *A. fumigatus*. In all of these studies the growth medium used was RPMI 1640 buffered with morpholinepropanesulfonic acid according to NCCLS M38-A recommendations (17). The minimum effective concentration (MEC), defined as the lowest caspofungin concentration that results in growth of *A. fumigatus* producing conspicuously aberrant hyphae, has been found to generate more consistent susceptibility data than the MIC (1, 9, 10).

Alterations in the growth medium, pH, incubation temperature, and inoculum size were found to cause caspofungin MIC variations in microdilution tests (4) but not in macrodilution tests (12). Most investigators have found that caspofungin MICs against all fungal types are lower when determined in Antibiotic Medium 3 (AM3) than in RPMI 1640 medium (2, 4, 20, 23).

To provide a prospective evaluation of interlaboratory agreement with caspofungin susceptibility tests, 17 laboratories collaborated to test 30 isolates of *Candida* spp. and 20 isolates of *Aspergillus* spp. under a variety of different conditions. The laboratories were located in the United States, Canada, Europe, and Australia. The test plates and growth media were supplied from a central source. The MIC and MEC end points were determined by visual inspection, and MICs were additionally determined from spectrophotometric data.

MATERIALS AND METHODS

Fungi. A panel of 30 isolates of *Candida* spp. and 20 *Aspergillus* spp. (Table 1) was assembled by Merck Research Laboratories (Rahway, N.J.). A blinded code number was assigned to each isolate, and the panels of fungi were distributed to the 17 participating laboratories. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included among the 30 yeast isolates as NCCLS-recommended QC strains for antifungal susceptibility testing by method M27-A2 (19). *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were included among the 20 mould isolates as reference strains for testing by method M38-A (17). The panel of yeast isolates also contained two strains known to be heterozygous for a mutation in the *FKS1* gene, the caspofungin target, and six strains known to be homozygous for mutations in *FKS1* (8). All eight of these yeasts have reduced susceptibility to caspofungin in vitro; the two heterozygous mutants less so than the six homozygous mutants.

For all isolates, inocula of yeasts and *Aspergillus* conidia were prepared by standard methods (17, 19) and diluted appropriately to the specified initial concentrations. The growth media and 96-well microdilution plates containing antifungal agents were prepared by a commercial supplier (Trek Diagnostic Systems, Inc., Cleveland, Ohio). The media used in the tests were morpholinepropanesulfonic acid-buffered RPMI 1640 (RPMI) (17, 19), RPMI 1640 with a 2% glucose concentration instead of the usual 0.2% (RP-G), and AM3.

Experimental design. For the 30 *Candida* spp. isolates, duplicate plates were set up with caspofungin dilutions from 32 to 0.032 μg/ml tested in RPMI and RP-G with starting yeast concentrations of 1 to 5×10^3 CFU/ml and in RP-G with a starting yeast concentration of 1×10^5 to 5×10^5 CFU/ml. For tests in AM3, the caspofungin dilution range was 8 to 0.008 μg/ml, and the initial yeast concentration was 10^3 CFU/ml. As a reference standard, fluconazole dilutions from 64 to 0.063 μg/ml were tested in RPMI with an initial yeast concentration of 10^3 CFU/ml (19).

Inoculated microplates were incubated at 35°C in a humid environment. After 24 h the plates were carefully shaken to distribute yeast growth evenly, and the MICs of caspofungin for *Candida* spp. were recorded according to two visual criteria: MIC_0, the lowest caspofungin concentration that supported no visible growth (a clear well), and MIC_2, the lowest caspofungin concentration that caused a significant diminution of growth below control growth levels (19). Each lab then used a microplate spectrophotometer to determine the turbidity (optical density [OD]) of each well at 530 to 500 nm. For each of the three growth media tested, one caspofungin plate was set up with uninoculated medium for determination of the mean background OD values. These values were subtracted from the experimental OD values, and two MIC end points were determined from the spectrophotometric data: IC_{50}, the lowest caspofungin concentration that reduced turbidity to <50% of the mean control turbidity, and IC_{90}, the lowest caspofungin concentration that reduced turbidity to <90% of the control.

For the 20 *Aspergillus* spp. isolates, duplicate microdilution plates contained a caspofungin concentration series from 16 to 0.016 μg/ml, tested in RPMI, RP-G, and AM3, all inoculated to an initial concentration of 1×10^4 to 5×10^4 CFU/ml as recommended by the NCCLS for mold testing (17). Itraconazole concentrations from 8 to 0.008 μg/ml were also tested as a reference controls. Visual and spectrophotometric MIC and IC readings were made as for the *Candida* spp., except that the plates were not shaken before they were read. In addition, the caspofungin MEC was recorded at 24 h; this concentration was defined by reference to a photograph as the lowest that led to the growth of small, rounded, compact colonies compared to the florid hyphal growth seen in controls.

After 48 h of incubation, the MIC_0, MIC_2, IC_{90}, and IC_{50} values were read for all fungi in the same way as at 24 h. After 48 h, MIC_2 was read for fluconazole, and the MIC_0 was read for itraconazole. The protocol also called for measurement of minimum fungicidal concentrations of caspofungin against *Candida* spp. at 48 h, but a high level of protocol violation in this test led to its being discounted for further analysis.

Analysis of data. All visually read susceptibility end points were typed into standardized result spreadsheets, and all spectrophotometric data were pasted into a standardized analysis template distributed to all participants. MIC and IC values that were off-scale were recorded as the lowest test concentration when all wells showed growth inhibition and as the next-highest concentration above the highest in a series when no well reached the end point being tested.

For each test condition the minimum, maximum, and modal MIC or IC value was determined. For each isolate tested, the number and percentage of results within ±1 dilution of the modal MIC or IC value was determined. "Excellent agreement" was defined as ≥90% of results or more within the mode ± one dilution range. "Moderate agreement" was defined as ≥80% of results within the mode ± one dilution range.

TABLE 1. Details of the panel of fungi tested in this study[a]

Isolate	Species	Description	Isolate	Species	Description
Y101	C. dubliniensis	Rex collection 34-504-032	A201	A. flavus	Nares isolate from Houston, Tex.
Y102	C. albicans	Biopsy sample from Colombia	A202	A. flavus	Sputum sample from Tucson, Ariz.
Y103	C. albicans	Merck collection MY1055	A203	A. flavus	Bone fragment from Brazil
Y104	C. albicans	CAI-4 (8)	A204	A. flavus	Sputum sample from Houston, Tex.
Y105*	C. albicans	CAI-4-R1 (8)	A205	A. fumigatus	Sputum sample from Belgium
Y106*	C. albicans	NR4 (8)	A206	A. fumigatus	BAL sample from Lexington, Ky.
Y107*	C. albicans	NR3 (8)	A207	A. fumigatus	Sputum sample from France
Y108*	C. albicans	T25 (8)	A208	A. fumigatus	Sputum sample from Rochester, N.Y.
Y109*	C. albicans	Merck collection strain 212	A209	A. fumigatus	Tracheal secretions sample from France
Y110	C. albicans	Rex collection 34-032-001	A210	A. fumigatus	Rinaldi collection 02-1835
Y111*	C. albicans	Blood isolate from Montreal, Quebec, Canada	A211	A. niger	Sputum sample from Rochester, Minn.
Y112*	C. albicans	Tissue isolate from Montreal, Quebec, Canada	A212	A. niger	BAL sample from Rochester, Minn.
Y113	C. glabrata	Blood isolate from Australia	A213	A. niger	BAL sample from Lexington, Ky.
Y114	C. glabrata	Blood isolate from Chile	A214	A. nidulans	Perlin collection CLF12
Y115	C. guilliermondii	Blood isolate from Belgium	A215	A. nidulans	Perlin collection CLF14
Y116	C. guilliermondii	Blood isolate from Russia	A216	A. terreus	Lung (post mortem) sample from Tucson, Ariz.
Y117	C. krusei	Blood isolate from Buffalo, N.Y.	A217	A. terreus	Left breast sample from Australia
Y118*	C. krusei	Stool isolate from Germany	A218	A. terreus	Left toe sample from Australia
Y119	C. parapsilosis	Blood isolate from Houston, Tex.	A219	A. fumigatus	ATCC 204305 (NCCLS reference isolate)
Y120	C. parapsilosis	Blood isolate from Belgium	A220	A. flavus	ATCC 204304 (NCCLS reference isolate)
Y121	C. parapsilosis	Biopsy isolate from Peru			
Y122	C. parapsilosis	Blood isolate from Brazil			
Y123	C. Iusitaniae				
Y124	C. tropicalis	Blood isolate from Canada			
Y125	C. tropicalis	Blood isolate from Brazil			
Y126	C. rugosa	Blood isolate from Canada			
Y127	C. lipolytica	Oropharyngeal swab from Peru			
Y128	C. kefyr	Biopsy isolate from Guatemala			
Y129	C. krusei	ATCC 6258 (NCCLS QC isolate)			
Y130	C. parapsilosis	ATCC 22019 (NCCLS QC isolate)			

[a] Yeast isolates of known low susceptibility to caspofungin are indicated by an asterisk.

RESULTS

Data returns, technical problems, and protocol violations. All 17 participating laboratories, designated A through Q here, returned data spreadsheets. The visually read MICs were complete according to protocol for all 17 laboratories, with the sole exception of laboratory P, which provided no caspofungin MEC readings for molds tested in RP-G and AM3. For the spectrophotometric data, the results from laboratory K contained many omissions and inexplicable inaccuracies and were rejected from analysis. Laboratory F provided spectrophotometric results only for one of each of the duplicate plates set up with *Candida* spp. and no spectrophotometric data for the *Aspergillus* spp.

Two complaints of technical problems arose commonly among the participants. One was of yeast isolates where growth was inhibited in wells in the central portion of the dilution series but returned at the two or three highest concentrations (spectrophotometric data verified that regrowth at the highest test concentrations occurred often for some of the isolates tested [see below]). The second was of the appearance of air bubbles in the plates, which hampered the reading of end points, particularly by spectrophotometry. In some laborato-

ries that experienced air bubble formation in the plates the plates were centrifuged to disperse bubbles before they were read. Where participants felt unable to judge a visual MIC end point (commonly because of inadequate control growth), the result was recorded as a blank for purposes of analysis.

Intralaboratory data agreement. With very few exceptions, the results showed excellent agreement for duplicate results obtained within each laboratory. For the 30 yeast isolates across 17 laboratories, 1.8 to 3.6% of the results showed intralaboratory differences beyond one twofold dilution, depending on the test conditions; these percentages were calculated from >1,000 data per test condition. For the 20 mold isolates across the 17 laboratories, 0.0 to 5.1% of results showed intralaboratory differences beyond one twofold dilution.

Fluconazole MICs for *Candida* spp. For QC strain *C. krusei* ATCC 6258, the current fluconazole QC limits according to the NCCLS are 16 to 128 μg/ml (19). From the 17 participating laboratories, the fluconazole modal MIC for this strain was 32 μg/ml, and all laboratories reported this value or 16 or 64 μg/ml, which is in perfect agreement with the reference MIC range. For QC strain *C. parapsilosis* ATCC 22019 the reference fluconazole MIC range is 1.0 to 4.0 μg/ml (19). In the present

TABLE 2. Interlaboratory agreement for caspofungin susceptibility data with 30 isolates of *Candida* spp.[a]

Test conditions	Endpoint	Visual readings		Spectrophotometer results	
		Agreement range (%)	Excellent agreement (%)	Agreement range (%)	Excellent agreement (%)
RPMI, 24 h	MIC_0 or IC_{90}	37.5–100.0	33.3	36.6–93.3	3.3
	MIC_2 or IC_{50}	64.7–100.0	73.3	26.6–100.0	53.3
RPMI, 48 h	MIC_0 or IC_{90}	44.1–100.0	36.7	33.3–86.7	0.0
	MIC_2 or IC_{50}	26.4–100.0	43.3	40.0–96.7	20.0
RP-G, 10^3 yeasts/ml, 24 h	MIC_0 or IC_{90}	29.4–94.1	20.0	33.3–100.0	16.7
	MIC_2 or IC_{50}	53.1–97.0	50.0	40.0–100.0	53.3
RP-G, 10^3 yeasts/ml, 48 h	MIC_0 or IC_{90}	41.1–94.1	40.0	26.6–90.0	10.0
	MIC_2 or IC_{50}	38.2–100.0	26.7	36.6–96.7	36.7
RP-G, 10^5 yeasts/ml, 24 h	MIC_0 or IC_{90}	38.2–100.0	36.7	30.0–100.0	20.0
	MIC_2 or IC_{50}	41.1–94.1	26.7	53.3–100.0	40.0
RP-G, 10^5 yeasts/ml, 48 h	MIC_0 or IC_{90}	35.2–100.0	60.0	46.6–96.7	16.7
	MIC_2 or IC_{50}	40.6–100.0	16.7	30.0–100.0	30.0
AM3, 24 h	MIC_0 or IC_{90}	56.2–100.0	50.0	40.0–92.9	6.7
	MIC_2 or IC_{50}	43.7–100.0	60.0	42.8–93.3	33.3
AM3, 48 h	MIC_0 or IC_{90}	48.3–100.0	40.0	43.3–96.7	6.7
	MIC_2 or IC_{50}	18.7–100.0	56.7	20.0–96.7	16.7

[a] Agreement range, percentage of results within excellent agreement range across all 30 isolates; excellent agreement, percentage of isolates with ≥90% interlaboratory agreement.

study the modal MIC for fluconazole was 2.0 μg/ml, and 30 of 33 (90.9%) of results fell within ±1 dilution of this mode and thus within the NCCLS specified limits. One laboratory reported duplicate off-range results of 8 μg/ml for ATCC 22019; however, exclusion of data from this laboratory had no effect on the results of the analyses of interlaboratory variation for fluconazole or caspofungin MICs.

Fluconazole modal MICs for the 30 *Candida* spp. isolates ranged from 0.13 to 32 μg/ml, showing that a full range of fluconazole susceptibilities was represented in the test panel. Across the 30 isolates, reproducibility within a three-dilution range varied from 62.9 to 100%. Excellent agreement was achieved for only 14 (47%) of the isolates, but moderate agreement was achieved for 25 (83%) of the isolates. Excellent agreement was less common (36.4% of 11 isolates) for *C. albicans* than for other *Candida* species (52.6% of 19 isolates), but this difference was not statistically significant (χ^2 test).

Caspofungin MICs for *Candida* spp. Levels of agreement for MIC and IC values obtained with caspofungin for the 30 *Candida* isolates tested in 17 laboratories for all growth media, inoculum sizes, incubation times, and end points are summarized in Table 2. For visual readings, the interlaboratory reproducibility of the MIC estimates was poor under most of the test conditions. Excellent interlaboratory agreement was achieved for ≥60% of the isolates tested only for MIC_2 in RPMI read at 24 h, MIC_0 in RP-G with 10^5 cells/ml read at 48 h, and MIC_2 in AM3 read at 24 h. Moderate agreement was achieved with visual end points for 93% of the *Candida* isolates with MIC_2 read at 24 h in RPMI and for 77% of the isolates with MIC_0 or MIC_2 read at 24 h in AM3 (details not shown).

For the two QC isolates, excellent agreement with published MIC ranges (3) was achieved under NCCLS-recommended conditions (MIC_2 read at 48 h in RPMI). For *C. parapsilosis* ATCC 22019 all data, except for a single result of 0.25 μg/ml fell in the QC range of 0.5 to 4.0 μg/ml (mode 1.0 μg/ml). For *C. krusei* ATCC 6258, 16 laboratories reported results within the QC range of 0.25 to 1.0 μg/ml (mode = 0.5 μg/ml), with one laboratory reporting 0.13 μg/ml.

Under many of the test conditions, the interlaboratory disparities in caspofungin visual MIC results for all 30 yeast isolates were considerable. MICs reported for some individual isolates included the high and low extremes of the concentration ranges tested. However, this effect was exacerbated by a minority of laboratories whose results occasionally differed substantially from those of the other participants. Scrutiny of the modal MICs for the panel of 30 *Candida* spp. isolates from each laboratory indicated outlying data (more than two dilutions higher or lower than the mode of values for all of the laboratories) under most test conditions. The results from four laboratories—C, D, F, and L—differed most often from the remainder by this assessment, but there was no consistent discrepancy, suggesting that data from any individual laboratories were persistently higher or lower than the rest to justify their exclusion from analysis.

The interlaboratory agreement for caspofungin IC_{50} and IC_{90} values determined from spectrophotometric data are also summarized in Table 2. When all data were included in the analysis the interlaboratory agreement for the spectrophotometric data was of a similar order as for visually read MICs. Excellent agreement for more than half of the test isolates was seen only in RPMI and in RP-G with the 10^3 yeasts/ml initial concentration.
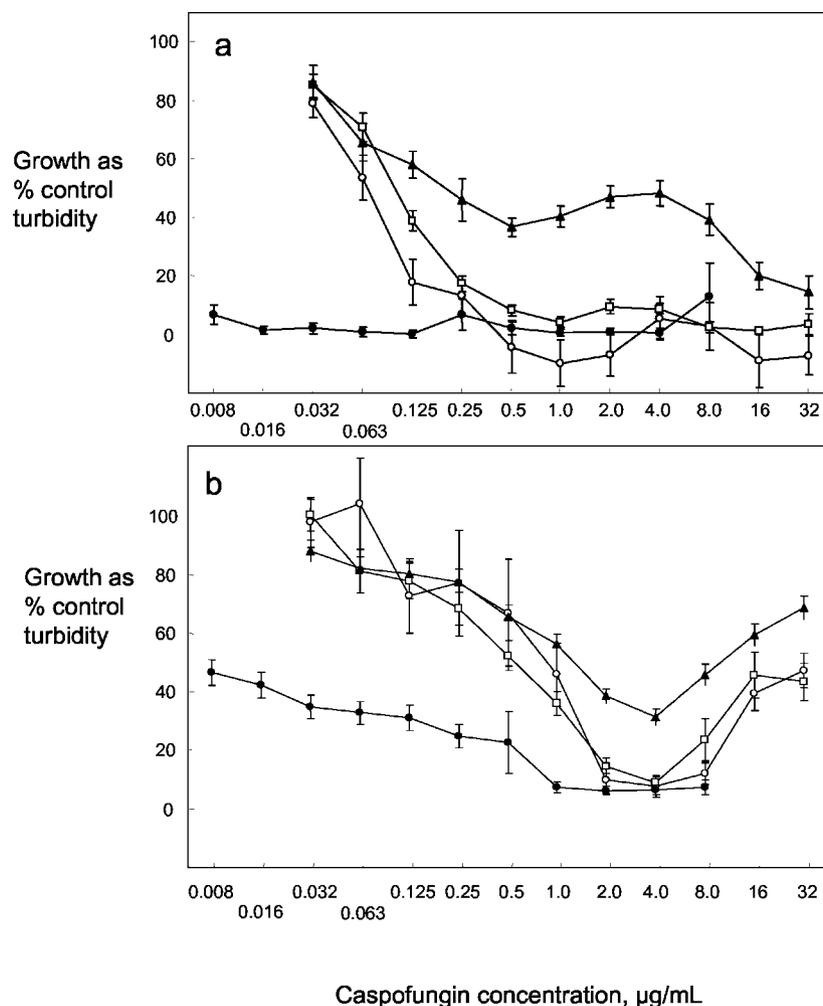
FIG. 1. Dose-response curves for caspofungin growth inhibition constructed from individual spectrophotometric data presented by 16 laboratories. Curves show growth of a *C. albicans* isolate as mean percent control in the presence of a caspofungin concentration series, with error bars showing the standard error of the mean ($n = 32$). Cultures were in RPMI (○), RP-G inoculated to 10^3/ml (□), RP-G inoculated to 10^5/ml (▲), and AM3 (●). (a) Y102 after 24 h of incubation; (b) Y106 after 48 h of incubation.

Figure 1 illustrates typical characteristics of the dose-response curves obtained from spectrophotometric means of growth turbidity relative to control growth in caspofungin dilution series under different conditions. For *C. albicans* isolate Y102 after 24 h of incubation (Fig. 1a), the dose-response curves in RPMI and RP-G with 10^3 cells/ml were similar. For RP-G with the higher inoculum, the curve showed an inflexion at middle-range caspofungin concentrations and then fell to <20% of control at 32 µg/ml. The curve in AM3 indicated considerable growth inhibition at all caspofungin concentrations. Interlaboratory agreement was good after 24 h, with low standard errors of the mean at all caspofungin concentrations. For *C. albicans* Y106 after 48 h (Fig. 1b) the dose-response curves showed a nonsigmoid appearance. In all three RPMI 1640-based media the curves indicated growth inhibition to be <40% of the control with caspofungin at 4 µg/ml and then regrowth at higher concentrations. The curve in AM3 showed no regrowth of the yeast at high caspofungin concentrations.

Test conditions to distinguish *Candida* isolates of high and low caspofungin susceptibility. The panel of yeast isolates in-

cluded eight yeasts known to have mutations in the *FKS1* gene that result in lower than normal caspofungin susceptibility. To determine whether the conditions that gave highest interlaboratory test reproducibility for caspofungin MIC and IC values could distinguish the two yeast susceptibility groups, the results obtained by MIC_2 and IC_{50} in RPMI and AM3 after 24 h were analyzed (Table 3). The distribution of MIC results reported from the 17 laboratories for the isolates with normal and reduced caspofungin susceptibility were analyzed by the χ^2 test. The larger the value of χ^2 for each paired data set of high versus low susceptibility, the greater the differentiation between the susceptibility groups (all of the values in Table 4 showed a statistically significant difference in the MIC distributions for high- and low-susceptibility isolates). The results suggested that the visually determined MIC_2 values discriminated better between the two groups than the spectrophotometrically determined IC_{50} values and that the data from tests in AM3 discriminated better than the data from tests in RPMI.

Itraconazole MICs for *Aspergillus* spp. The itraconazole MICs for the panel of 20 *Aspergillus* spp. isolates tested in the

TABLE 3. Percentages of MIC$_2$ or IC$_{50}$ results recorded in 17 laboratories for 8 *Candida* spp. isolates with known low susceptibility to caspofungin and 22 isolates with normal susceptibility

Caspofungin concn (μg/ml)	% MIC$_2$ or IC$_{50}$[a]							
	RPMI (MIC$_2$)		RPMI (IC$_{50}$)		AM3 (MIC$_2$)		AM3 (IC$_{50}$)	
	Normal ($n = 739$)	Low ($n = 265$)	Normal ($n = 535$)	Low ($n = 193$)	Normal ($n = 733$)	Low ($n = 270$)	Normal ($n = 513$)	Low ($n = 198$)
0.008	0.0	0.0	0.0	0.0	56.5	6.7	71.2	8.6
0.016	0.0	0.0	0.0	0.0	8.9	0.4	9.6	2.5
0.032	4.6	0.4	9.3	3.6	22.0	1.5	11.1	2.5
0.063	21.2	0.0	26.5	1.6	7.6	1.9	5.8	2.5
0.13	20.7	0.0	23.6	5.7	2.7	3.3	0.4	5.1
0.25	18.4	1.9	18.1	2.1	0.5	7.4	0.0	4.5
0.5	21.1	2.3	14.6	3.6	0.1	21.5	0.4	15.2
1	11.1	14.0	6.5	13.5	0.0	28.1	0.0	33.3
2	1.8	46.8	1.1	37.8	0.0	17.8	0.0	15.7
4	0.0	25.3	0.0	20.2	0.0	6.7	0.0	6.1
8	0.1	4.5	0.0	5.7	0.0	0.7	0.2	2.0
16	0.5	2.3	0.2	4.7	1.6	4.1	1.4	2.0
32	0.4	1.5	0.0	1.0	0.0	0.0	0.0	0.0
>32	0.0	1.1	0.0	0.5	0.0	0.0	0.0	0.0

[a] n = the number of results from 17 laboratories. The χ^2 values for RPMI (MIC$_2$), RPMI (IC$_{50}$), AM3 (MIC$_2$), and AM3 (IC$_{50}$) were 727, 467, 784, and 529, respectively.

17 participating laboratories were read as the MIC$_0$ after 48 h growth of the molds. The data varied only over a narrow range of values, from 0.13 to 1.0 μg/ml, with a modal MIC for most isolates of 0.5 μg/ml. For the two NCCLS reference isolates, *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305, the modal itraconazole MICs of 0.50 and 1.0 μg/ml fell within the prescribed reference ranges for method M38-A (17). The per-centage of data within the prescribed ranges was 90.9% (30 of 33 readings) and 100% for the two strains, respectively.

Caspofungin MICs for *Aspergillus* spp. Agreement between 17 laboratories for MIC and IC values of caspofungin for the 20 *Aspergillus* isolates for all growth media, inoculum sizes, incubation times, and end points is summarized in Table 4. The agreement between results obtained with the spectrophotom-

TABLE 4. Interlaboratory agreement for caspofungin susceptibility data with 20 isolates of *Aspergillus* spp.[a]

Test conditions	Endpoint	Visual readings		Spectrophotometer results	
		Agreement range (%)	Excellent agreement (%)	Agreement range (%)	Excellent agreement (%)
RPMI, 24 h	MIC$_0$ or IC$_{90}$	73.5–100.0	75.0	34.4–93.1	5.0
	MIC$_2$ or IC$_{50}$	46.8–91.2	5.0	27.5–86.2	0.0
	MEC	47.0–90.6	5.3		
	MEC (modified)[b]	91.1–100.0	100.0		
RPMI, 48 h	MIC$_0$ or IC$_{90}$	38.2–79.4	0.0	48.0–96.6	20.0
	MIC$_2$ or IC$_{50}$	50.0–96.4	10.0	20.0–72.0	0.0
RP-G, 24 h	MIC$_0$ or IC$_{90}$	82.3–100.0	70.0	37.9–93.1	5.0
	MIC$_2$ or IC$_{50}$	37.5–96.9	15.0	24.1–82.8	0.0
	MEC	50.0–96.9	0.0		
	MEC (modified)	85.2–100.0	75.0		
RP-G, 48 h	MIC$_0$ or IC$_{90}$	52.9–88.2	0.0	52.1–95.7	55.0
	MIC$_2$ or IC$_{50}$	57.6–100.0	30.0	24.0–80.0	0.0
AM3, 24 h	MIC$_0$ or IC$_{90}$	57.6–100.0	70.0	41.3–93.1	35.0
	MIC$_2$ or IC$_{50}$	32.3–94.1	10.0	31.0–89.7	0.0
	MEC	60.0–96.7	15.0		
	MEC (modified)	81.2–100.0	85.0		
AM3, 48 h	MIC$_0$ or IC$_{90}$	26.4–91.2	10.0	44.8–93.1	5.0
	MIC$_2$ or IC$_{50}$	69.2–100.0	65.0	37.9–89.	0.0

[a] High levels of agreement reflect predominantly off-scale readings. Agreement range, percentage of results within excellent agreement range across all 30 isolates; excellent agreement, percentage of isolates with ≥90% interlaboratory agreement.
[b] MEC (modified), data from laboratories E, H, and J were excluded from analysis.

eter was impossibly poor under all conditions. For the visually read end points, the best agreement was obtained (≥70% of isolates showing excellent agreement) for MIC_0 readings at 24 and 48 h for all growth media. However, this is a misleading observation, since almost all of the isolates were scored as resistant to the highest concentration of caspofungin tested (MIC > 16 µg/ml) in terms of MIC_0 in most laboratories, and agreement with so many off-scale data was to be expected. The interlaboratory agreement for the tests with *Aspergillus* spp. isolates was therefore interpreted as poor under all conditions. Spectrophotometric IC determinations with the molds were unhelpful with the *Aspergillus* spp. isolates.

Caspofungin MEC values for *Aspergillus* spp. Measurement of the 24-h MECs did not enhance the poor interlaboratory reproducibility of the *Aspergillus* susceptibility data; results tended to parallel those obtained with the MIC_2 readings (Table 4). However, MEC data from laboratories E, H, and J tended to be much higher than those from other laboratories, suggesting possible problems with MEC readings from these three participants. Removal of these sets of data from the analysis improved the interlaboratory agreement under all three test conditions (Table 4). The percentage of 20 isolates for which excellent agreement was achieved rose from only 5 to 15%, with all results included, to 100, 75, and 85% in RPMI, RP-G, and AM3, respectively, with the data from laboratories E, H, and J omitted.

MEC results also produced the lowest susceptibility readings for the *Aspergillus* isolates. In RPMI, RP-G, and AM3, respectively, 93, 91, and 87% of the reported MECs were <1 µg/ml, rising to 100, 98, and 98%, respectively, when the results from laboratories E, H, and J were omitted.

DISCUSSION

This study involved analysis of more than 52,000 individual datum points determined from 17 laboratories located in North America, Europe, and Australasia and represents one of the most extensive surveys of methodology for susceptibility testing with an antifungal agent ever undertaken. The results provide reasons for both optimism and concern over susceptibility testing with caspofungin in vitro. We set our criterion for excellent reproducibility as a requirement for ≥90% of MIC or IC data for a single fungal isolate to be within a mode ± one dilution range. This matches the levels of agreement obtained in, for example, NCCLS QC studies with antifungal agents (3). It may be argued that the criterion is too stringent, but it represents the normally accepted standard.

A positive finding of our study is that two test conditions, MIC_2 or IC_{50} read at 24 h for cultures in RPMI 1640 medium or AM3, gave excellent interlaboratory agreement for a majority of the panel of 30 isolates of *Candida* spp., both by visual and spectrophotometric end point determination. Under these conditions a high level of discrimination was achieved between strains known to have lowered caspofungin susceptibility and normally susceptible isolates. These conditions therefore emerge as strong contenders for refinement of methodology to give as-near-as-possible acceptable interlaboratory reproducibility for caspofungin testing—an obvious prerequisite before clinically relevant resistance breakpoints can be set for this new antifungal agent.

However, it is a matter of concern that the reproducibility in caspofungin susceptibility tests with isolates of *Aspergillus* spp. was of such a low order. Under all conditions tested, some growth of these molds occurred in the presence of all concentrations of caspofungin, so that a susceptibility test end point based on complete inhibition of growth is impractical, as has been observed previously (1, 10, 13). In contrast, an MIC end point based on "prominent growth reduction relative to control" (MIC_2) gave results at the very low end of the caspofungin concentration range tested, suggesting a high susceptibility of *Aspergillus* spp. to this agent. However, even for MIC_2 data, the proportion of isolates for which 90% of the results fell within the range of mode ± one dilution was unacceptably low. The MEC, an end point proposed in previous studies that also measures partial, rather than complete growth inhibition (1, 10, 13), greatly improved interlaboratory reproducibility over MIC readings in the present study when data from 3 of the 17 participating laboratories were excluded. The reason why three laboratories that would generally be regarded as experienced with antifungal susceptibility testing should produce aberrant data is unclear. The MEC end point requires some caution in its interpretation, as it represents a subjective assessment of the appearance of growth, but our findings generally confirm the utility of this end point for testing caspofungin against *Aspergillus* spp.

For both the *Candida* and the *Aspergillus* spp. tested, the data for fluconazole or itraconazole against QC and reference isolates fell almost perfectly within expected limits. This finding confirmed the competence of the 17 laboratories to undertake antifungal susceptibility testing. Although it was possible to define MEC data from 3 of 17 laboratories as unacceptably divergent, the same was not true for caspofungin MICs against *Candida* or *Aspergillus* spp., where apparently outlying data sets were typically seen as occasional idiosyncracies under just one of the conditions tested, rather than as consistently "maverick" results from one or more laboratories.

The results for tests with fluconazole versus *Candida* spp. gave a higher level of interlaboratory discordance than anticipated. These tests were done by the reference microdilution methodology prescribed by the NCCLS (19); yet, for 5 of the 30 isolates tested, fewer than 80% of the results from the 17 laboratories agreed within a mode ± one dilution range. A recent New York State proficiency testing study found an overall level of agreement with reference values of just 75% for fluconazole MICs determined in seven laboratories with five isolates of *Candida* spp. (24). No *Candida* isolate from an infected patient is routinely subjected to multiple-laboratory testing, and the possibility of over- or underestimation of susceptibility in a single laboratory test appears high enough to justify future attention.

Susceptibility testing with any microorganism is a procedure with a high level of inherent run-to-run variability, and any prescribed set of test conditions represents a compromise between choices of test variables. Some isolates of fungi seem to generate consistent MIC data regardless of variations in test conditions, whereas others show high vulnerability even to relatively minor variations (4, 25). The objective of the design of susceptibility test conditions should be to devise a methodology that is least affected by small differences in technical parameters. Our study shows that, for tests with caspofungin

against *Candida* spp., the present NCCLS method M27-A gives reasonable interlaboratory reproducibility, provided MICs are read as MIC_2 after 24 h of incubation. However, there is still room for improvement in the methodology to raise the level of interlaboratory agreement to a higher standard. For caspofungin testing with *Aspergillus* spp., the MEC currently offers an end point that can give generally reproducible results in many laboratories, but the finding of 3 laboratories of 17 generating aberrant data even by MEC suggests that further refinement of caspofungin test methodology for *Aspergillus* spp. is still required.

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