ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2003, p. 2100–2107 Vol. 47, No. 7
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.7.2100–2107.2003

Influences of Methodological Variables on Susceptibility Testing of Caspofungin against *Candida* Species and *Aspergillus fumigatus*

C. Bartizal† and F. C. Odds*

Department of Molecular & Cell Biology, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom

Received 30 January 2003/Returned for modification 11 March 2003/Accepted 21 March 2003

The influences of test variables on the outcome of susceptibility testing with caspofungin were tested with isolates of *Candida* spp. and *Aspergillus fumigatus*. Among six growth conditions tested with a range of inoculum sizes, the highest control growth yields were obtained in Sabouraud broth for all fungi, followed by RPMI 1640 (pH 7) for *Candida* spp. and antibiotic medium 3 (AM3) for *A. fumigatus*. RPMI 1640 gave unacceptably low growth yields with *A. fumigatus*. The caspofungin MICs under these various conditions ranged over more than 4 twofold dilutions for 7 of 16 fungi tested when a 50% inhibition (50% inhibitory concentration [IC_{50}]) endpoint was used and for 12 of 16 fungi tested when an 80% inhibition (IC_{80}) endpoint was used. A multifactorial design to study the influences of six test variables on control growth and the MIC showed that, for 14 isolates of *Candida* spp., the glucose concentration and the medium composition were the most common factors significantly influencing both control growth yields and the MIC. For eight *A. fumigatus* isolates, incubation time (24 versus 48 h) and temperature (30 versus 35°C) significantly affected control optical density (OD) values, while growth medium (AM3 versus Sabouraud broth) was the most common process variable affecting the MICs. Tests with AM3 from three suppliers showed significant variations in control OD values related to supplier, but IC_{50}s fell within a 2- or 3-dilution range for 19 (86%) of the 22 isolates tested. We recommend that, at present, AM3 is superior to RPMI 1640 for testing of the susceptibilities of both yeasts and filamentous fungi to caspofungin and that a minimum incubation time of 48 h is necessary to test *A. fumigatus* adequately.

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 the European Union for the treatment of invasive aspergillosis refractory to other antifungal treatments and esophageal *Candida* infections and approved in the European Union and pending approval in the United States for the treatment of invasive *Candida* infections (10). Caspofungin inhibits the synthesis of β-1,3-D-glucan in fungal cell walls, a property that results in fungicidal effects against species in which this polysaccharide is vital to cell wall integrity.

Preclinical evaluations of caspofungin in susceptibility tests with *Candida* species have all been done under the conditions of National Committee for Clinical Laboratory Standards (NCCLS) method M27-A (16), approved for susceptibility testing of yeasts with azoles, flucytosine, and polyene antifungal agents (amphotericin B), or close derivatives of this method; and they have shown reasonable interlaboratory reproducibilities. Published caspofungin MICs for *Candida albicans* in studies based on panels with from 4 to 486 isolates all fall in the range of 0.015 to 4.0 μg/ml, with statistics indicating a central tendency (MICs for 50% of isolates and geometric means) in the narrow range of 0.063 to 0.5 μg/ml (2, 4, 8, 11, 13, 14, 17, 18). Quality control (QC) limits have been established for caspofungin in tests with reference isolates of *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) (3).

For *Aspergillus* species, notably, *Aspergillus fumigatus*, the level of agreement by MIC testing is far less impressive. For tests with 26 *A. fumigatus* isolates, Arikan et al. (1) measured geometric mean MICs of caspofungin of 0.43 μg/ml after 24 h of incubation and >16 μg/ml after 72 h of incubation. These results were determined by use of the methodological recommendations for filamentous fungi with azoles, flucytosine, and polyenes published by NCCLS (M38-A) (15). By comparison, 72-h geometric mean MICs for *A. fumigatus* were determined to be ≤0.09 μg/ml by Del Poeta and colleagues (6) and 2.15 μg/ml by Espinel-Ingroff (8). In the laboratory of Pfaller and colleagues (19), the 72-h caspofungin MICs for 12 isolates of *A. fumigatus* were all 0.06 and 0.12 μg/ml. In all these studies the growth medium used was RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS), according to NCCLS M38-P recommendations (15), although details such as inoculum size and the temperature of incubation differed slightly between studies.

The minimal effective concentration, defined as the lowest caspofungin concentration that results in the growth of *A. fumigatus* with conspicuously aberrant hyphae, has been suggested as a measurement that gives more consistent results than those found by MIC testing (1, 12). Use of alternative growth media such as antibiotic medium 3 (AM3) for caspofungin susceptibility testing leads to a lowering of MIC endpoints in tests with *Aspergillus* and *Candida* species (1, 11, 17), but the reduction was small for *Candida* spp., even when medium variations were combined with systematic alterations in

* Corresponding author. Mailing address: Department of Molecular & Cell Biology, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom. Phone and fax: (44) 1224 555828. E-mail: f.odds@abdn.ac.uk.

† Present address: University of Notre Dame, Notre Dame, Ind.

pH and incubation temperature (11). It is evident that the optimum conditions for reproducible testing of the susceptibilities of *Candida* and *Aspergillus* spp. to caspofungin remain to be established.

The influences of performance variables on the outcomes of antifungal susceptibility tests can be tested statistically in prospectively designed studies. For itraconazole, such a fractional factorial approach revealed that each of 18 fungal isolates individually differed in sensitivity to alterations in 10 process variables, confirming how complex it is to design a single susceptibility test system applicable to all yeasts or all filamentous fungi (20). The present study was designed along similar factorial lines, but with caspofungin as the test agent. Because this compound is water soluble, the influence of the solvent used to prepare stock solutions was omitted from the design. Additionally, the influence of the endpoint used for MIC determination was also assessed.

MATERIALS AND METHODS

Fungi and inoculum preparation. All fungal isolates were preserved at −80°C in 10% (vol/vol) glycerol and were maintained by subculture on Sabouraud agar (Oxoid, Basingstoke, United Kingdom) for the duration of the study. The following isolates were originally obtained from clinical specimens: *C. albicans* B56930, J990578, J981364, and J981371; *Candida glabrata* J981216; and *A. fumigatus* J980659, J980561, J970398, J970377, J960180, J960330, J940362, and J931199. *C. albicans* isolates NR2, NR3, T25, NR4, T32, T28, and T26 were laboratory-derived mutants that differed in their caspofungin susceptibilities (7) and were provided by Merck Research Laboratories (Rahway, N.J.). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were QC strains designated for antifungal susceptibility testing by the NCCLS (16).

Inocula for *Candida* spp. were grown for 18 h at 30°C in 5-ml volumes of NGY medium, comprising 0.1% neopeptone (Difco Laboratories, Detroit, Mich.), 0.4% glucose, and 0.1% yeast extract (Difco), with shaking at 200 rpm. This procedure resulted in suspensions containing a mean of 2×10^7 yeast CFU/ml, with a variation of ± 0.5 $\log_{10}$. For *A. fumigatus* isolates, cultures were grown on potato-dextrose agar at 30°C for 2 to 7 days until large numbers of conidia had formed. Conidial suspensions were harvested in sterile 0.5% sodium dodecyl sulfate adjusted to give an optical density (OD) of 1.0 at 570 nm, divided into small lots, and stored at −20°C. For use in susceptibility tests, the conidial suspensions were thawed and adjusted spectrophotometrically to the appropriate dilution. Viable counts were used to confirm that the initial fungal concentrations fell within the expected limits.

Caspofungin. Caspofungin was supplied by Merck Research Laboratories as a pure powder. It was dissolved in sterile distilled water to provide a stock solution containing 16 mg/ml and was stored frozen in small lots at −20°C.

Culture media. Four basic culture media were used in the experiments. All were prepared at twice their final concentrations for experimental use. Gibco RPMI 1640 was purchased as a 10× concentrated solution without glutamine or bicarbonate (Becton Dickinson Co., Sparks, Md.). To 1 volume of this concentrate was added 1 volume of concentrated glucose solution (the exact concentration was dependent on the experimental conditions), 1 volume of 1.65 M MOPS (pH 7.2), 0.1 volume of a 200 mM L-glutamine solution, and 1.9 volumes of water. The mixture was filter sterilized and had a final pH of 7.0. For some experiments the final mixture was adjusted to pH 5.0 by addition of concentrated HCl before filter sterilization.

Difco AM3 was purchased as dry powder from Becton Dickinson Co. and was dissolved in water together with extra glucose when necessary and filter sterilized. For some experiments the pH of this medium was adjusted to 5.0 by addition of concentrated HCl. To compare the results obtained with AM3 from different suppliers, AM3 was also purchased from Oxoid and VWR International Laboratories (Poole, United Kingdom). Mueller-Hinton (MH) broth was purchased as a dry powder from Invitrogen Co. (Paisley, Scotland), dissolved in water, and sterilized by autoclaving. Sabouraud broth (SAB) contained Oxoid Mycological Peptone (Unipath, Basingstoke, United Kingdom) at 10 g/liter and glucose at 40 g/liter and was sterilized by autoclaving.

General conditions for susceptibility testing. In all experiments, microdilution plates were filled with 100-μl volumes of aqueous caspofungin solutions at twice their final concentrations. Sterile distilled water was used in the control wells. The test culture media were prepared at twice their final concentrations, inoculated by addition of an appropriate small volume of yeast or conidium suspension, and dispensed in 100-μl volumes into the microdilution plate wells. The final test concentrations of caspofungin in all experiments were in a twofold dilution series ranging from 16 to 0.032 μg/ml.

The test plates were placed in plastic boxes with airtight lids and a beaker of water to ensure the presence of humidity. They were incubated according to the conditions of the variable experimental design, and then the OD values of the turbidities of the cultures in the wells were measured at 405 nm with a microplate reader (600 nm for cultures in SAB, which gave the lowest background OD at this wavelength). The background OD was taken as the average for the 96 wells in a plate to which uninoculated culture medium was added. The raw OD values were corrected for background absorbance and converted to indicate growth turbidity as a percentage of that for control (drug-free) growth with the aid of a computer spreadsheet template. MICs were determined by the use of two endpoint criteria from dose-response curves of percent control growth versus the caspofungin concentration. The 50% inhibitory concentration (IC_{50}) and IC_{80} were the lowest caspofungin concentrations that reduced the turbidities below 50 and 80% of that for the control, respectively. When the OD for control growth was less than 0.15, the results were excluded from analysis.

For statistical analyses of the data, IC_{50}s or IC_{80}s above 16 μg/ml were recorded as 32 μg/ml; for a series in which growth was <50 or <80% of control at 0.032 μg/ml, the inhibitory concentration (IC) was recorded as 0.016 μg/ml. All MICs were expressed to two significant digits.

Selection of culture medium. In the absence of recommended standard media for caspofungin susceptibility testing, six culture media were evaluated in preliminary experiments. These were RPMI 1640 with 2% glucose at pH 7.0 and pH 5.0, AM3 at pH 7.0 and pH 5.0, SAB, and MH broth. The initial concentrations of fungi for each medium were 2×10^3, 2×10^4, and 2×10^5 yeasts/ml or 2×10^3 and 2×10^4 *A. fumigatus* conidia/ml. The test plates were incubated for 48 h at 35°C.

Multifactorial experimental design. Six performance variables were investigated: culture medium, glucose concentration in the medium, inoculum size, the use versus the nonuse of an adhesive sticker to seal the plate, temperature of incubation, and duration of incubation. For the *Candida* sp. isolates, AM3 at pH 7.0 was compared with RPMI 1640 with the initial pH adjusted to 5.0; initial glucose concentrations were 0.2 or 2.0%, initial yeast concentrations were 2×10^4 and 2×10^5 yeasts/ml, incubation temperatures were 30 and 35°C, and incubation times were 24 and 48 h. For the *A. fumigatus* isolates, AM3 was compared with SAB; initial conidium concentrations were 2×10^3 and 2×10^4/ml, and the other variables were the same as those for the *Candida* spp.

Data for the control OD and for the log-transformed IC_{50} or IC_{80} for each fungal isolate under each combination of the six performance variables were analyzed by univariate analysis of variance (ANOVA) to determine the individual variables that contributed significantly to a variation in any parameter for each strain tested.

RESULTS

Selection of culture medium for multifactorial variable experiments. In a pilot experiment, eight isolates of *Candida* spp. (*C. albicans* B59630, J990578, NR2, NR3, and T25; *C. glabrata* J981216; *C. krusei* ATCC 22019; and *C. parapsilosis* ATCC 6258) and the eight *A. fumigatus* isolates were inoculated at different starting concentrations into each of six growth media. The control OD values (Table 1) showed that the *Candida* species gave the highest yields in SAB, followed by RPMI 1640 at pH 7 and RPMI 1640 at pH 5. The growth yields in MH broth, AM3 at pH 5, and AM3 at pH 7 were much lower, with the OD seldom exceeding 0.5 in AM3 at either pH. The mean control OD values tended to rise with increasing inoculum size, although the variation was small except in MH broth.

A different relation between the control OD and growth medium was found for the *A. fumigatus* isolates (Table 1). While SAB still gave maximum yields in terms of the mean OD, the next highest control OD values were seen in MH broth, followed by AM3 at pH 5 and AM3 at pH 7. In all media tested, the control OD value for the *A. fumigatus* isolates often

TABLE 1. Effects of variations in medium and inoculum on control OD and caspofungin MIC data for eight isolates each of *Candida* spp. and *A. fumigatus*[a]

Medium	pH	Inoculum (CFU/ml)	Mean ± SD control OD		No. of isolates for which ODs were <0.150		IC_{50} (μg/ml)				IC_{80} (μg/ml)			
			Candida spp.	*A. fumigatus*	*Candida* spp.	*A. fumigatus*	*Candida* spp.		*A. fumigatus*		*Candida* spp.		*A. fumigatus*	
							Range	Gmean[b]	Range	Gmean	Range	Gmean	Range	Gmean
AM3	5.0	2×10^3	0.339 ± 0.115	1.165 ± 0.224	0	0	0.016–4.0	0.23	0.063–0.25	0.19	0.063–4.0	0.33	0.25–4.0	0.59
		2×10^4	0.326 ± 0.050	1.279 ± 0.256	0	0	0.063–4.0	0.33	0.016–0.25	0.11	0.063–>16	1.01	0.063–16	1.20
		2×10^5	0.393 ± 0.064		0		0.063–4.0	0.55			0.063–>16	0.93		
	7.0	2×10^3	0.215 ± 0.054	0.903 ± 0.312	1	0	0.063–2.0	0.28	0.13–1.0	0.20	0.063–2.0	0.41	0.13–>32	1.10
		2×10^4	0.276 ± 0.049	1.244 ± 0.312	0	0	0.016–2.0	0.28	0.016–0.13	0.059	0.063–2.0	0.39	0.016–16	0.78
		2×10^5	0.330 ± 0.077		0		0.016–4.0	0.33			≤0.032–4.0	0.39		
MH	7.0	2×10^3	0.249 ± 0.061	1.686 ± 0.441	0	0	0.016–4.0	0.35	0.063–0.25	0.16	0.25–4.0	0.59	0.13–0.5	0.27
		2×10^4	0.278 ± 0.055	1.519 ± 0.571	0	0	0.13–4.0	0.47	0.016–0.25	0.12	0.25–4.0	0.77	0.13–1.0	0.28
		2×10^5	0.488 ± 0.369		0		0.063–4.0	0.60			0.063–4.0	0.60		
RPMI 1640	5.0	2×10^3	0.673 ± 0.208	0.033 ± 0.024	0	8	0.25–>16	1.4			0.25–>16	2.38		
		2×10^4	0.819 ± 0.165	0.058 ± 0.073	0	7	0.25–>16	2.0			0.5–>16	5.66		
		2×10^5	0.851 ± 0.154		0		0.25–>16	2.4			0.25–>16	4.00		
	7.0	2×10^3	0.782 ± 0.135	0.243 ± 0.083	0	0	0.25–8.0	1.2	0.25–>16	1.41	0.25–8.0	1.54	1–>16	13.5
		2×10^4	0.892 ± 0.116	0.313 ± 0.131	0	1	0.25–16	1.7	0.13–1.0	0.46	0.5–>16	4.00	2–>16	19.5
		2×10^5	0.915 ± 0.084		0		0.25–16	1.4			0.25–>16	2.00		
SAB	5.2	2×10^3	1.185 ± 0.278	3.188 ± 0.396	0	0	0.063–2.0	0.25	0.13–1.0	0.39	0.13–8.0	0.55	0.25–2.0	0.65
		2×10^4	1.325 ± 0.090	3.263 ± 0.095	0	0	0.063–2.0	0.36	0.13–0.25	0.19	0.13–16	0.93	0.25–0.5	0.28
		2×10^5	1.295 ± 0.096		0		0.13–16	1.0	0.13		0.13–16	1.01		

[a] The test plates were incubated for 48 h at 35°C.
[b] Gmean, geometric mean.

TABLE 2. Ranges of caspofungin IC_{50} and IC_{80}s for eight isolates each of *Candida* spp. and *A. fumigatus* determined in six different media and with two or three different starting fungus concentrations[a].

Isolate	Range IC_{50} (μg/ml)	No. of dilns[b]	Mode IC_{50} (μg/ml)	Range IC_{80} (μg/ml)	No. of dilns	Mode IC_{80} (μg/ml)
C. krusei ATCC 6258	0.016–2.0	7	0.25	0.063–2.0	5	0.25
C. parapsilosis ATCC 22019	0.25–2.0	3	0.50	0.25–>16	7	0.50
C. albicans B59630	≤0.032–0.50	5	0.063	≤0.032–>16	11	0.063
C. albicans J990578	0.063–>16	9	0.25	0.25–>16	7	0.25
C. albicans NR2	0.25–4.0	4	0.50	0.5–>16	6	0.50
C. albicans NR3	1.0–8.0	3	4.0	2–>16	4	2.0
C. albicans T25	2.0–>16	4	4.0	2–>16	4	4.0
C. glabrata J981216	0.063–1.0	4	0.13	0.063–1.0	4	0.13
A. fumigatus J980659	0.063–1.0	4	0.25	0.5–>16	6	0.50
A. fumigatus J980561	0.063–0.50	3	0.25	0.13–2.0	4	0.25
A. fumigatus J970398	≤0.032–>16	11	0.13	0.13–>16	8	0.25
A. fumigatus J970377	0.063–2.0	5	0.25	0.25–>16	7	>16
A. fumigatus J960180	0.016–0.25	4	0.016	≤0.032–1.0	6	0.25
A. fumigatus J960330	0.13–0.50	2	0.13	0.13–>16	8	0.25
A. fumigatus J940362	≤0.032–1.0	6	0.25	0.13–>16	8	0.25
A. fumigatus J931199	0.063–>16	9	0.13	0.25–>16	7	0.25

[a] See Table 1.
[b] No. of dilns, number of twofold dilutions between extremes of range.

exceeded 1.0. By contrast, all but one control OD value was <0.4 in RPMI 1640 at pH 7, and most control OD values were below the nominal threshold of acceptability, 0.150, in RPMI 1640 at pH 5.

Caspofungin MICs determined in the various broth media were not directly related to the control OD. For the eight *Candida* sp. isolates, the highest MICs (judged by geometric mean data; Table 1) were seen in tests performed in RPMI 1640 at pH 5, followed by RPMI 1640 at pH 7, regardless of the choice of 50 or 80% growth inhibition endpoints. For several isolates an "eagle" effect in dose-response curves was noted, in which the turbidity rose at the two or three highest caspofungin concentrations. This effect was ignored for determination of the IC_{50}s and IC_{80}s. The lowest geometric mean ICs for the yeasts were determined in AM3 at pH 7, followed by AM3 at pH 5 and MH broth. IC_{50}s and IC_{80}s in SAB showed considerable variation with the initial yeast concentration (Table 1). The geometric mean caspofungin IC_{50}s for the eight *A. fumigatus* isolates showed less variation with test conditions than those for the yeasts (Table 1); however, the IC_{80}s showed considerably more variation between media. For each medium and endpoint, only minor differences were noted between the two inoculum sizes. Geometric mean IC_{50}s determined in AM3 at pH 5, AM3 at pH 7, and MH broth were of the order of 0.1 to 0.2 μg/ml at both inoculum sizes. The highest MICs were those determined in RPMI 1640 at pH 7, and the control growth in RPMI 1640 at pH 5 for all eight isolates was below the limit for acceptable analysis. Differences between geometric mean IC_{80}s and IC_{50}s were often considerable for the *A. fumigatus* isolates when the inoculum size was 2×10^4 conidia/ml: more than 10-fold in AM3 at both pH values and in RPMI 1640 at pH 7.

The ranges of caspofungin IC_{50}s and IC_{80}s determined for each isolate are shown in Table 2. For the *C. parapsilosis* QC isolate, the modal caspofungin IC at both endpoints and most of the individual values were within the normal 48-h range of 0.5 to 4.0 μg/ml determined by the NCCLS Antifungal Subcommittee (3). The sevenfold dilution range of the IC_{80} for this isolate resulted from a single, aberrantly high result. For the *C. krusei* QC isolate, the modal value of 0.25 μg/ml at both endpoints fell within the NCCLS normal range of 0.25 to 1.0 μg/ml; among the total of 18 results, 3 IC_{50}s and 6 IC_{80}s fell 1 dilution outside the normal range, with the extremes in the range represented by single values only. The data in Table 2 indicate that individual isolates of *Candida* spp. and *A. fumigatus* differed considerably in the extent to which the caspofungin susceptibility result varied with changes in medium composition and inoculum size. IC_{80}s in particular showed high variabilities in these experiments. NCCLS publications on antifungal susceptibility testing allow a 3- or 4-dilution MIC variation as acceptable, depending on the agent and fungus tested (3, 15, 16). From Table 2, variability in the caspofungin IC_{50} was above this level for three of the eight *Candida* spp. and four of the eight *A. fumigatus* isolates. The variability in the IC_{80} was even greater, with the IC_{80}s for five yeasts and seven *A. fumigatus* isolates varying beyond the 4-dilution range.

Multifactorial investigation of MIC performance variables. The data in Table 1 indicate that RPMI 1640 with 2% glucose gave adequate control growth for caspofungin testing with *Candida* spp. but not with *A. fumigatus*. AM3 at pH 7 was therefore chosen as the common medium for the testing of both fungal types. To provide a contrast to this medium with its neutral pH in the multifactorial design, RPMI 1640 at pH 5 was chosen as the comparator for tests with *Candida* spp. and SAB, with a pH of 5.6, was chosen as the comparator for *A. fumigatus*.

The results of the multifactorial variable tests are summarized in Table 3. For the 14 isolates of *Candida* spp. the culture medium and the glucose concentration repeatedly emerged as significant contributors to variations in the control OD. Regardless of the other variables, the control OD values in cultures of AM3 at pH 7 tended to be higher than those in RPMI 1640 at pH 5; and for each medium, the cultures containing 2% glucose generally gave higher control OD values than the cultures containing 0.2% glucose. Incubation time, temperature, and starting inoculum were significant variables influencing the

TABLE 3. Multivariate analysis of effects of performance variables on control growth OD and MIC (IC_{50} and IC_{80}) for 13 isolates of *Candida* spp. and 8 isolates of *A. fumigatus*

Isolate	Control OD		No. of isolates for which OD was <0.100	Significant variables[a]	IC_{50} (μg/ml)		Significant variable(s)[a]	IC_{80} (μg/ml)		Significant variable(s)[a]
	Range	Median			Range	Gmean[b]		Range	Gmean	
C. albicans B59630	0.279–1.558	0.629	0	M, G	0.016–16	0.096	M, G	0.016–>16	2.5	G
C. albicans J981364	0.422–1.555	0.923	0	G, T	0.063–>16	0.24	M, G	0.063–>16	2.6	G
C. albicans J981371	0.461–1.569	0.909	0	G, T	≤0.032–>16	0.24	M, G, I	0.063–>16	4.6	M, G
C. albicans J990578	0.391–1.472	0.701	0	M, G	0.063–>16	0.77	G, I, H	≤0.016–>16	9.7	G, I
C. albicans NR2	0.220–1.536	0.563	0	M, G	0.13–>16	2.2	M, I, T	0.13–>16	8.4	M, G, T
C. albicans NR3	0.218–1.544	0.528	0	M, G	1.0–>16	3.5	M, G, I, T	1.0–>16	9.0	G, T
C. albicans NR4	0.343–1.514	0.813	0	M, G	0.063–>16	2.4	M, G, I, T	0.50–>16	7.3	G, T
C. albicans T25	0.272–1.429	0.556	0	M, G	2.0–>16	9.3	M, I, T, H	4.0–>16	17	M, G
C. albicans T26	0.315–1.433	0.668	0	M, G, I	2.0–>16	8.4	M, I, T, H	2.0–>16	16	G, I, T
C. albicans T28	0.346–1.385	0.614	0	M, G	0.063–>16	0.55	M, I, H	0.25–>16	7.8	G, I, H
C. albicans T32	0.369–1.459	0.752	0	M, G	≤0.032–>16	0.51	M, G, H	0.063–>16	6.0	M, G
C. glabrata J981216	0.210–1.714	0.726	0	M, G	0.063–0.50	0.16	M, G	0.063–1.0	0.28	M, G
C. krusei ATCC 6258	0.163–1.676	0.520	0	M, G, H	0.13–1.0	0.25	M, I, H	0.13–>16	0.90	M, G
C. parapsilosis ATCC 22019	0.195–1.447	0.471	0	M, G, H	0.13–2.0	0.44	M, G, I, H	0.25–>16	2.9	G, T
A. fumigatus J931199	0.105–3.748	0.817	0	T, H	0.063–>16	0.8	I, H	0.13–>16	5.7	M
A. fumigatus J940362	0.087–3.990	0.809	5	T, H	0.063–>16	0.8	T, H	0.13–>16	5.6	M
A. fumigatus J960180	0.101–3.990	0.937	0	T, H	≤0.032–>16	1.0	T, H	0.13–>16	4.6	M
A. fumigatus J960330	0.085–3.644	0.552	6	T, H	≤0.032–>16	1.3	M, H	0.063–>16	5.5	M
A. fumigatus J970377	0.103–3.990	0.737	0	M, T, H	≤0.032–>16	1.3	M, H	0.13–>16	6.6	M, T
A. fumigatus J970398	0.089–3.304	0.582	3	T, H	≤0.032–>16	1.5	M, H	0.13–>16	5.8	M
A. fumigatus J980561	0.033–3.990	0.553	7	T, H	≤0.032–>16	0.9	H	0.13–>16	4.1	M
A. fumigatus J980659	0.106–3.976	0.783	0	G, T, H	0.13–>16	1.3	T, H	0.13–>16	7.3	M

[a] G, glucose concentration; I, initial cell concentration; M, growth medium; S, plates sealed with sticker; T, incubation temperature; H, incubation time. For inclusion in the table, the variable must have been shown to be a significant contributor to OD or MIC variation by ANOVA at the level of $P < 0.01$.
[b] Gmean, geometric mean.

control OD for only a minority of the *Candida* spp., and the use of an adherent sticker to seal the plates was never a significant factor influencing control OD.

For the *A. fumigatus* isolates, incubation time and temperature showed consistently significant influences on control OD values, with higher OD values associated with an incubation time of 48 h as opposed to an incubation time of 24 h.

Multivariate analysis of the caspofungin IC_{50} data showed that more of the variables significantly influenced this parameter than the control OD for the *Candida* sp. isolates (Table 3), while IC_{80}s were generally affected mainly by the medium and glucose concentration, as was control growth. The choice of medium was again the most common significant factor influencing the IC_{50}s for yeasts, with values determined in RPMI 1640 pH 5 generally being considerably higher than those determined in AM3 cultures. With the different glucose concentrations, there was a trend toward higher ICs in 2% glucose than in 0.2% glucose.

For the *A. fumigatus* isolates, the incubation time was the most common factor statistically significantly influencing the IC_{50}s, while the IC_{80}s were most often influenced by the growth medium. The latter result was largely attributable to control growth OD values below 0.15 after 24 h of incubation in AM3. Caspofungin ICs at both endpoints were commonly read as >16 at 24 h and were much lower at 48 h: this phenomenon indicated that the inhibitory effects of the agent after 24 h were inapparent relative to the growth of the weakly grown controls.

Variability of ICs in AM3 from different sources. From the results obtained in this study, AM3 appears to offer the greatest robustness to variation in process variables in caspofungin MIC determinations. However, previous studies have shown that different batches or sources of AM3 have given various results when it is used in antifungal susceptibility tests (21). We therefore applied the multifactorial design approach to study the variation in control ODs and ICs for caspofungin with 14 isolates of *Candida* spp. and 8 isolates of *A. fumigatus* tested in AM3 purchased from three separate suppliers. The variables tested, in addition to the manufacturer of AM3, were pH (unadjusted, therefore, ~7.0 versus a pH adjusted to 5.0), inoculum size (high or low, as in the previous experiments), and incubation time (48 versus 72 h, to overcome the problem of slow growth among isolates of *A. fumigatus*).

For the *Candida* spp., control OD values were little affected by the test variables. The manufacturer of AM3 emerged as a significant variable affecting the control OD for 6 of the 14 isolates tested; however, the level of variation overall was small: the mean ± standard deviation control ODs for the 14 isolates were 0.52 ± 0.14, 0.58 ± 0.17, and 0.68 ± 0.19 for AM3 from Difco, Oxoid, and VWR, respectively. Incubation time significantly affected control OD values for 4 of the 14 *Candida* isolates. For the eight *A. fumigatus* isolates, the manufacturer of AM3 was the main variable influencing control OD values; the ANOVA showed P values <0.001 for all eight isolates. The considerable quantitative difference in the control ODs was reflected by the mean ± standard deviations for all the isolates, regardless of the other variables. These were 1.05 ± 0.22, 1.10 ± 0.15, and 1.92 ± 0.19 for AM3 from Difco, Oxoid, and VWR, respectively. Under all conditions, the control OD for *Candida* isolates and *A. fumigatus* tended to be notably higher

TABLE 4. IC_{50}s and IC_{80}s of caspofungin for 22 fungal isolates in AM3 medium from three different suppliers[a]

Isolate	AM3 from Difco				AM3 from Oxoid				AM3 from VWR			
	48 h		72 h		48 h		72 h		48 h		72 h	
	IC_{50} (μg/ml)	IC_{80} (μg/ml)	IC_{50} (μg/ml)	IC_{80} (μg/ml)	IC_{50} (μg/ml)	IC_{80} (μg/ml)	IC_{50} (μg/ml)	IC_{80} (μg/ml)	IC_{50} (μg/ml)	IC_{80} (μg/ml)	IC_{50} (μg/ml)	IC_{80} (μg/ml)
C. albicans B59630	0.5	0.5	1	1	0.5	1	1	1	0.5	0.5	0.5	1
C. albicans J981309	0.5	0.5	0.5	0.5	0.5	0.5	1	1	0.5	0.5	0.5	0.5
C. albicans J981364	0.5	0.5	0.5	0.5	0.5	0.5	1	1	0.5	0.5	0.5	1
C. albicans J981371	0.5	>16	0.5	0.5	0.5	1	0.5	1	0.5	0.5	1	2
C. albicans J990578	0.5	>16	1	1	0.5	0.5	1	2	1	1	1	1
C. albicans NR2	2	4	2	4	2	2	4	4	4	8	2	8
C. albicans NR3	4	4	4	8	2	4	4	4	2	4	4	>16
C. albicans T25	8	8	8	8	4	4	4	4	4	8	8	8
C. albicans T26	8	8	4	8	4	4	1	4	4	8	4	8
C. albicans T28	>16	>16	2	2	0.5	0.5	0.5	0.5	0.5	0.5	0.5	2
C. albicans T32	>16	>16	1	1	1	1	1	1	0.5	0.5	1	1
C. glabrata J981216	0.5	0.5	0.5	1	0.5	0.5	1	1	0.5	0.5	2	2
C. krusei ATCC 6258	0.5	0.5	0.5	1	0.5	1	1	1	1	1	1	1
C. parapsilosis ATCC 22019	1	1	1	1	1	1	1	2	1	1	1	1
A. fumigatus J931199	0.5	8	1	8	1	4	1	2	1	4	1	8
A. fumigatus J940362	1	2	1	4	2	4	1	8	1	2	4	4
A. fumigatus J960180	0.5	4	1	8	1	1	1	2	0.5	8	2	>16
A. fumigatus J960330	0.5	8	1	2	0.5	1	1	4	1	1	1	1
A. fumigatus J970377	1	16	1	>16	1	>16	1	>16	1	2	16	>16
A. fumigatus J970398	0.5	1	1	1	1	1	1	2	0.5	1	1	1
A. fumigatus J980561	0.5	2	0.5	1	1	1	1	2	1	1	1	1
A. fumigatus J980659/3	1	>16	1	>16	0.5	>16	1	>16	1	1	1	>16

[a] The endpoints were read after 48 and 72 h of incubation.

in AM3 from VWR than in AM3 from Difco or Oxoid. Prolongation of the incubation time to 72 h also led to a significant increase in the control ODs for five of the eight *A. fumigatus* isolates; for each brand of AM3, the mean increase was of the order of 0.2 to 0.3 OD units.

Despite the significant influences of AM3 from different manufacturers (and, less often, incubation time) on control OD values, the caspofungin IC_{50}s and IC_{80}s determined for each strain were seldom affected by any of the four variables examined. For two isolates of *Candida* spp., the ANOVA revealed inoculum size as a significant factor influencing the IC_{50}s. The incubation time significantly affected the IC_{50}s for two of the *A. fumigatus* isolates. No other process variables were found to affect the result for any isolate. Similarly, for the caspofungin IC_{80} data, one *A. fumigatus* isolate was significantly affected by the inoculum size and the incubation time, but no other effects of the process variables on the IC_{80}s for *A. fumigatus* were seen. For the *Candida* spp., pH and inoculum size were significant variables for two isolates each, and the brand of AM3 was a significant variable for one isolate.

The high robustness of the caspofungin IC_{50}s and IC_{80}s in AM3 from different sources is illustrated in Table 4, in which the results obtained after 48 and 72 h of incubation in unmodified AM3 from three suppliers are shown. For 12 of the 14 *Candida* isolates and 7 of the 8 *A. fumigatus* isolates, IC_{50}s varied within a mode ±1 dilution range; for 11 *Candida* isolates and 5 *A. fumigatus* isolates there was only a 1-dilution difference across the six results for three sources of AM3 at two incubation times (Table 4). For the two *C. albicans* isolates for which single aberrant IC_{50}s were recorded, they both arose on the Difco AM3. For *A. fumigatus* J970377, for which the consistency of IC_{50}s was marred by a single value of 16 μg/ml, this value arose in VWR AM3. The variability between replicate IC_{80}s was generally much greater, regardless of the source of AM3.

The data in Table 4 consistently show higher modal IC_{50}s (2 to 4 μg/ml) for *C. albicans* NR2, NR3, T25, and T26 than for the other isolates tested, with modal IC_{50}s of 0.5 or 1.0 μg/ml.

DISCUSSION

As in a previous study with itraconazole (20), the present investigation of multiple combinations of performance variables and their influences on control growth and MICs in microdilution susceptibility tests with caspofungin has confirmed how difficult it is to contrive a single set of consensus conditions that constitute a protocol suitable for all fungal isolates. The NCCLS Antifungal Subcommittee has already acknowledged this difficulty, to the extent that it has published separate reference methods for clinically important yeasts (16) and filamentous fungi (15). Our data suggest, however, that more investigation of performance variables for the testing of caspofungin susceptibility in vitro will be necessary before reference conditions for this agent can be confirmed.

Some of the findings of this study give cause for concern, in particular, that RPMI 1640 buffered at pH 7, recommended for susceptibility testing with both yeasts and molds (15, 16), sustains only poor growth of *A. fumigatus* isolates. We also showed (Table 3) that the incubation time was a significant cause of variation in the caspofungin MIC for *A. fumigatus*, and this effect was related to the poor control growth that was seen for this species at 24 h, regardless of the growth medium. Since caspofungin, like all antifungals in clinical use, exerts its effects exclusively on actively growing fungi (5), it is rational, given the

clinical need for rapid tests, to discount conditions that support at best only slow rates of growth of a test fungus. Moreover, when drug-free controls are only weakly turbid, it is difficult to assess differences in turbidity indicative of growth inhibition, whether this is done visually or by spectrophotometry. We consider that the 48-h incubation period recommended by NCCLS method M38-P for *A. fumigatus* (15) is a minimum acceptable time for testing with caspofungin, since many isolates of this species achieve only slight OD values for turbidity by 24 h, regardless of the choice of medium. In common with other studies (1, 11, 17), we found that the caspofungin MICs determined in AM3 at its unadjusted, unbuffered, native pH of 7.0 tended to be lower for yeasts and *A. fumigatus* than the MICs determined in RPMI 1640 (Table 1). On the basis of all the available data, we generally regard AM3 to be superior to RPMI 1640 for caspofungin susceptibility testing. RPMI 1640 undoubtedly gives satisfactory results for the testing of the susceptibilities of yeasts, but it is frequently unable to provide adequate levels of control growth for *A. fumigatus.* We regard it as undesirable in routine practice, such that different media need to be used, according to the type of fungus tested. However, our data also suggest that the ideal medium for caspofungin susceptibility testing with all types of fungi has yet to be devised.

RPMI 1640 at pH 5, rather than the customary pH 7, was chosen as a comparator medium for AM3 in the multifactorial tests, so that media at pHs below 7 were compared with AM3 for both the *Candida* spp. and the *A. fumigatus* isolates. The primary objective of multifactorial experimental designs is to identify major sources of variation that influence quantitative endpoints. Where the choice of medium emerged as a common factor that significantly influenced susceptibility test outcomes, as it did for the yeasts but not the molds in the study, this result may point to the pH as well as to the medium composition as a factor for inclusion in future efforts for optimization of caspofungin testing in vitro.

Concerns of variability in the growth characteristics of fungi on AM3 from different sources or batches (21) were confirmed with control OD values but not with ICs in our multivariate assessment of the media from three commercial sources; AM3 from VWR gave much higher control OD values for *A. fumigatus* isolates than AM3 from two other suppliers, and the source of medium emerged from ANOVA applied to the control OD data as the most significant source of variation. However, all the fungi tested grew well on AM3, regardless of the supplier, initial pH, inoculum size, or an incubation time of 48 or 72 h, with no data rejected for low control OD values. The caspofungin IC_{50}s determined with variations in AM3 supplier and other process parameters were remarkably robust (Table 4), with ANOVA showing the supplier to be a significant source of variation in the IC_{80}s for just 1 of the 22 fungi tested and with only occasionally significant effects indicated for other process variables as well. Whether this level of robustness in caspofungin MICs would stand a test of interlaboratory replication remains to be determined.

The previously published process analysis of itraconazole susceptibility testing (20) examined more variables than the present study did. These were the nature of the solvent used to dissolve the agent, the shape of the microplate well (flat or round bottomed), the supplier of the growth medium, the medium used to prepare the inoculum, and the incubation atmosphere (CO_2 enriched or free air). Because caspofungin is highly soluble in water, there was no concern about the stock solution solvent used. The previous study showed that the microplate well shape, the supplier of the medium, the source of the inoculum, and the incubation atmosphere seldom or never emerged as variables significantly affecting either control ODs or MICs, so these variables were not reexamined in the present study.

In the multifactorial design, inoculum size rarely had a significant influence on the control OD for *Candida* spp. or *A. fumigatus*, a finding consistent with the results of the experiment whose results are shown in Table 1, in which six different growth media were used for caspofungin susceptibility testing. The NCCLS reference methods for yeasts and filamentous fungi differ in their recommendations for inoculum size, with a starting concentration of 500 to 5,000 cells/ml preferred for yeasts (16) and a higher inoculum of 4,000 to 50,000 conidia/ml preferred for molds (15). Our data suggest that caspofungin ICs are reasonably robust to inoculum variations, although recent unpublished findings from our laboratory suggest that particularly low inoculum sizes may have more serious influences on caspofungin MICs than the levels of inocula used in the present study.

Incubation temperature and time appeared with moderate frequency as significant influences on control OD and ICs (Table 3). For the *A. fumigatus* isolates, both variables significantly affected control OD values, while incubation time significantly affected caspofungin IC_{50}s, but not IC_{80}s, for *A. fumigatus.* One or both parameters also significantly affected the IC_{50}s for several of the *Candida* isolates. In general, the yeasts grew better at 30°C than at 35°C and after longer (48 to 72 h) rather than shorter (24 to 48 h) incubation times. Laboratories are normally reluctant to use incubation temperatures below 35°C for susceptibility testing, because lower temperatures are perceived as removed from physiologic norms. Shorter incubation times are more likely to result in the determination of MIC endpoints during exponential growth phases, a time that was shown for other agents to result in lower variabilities in the MICs than readings determined during the stationary phase (9). Only prospective testing with large numbers of yeast isolates from patients for which the full caspofungin treatment history is known will provide a scientifically adequate basis for determination of the optimum temperatures and times for susceptibility determinations. We consider, however, that, in addition to the observation that many antifungal agents work only against actively growing fungi, it is generally preferable in practice to test for susceptibility under conditions that ensure reasonably high control growth yields; otherwise, the differential between inhibited and uninhibited growth can become too small to give reproducible results, whether growth is judged visually or by spectrophotometry.

The choice of inhibition endpoint for antifungal susceptibility tests is well known to be an important variable. The NCCLS recommendation for broth microdilution tests with azole antifungals and flucytosine with yeasts is to use an endpoint of a "prominent decrease in turbidity," which, in practice, correlates with a 50% reduction in growth, as determined by spectrophotometry (16). For amphotericin B against yeasts, the endpoint is an optically clear well. For tests with filamentous

fungi, similar rules apply; 50% growth reduction is the recommended endpoint for flucytosine, fluconazole, and ketoconazole, while 100% reduction is recommended for other azoles and amphotericin B. Our data with caspofungin suggest that while both the 50 and 80% spectrophotometric endpoints give predominantly on-scale MICs for putatively susceptible yeasts, regardless of other process variables, the 50% endpoint gave less variability for *A. fumigatus* (Tables 2 and 3), and the results in Table 4 show that the 50% endpoint gave more consistent MICs than the 80% endpoint for all isolates tested. For any agent the 50% endpoint is most likely to detect the point of maximum infection on an antifungal dose-response curve, which makes it a more practical endpoint for spectrophotometric determinations.

The IC_{50}s for *C. albicans* strains NR2, NR3, T25, T26, T28, and T32 in this study (Table 4) confirm the initial description of the susceptibilities of these strains to echinocandins (7), with the first four showing lower levels of susceptibility in vitro. NR3, T25, and T26 also showed reduced susceptibilities to echinocandin treatment in a mouse model of disseminated *C. albicans* inflection (7). However, the levels of resistance to caspofungin of NR2, NR3, T25, and T26 in tests in AM3 were only 1 or 2 dilutions higher than those of more susceptible isolates in terms of the IC_{50}. This situation resembles that for amphotericin B, for which similarly low differences in MICs were found between resistant and susceptible isolates (21).

Our study confirms that, with caspofungin, as with itraconazole (20), every individual isolate of yeast and *A. fumigatus* effectively has its own optimum requirements for susceptibility testing, in the sense that some fungal isolates are clearly more sensitive than others to changes in test protocol variables (Tables 2 and 3). AM3 emerges from the present study as a reasonable choice of medium that yields excellent control growth with the whole range of fungal species that we tested (regardless of the glucose concentration) and consistent IC_{50}s for the majority (86%) of the isolates tested. However, the variability in control growth determined for AM3 from different sources and the low level of discrimination between *C. albicans* isolates with less susceptibility to echinocandins in vivo suggest that AM3 may not be the ultimate medium of choice for caspofungin susceptibility testing. With AM3 it should be possible to optimize other test conditions to derive caspofungin MICs with the maximum possible intra- and interlaboratory consistencies, but we regard the question of optimum conditions for caspofungin susceptibility testing as still unresolved. For tests with *A. fumigatus*, a minimum incubation time of 48 h should be used.

ACKNOWLEDGMENTS

This study was supported by a grant from Merck & Co., Inc.
We thank Amanda Davidson for skilled technical assistance.

REFERENCES

1. **Arikan, S., M. Lozano Chiu, V. Paetznick, and J. H. Rex.** 2001. In vitro susceptibility testing methods for caspofungin against *Aspergillus* and *Fusarium* isolates. Antimicrob. Agents Chemother. **45:**327–330.
2. **Barchiesi, F., A. M. Schimizzi, A. W. Fothergill, G. Scalise, and M. G. Rinaldi.** 1999. In vitro activity of the new echinocandin antifungal, MK-0991, against common and uncommon clinical isolates of *Candida* species. Eur. J. Clin. Microbiol. Infect. Dis. **18:**302–304.
3. **Barry, A. L., M. A. Pfaller, S. D. Brown, A. Espinel-Ingroff, M. A. Ghannoum, C. Knapp, R. P. Rennie, J. H. Rex, and M. G. Rinaldi.** 2000. Quality control limits for broth microdilution susceptibility tests of ten antifungal agents. J. Clin. Microbiol. **38:**3457–3459.
4. **Bartizal, K., C. J. Gill, G. K. Abruzzo, A. M. Flattery, L. Kong, P. M. Scott, J. G. Smith, C. E. Leighton, A. Bouffard, J. F. Dropinski, and J. Balkovec.** 1997. In vitro preclinical evaluation studies with the echinocandin antifungal MK-0991 (L-743,872). Antimicrob. Agents Chemother. **41:**2326–2332.
5. **Bowman, J. C., P. S. Hicks, M. B. Kurtz, H. Rosen, D. M. Schmatz, P. A. Liberator, and C. M. Douglas.** 2002. The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus* in vitro. Antimicrob. Agents Chemother. **46:**3001–3012.
6. **Del Poeta, M., W. A. Schell, and J. R. Perfect.** 1997. In vitro antifungal activity of pneumocandin L-743,872 against a variety of clinically important molds. Antimicrob. Agents Chemother. **41:**1835–1836.
7. **Douglas, C. M., J. A. Dippolito, G. J. Shei, M. Meinz, J. Onishi, J. A. Marrinan, W. Li, W. G. K. Abruzzo, A. Flattery, K. Bartizal, A. Mitchell, and M. B. Kurtz.** 1997. Identification of the *FKS1* gene of *Candida albicans* as the essential target of 1,3-β-D-glucan synthase inhibitors. Antimicrob. Agents Chemother. **41:**2471–2479.
8. **Espinel-Ingroff, A.** 1998. Comparison of in vitro activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743,872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. J. Clin. Microbiol. **36:**2950–2956.
9. **Galgiani, J. A., and D. A. Stevens.** 1976. Antimicrobial susceptibility testing of yeasts: a turbidimetric technique independent of inoculum size. Antimicrob. Agents Chemother. **10:**721–726.
10. **Keating, G. M., and B. Jarvis.** 2001. Caspofungin. Drugs **61:**1121–1129.
11. **Krishnarao, T. V., and J. N. Galgiani.** 1997. Comparison of the in vitro activities of the echinocandin LY303366, the pneumocandin MK-0991, and fluconazole against *Candida* species and *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **41:**1957–1960.
12. **Kurtz, M. B., I. B. Heath, J. Marrinan, S. Dreikorn, J. Onishi, and C. Douglas.** 1994. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. **38:**1480–1489.
13. **Lozano-Chiu, M., P. W. Nelson, V. L. Paetznick, and J. H. Rex.** 1999. Disk diffusion method for determining susceptibilities of *Candida* spp. to MK-0991. J. Clin. Microbiol. **37:**1625–1627.
14. **Marco, F., M. A. Pfaller, S. A. Messer, and R. N. Jones.** 1998. Activity of Mk-0991 (L-743,872), a new echinocandin, compared with those of LY303366 and four other antifungal agents tested against blood stream isolates of *Candida* spp. Diagn. Microbiol. Infect. Dis. **32:**33–37.
15. **National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi; approved standard, M38-A, vol. 18 no. 3. National Committee for Clinical Laboratory Standards, Wayne, Pa.
16. **National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard, 2nd ed. M27-A2. National Committee for Clinical Laboratory Standards, Wayne, Pa.
17. **Nelson, P. W., M. Lozano-Chiu, and J. H. Rex.** 1997. In vitro growth-inhibitory activity of pneumocandins L-733,560 and L-743,872 against putatively amphotericin B- and fluconazole-resistant *Candida* isolates—influence of assay conditions. J. Med. Vet. Mycol. **35:**285–287.
18. **Pfaller, M. A., R. N. Jones, G. V. Doern, A. C. Fluit, J. Verhoef, H. S. Sader, S. A. Messer, A. Houston, S. Coffman, and R. J. Hollis.** 1999. International surveillance of blood stream infections due to *Candida* species in the European SENTRY program: species distribution and antifungal susceptibility including the investigational triazole and echinocandin agents. Diagn. Microbiol. Infect. Dis. **35:**19–25.
19. **Pfaller, M. A., F. Marco, S. A. Messer, and R. N. Jones.** 1998. In vitro activity of two echinocandin derivatives, LY303366 and MK-0991 (L-743,792), against clinical isolates of *Aspergillus*, *Fusarium*, *Rhizopus*, and other filamentous fungi. Diagn. Microbiol. Infect. Dis. **30:**251–255.
20. **Rambali, B., J. A. Fernandez, L. Van Nuffel, F. Woestenborghs, L. Baert, D. L. Massart, and F. C. Odds.** 2001. Susceptibility testing of pathogenic fungi with itraconazole: a process analysis of test variables. J. Antimicrob. Chemother. **48:**163–177.
21. **Rex, J. H., M. A. Pfaller, T. J. Walsh, V. Chaturvedi, A. Espinel-Ingroff, M. A. Ghannoum, L. L. Gosey, F. C. Odds, M. G. Rinaldi, D. J. Sheehan, and D. W. Warnock.** 2001. Antifungal susceptibility testing: practical aspects and current challenges. Antimicrob. Agents Chemother. **14:**643–658.