Interlaboratory Reproducibility of Etest Amphotericin B and Caspofungin Yeast Susceptibility Testing and Comparison with the CLSI Method

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S. Ranque,[a] L. Lachaud,[b] M. Gari-Toussaint,[c] A. Michel-Nguyen,[a] M. Mallié,[d] J. Gaudart,[e] and S. Bertout[d]

Parasitology and Mycology, CHU Timone, Aix-Marseille Université, Marseille, France[a]; Laboratoire de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Nîmes, Université Montpellier I, Montpellier, France[b]; Parasitology and Mycology, Archet 2 Hospital, Nice, France[c]; UMI 233 Université Montpellier I/IRD/UCAD/UY1, TransVIHMI, UFR Sciences Pharmaceutiques et Biologiques, Montpellier, France[d]; and Aix-Marseille Université, LERTIM EA 3283, Marseille, France[e]

This study aimed to assess the interlaboratory reproducibility at four university hospital laboratories in the southeast region of France of the Etest technique for the determination of caspofungin (CAS) and amphotericin B (AMB) MICs and to compare it to the CLSI broth microdilution reference method. Consecutive clinical yeast isolates ($n = 198$) were included in the study. AMB and CAS MICs were read at 24 and 48 h. Interlaboratory reproducibility was estimated by using (i) an intraclass correlation coefficient (ICC), (ii) essential agreement (EA), and (iii) categorical agreement (CA). For Etest interlaboratory reproducibility for CAS, ICCs were 0.80 (95% confidence interval [CI], 0.76 to 0.84) and 0.81 (95% CI, 0.77 to 0.85) at 24 and 48 h, respectively. For AMB, the ICCs were 0.51 (95% CI, 0.43 to 0.58) and 0.69 (95% CI, 0.63 to 0.74) at 24 and 48 h, respectively. At 48 h, the between-center EAs ranged from 94.4 to 99.0% for both antifungals. For the comparison of the CLSI method and the Etest, the between-technique ICCs were 0.69 (95% CI, 0.63 to 0.74) and 0.62 (95% CI, 0.55 to 0.68) for CAS and AMB, respectively. The EAs ranged from 76.5 to 98.5% for CAS and from 90.3 to 97.4% for AMB according to the centers. CAs ranged from 87.9% to 91.4%, with four very major errors for 2 strains (1 *Candida albicans* strain and 1 *Candida krusei* strain), for CAS and from 97.5 to 99.5%, with four major errors, for AMB. In conclusion, the Etest showed a good interlaboratory reproducibility and a good correlation with the CLSI technique. It is well suited for the routine clinical laboratory and can thus be used to monitor clinical yeast isolates' *in vitro* susceptibilities in this setting.

Since the 1990s, knowledge about the diversity of yeast species involved in human infections, the incidence of drug-resistant isolates, and antifungal drug resistance mechanisms has significantly increase (6, 10, 16, 24). *In vitro* susceptibility tests are based on the measurement of growth with different drug concentrations so as to determine the MIC for the population of a given isolate, an *in vitro*-determined value that helps predict therapeutic efficacy (1). This has been achieved with some degree of confidence by using *in vivo* models to determine clinical breakpoints in invasive yeast infections, providing a useful indicator to guide therapeutic choices (20). The reference tests for susceptibility testing are the broth microdilution assays devised by the Clinical and Laboratory Standards Institute (CLSI) and by the European Committee on Antibiotic Susceptibility Testing (EUCAST) (5, 23). These reference methods are robust and reproducible; however, they remain time-consuming and poorly suited for the routine clinical laboratory setting. Moreover, the MIC values for amphotericin B are tightly clustered, and these methods rarely detect MIC values above 1 mg/liter (2). To overcome these limitations, many commercially available methods, such as the Etest, Sensititre YeastOne, or disk diffusion methods, that are easy to use in the routine setting have been developed. These methods have been recently incorporated into routine clinical laboratory practice and thus generate a considerable amount of antifungal MIC data from clinical fungal isolates. Presently, the monitoring of antifungal drug susceptibility is usually restricted to national reference laboratories that use broth microdilution assays to test clinical isolates referred from collaborating clinical laboratories. These laboratories thus collect invaluable data for the monitoring of susceptibility trends on national and international scales. However, there is a need to develop antifungal susceptibility monitoring at a local or regional scale. This complementary approach to the national reference centers could also improve patient care and generate significant cost reductions given the prevalence of yeast infections, their morbidity, and the costly protracted treatments required.

As a first step toward setting up a regional survey of *in vitro* antifungal susceptibility in the southeast region of France, the primary aim of the present study was to assess the interlaboratory reproducibility of MICs determined with the commercially available and routinely used Etest method for yeast isolated in first-line clinical mycology laboratories of the four regional teaching hospitals. The secondary aims were to validate the correlation of the MICs of amphotericin B and caspofungin obtained with the Etest and CLSI assays at 24 h and 48 h.

MATERIALS AND METHODS

The study was conducted by the Groupe d'Etude en Mycologie du Sud de la France (GEMSUD), a study group in medical mycology bringing together the parasitology and mycology laboratories of the four teaching hospitals located in the southeast region of France, namely, Marseille, Montpellier, Nice, and Nîmes.

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Address correspondence to S. Ranque, stephane.ranque@ap-hm.fr.

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TABLE 1 Characteristics of the yeast isolates collected in different centers

Isolate characteristic	No. (%) of isolates collected in:				Total no. (%) of isolates collected
	Marseille	Montpellier	Nice	Nîmes	
Species					
C. albicans	29 (58)	31 (62)	37 (74)	21 (42)	118 (59)
C. glabrata	4 (8)	5 (10)	5 (10)	15 (30)	29 (15)
C. parapsilosis	6 (12)	4 (8)	0 (0)	6 (12)	16 (8)
C. tropicalis	3 (6)	4 (8)	2 (4)	4 (8)	13 (7)
C. krusei	3 (6)	3 (6)	3 (6)	1 (2)	10 (5)
C. lusitaniae	1 (2)	2 (4)	0 (0)	1 (2)	4 (2)
C. kefyr	1 (2)	0 (0)	2 (4)	0 (0)	3 (2)
Others	3 (6)	1 (2)	1 (2)	2 (4)	7 (4)
C. famata	1				1
C. rugosa	1				1
C. guilliermondii	1				1
C. dublininensis			1		1
C. inconspicua				1	1
C. sake		1			1
C. sphaerica				1	1
Wards					
Medicine	20 (40)	18 (36)	16 (32)	16 (32)	70 (35)
Intensive care	8 (16)	15 (30)	5 (10)	15 (30)	43 (22)
Oncology-hematology	9 (18)	2 (4)	11 (22)	4 (8)	26 (13)
Surgery	9 (18)	7 (14)	6 (12)	3 (6)	25 (13)
Gynecology	0 (0)	2 (4)	4 (8)	7 (14)	13 (7)
Infectious diseases	0 (0)	3 (6)	4 (8)	5 (10)	12 (6)
Pediatrics	4 (8)	3 (6)	4 (8)	0 (0)	11 (6)
Specimens					
Lower respiratory tract	30 (60)	16 (32)	20 (40)	5 (10)	71 (36)
Urine	2 (4)	17 (34)	8 (16)	16 (32)	43 (22)
Upper respiratory tract	6 (12)	4 (8)	5 (10)	9 (18)	24 (12)
Stools	4 (8)	3 (6)	7 (14)	6 (12)	20 (10)
Skin	2 (4)	3 (6)	2 (4)	5 (10)	12 (6)
Vaginal	0 (0)	0 (0)	4 (8)	6 (12)	10 (5)
Digestive tract	2 (4)	1 (2)	2 (4)	2 (4)	7 (4)
Normally sterile fluids	1 (2)	3 (6)	2 (4)	1 (2)	7 (4)
Tissues (biopsy specimens)	2 (4)	2 (4)	0 (0)	0 (0)	3 (2)
Blood	1 (2)	1 (2)	0 (0)	0 (0)	2 (1)

Yeast isolates. From September to November 2008, each participating laboratory prospectively collected 50 yeast isolates cultured on Sabouraud dextrose agar with antibacterials (gentamicin and chloramphenicol), from distinct consecutive patients. Purity was checked by subculturing on Chromagar Candida medium (Becton Dickinson, France), and the yeast anamorphs were routinely identified by using routine methods. Finally, 200 *Candida* isolates were collected from different patient samples (Table 1). A total of 118 (59%) isolates were identified as *Candida albicans*, and 29 (15%) isolates were identified as *Candida glabrata*.

Antifungal susceptibility methods. The MICs of amphotericin B and caspofungin were determined by using both the CLSI and Etest assays. The CLSI method was performed in one center (Faculty of Pharmacy, Montpellier), while the Etest was performed in each of the four participating laboratories to determine amphotericin B and caspofungin MICs for the 200 yeasts isolates. The isolates were anonymized by using distinct labels for each center, thus allowing blinding to other centers' test results. The CLSI assay was performed as recommended previously (3, 5). The Etest assay was performed as recommended by the manufacturer (AB Biodisk, Sweden). Each assay was validated by using quality control strains ATCC 22019 (*Candida parapsilosis*) and ATCC 6258 (*Candida krusei*).

Statistical analysis. MIC data are presented as the range, MIC_{50}, and MIC_{90} for each species.

The interlaboratory reproducibility of the Etest assay was evaluated by calculating the intraclass correlation coefficient (ICC) and its 95% confidence interval (CI), which measure the consistency of the MIC values between the centers, as well as the essential agreement (EA) within $\pm 2 \log_2$ dilutions for both amphotericin B and caspofungin. The MICs were log transformed to approximate a normal distribution. The ICC is a reverse measurement of the variability of a quantitative variable; it has a maximum value of 1 if there is a perfect correlation and a minimum value of 0 if there is a complete absence of a correlation. The ICC is the coefficient which has the highest statistical power for correlation studies (14).

The overall agreement between the results of the Etest and the CLSI assays at 48 h was measured by using the ICC and the categorical agreement (CA). The interpretative breakpoints used for amphotericin B were susceptible at <1 mg/liter and resistant at ≥ 1 mg/liter (2). For caspofungin, the new interpretative breakpoints proposed by Pfaller et al. (19) for *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. guilliermondii* were used for the CLSI method; for other species, and as recommended by the manufacturer for the Etest method, a 2-mg/liter breakpoint was used (susceptible at ≤ 2 mg/liter and not susceptible at >2 mg/liter) (3). A very major error (VME) occurred when an isolate that was determined to be resistant by the reference CLSI assay was categorized as susceptible by the Etest assay in at least one center. A major error (ME) occurred when an isolate that was determined to be susceptible by the

TABLE 2 Amphotericin B and caspofungin MIC_{50}s and MIC_{90}s obtained with the CLSI method and the Etest method in the four centers at the 48-h endpoint reading according to *Candida* species

Drug and *Candida* sp.	No. of isolates	MIC value (mg/liter)															
					Etest												
		CLSI test at 48 h			Marseille			Montpellier			Nice			Nîmes			
		MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
Amphotericin B																	
C. albicans	117	0.125–1	0.25	0.5	0.047–0.25	0.125	0.19	0.047–0.38	0.19	0.25	0.032–1	0.19	0.25	0.047–0.38	0.19	0.25
C. glabrata	28	0.125–1	0.5	1	0.064–0.5	0.25	0.5	0.125–1	0.5	0.75	0.094–1.5	0.38	1	0.125–0.75	0.38	0.5
C. krusei	10	0.5–1	1	1	0.5–1.5	0.75	1	1.5–3	1.5	3	0.064–3	1	2	0.38–2	0.5	1.5
C. parapsilosis	16	0.25–1	0.5	1	0.125–0.75	0.38	0.5	0.25–4	0.5	1	0.064–0.75	0.25	0.75	0.25–1	0.5	1
C. tropicalis	13	0.06–1	1	1	0.094–.38	0.125	0.38	0.094–1.5	0.38	0.75	0.064–0.5	0.19	0.38	0.032–0.5	0.19	0.38
Others	14	0.03–1	0.25	0.5	0.047–0.38	0.125	0.38	0.047–0.5	0.125	0.5	0.016–0.5	0.125	0.38	0.032–0.5	0.125	0.38
All species	198	0.03–1	0.25	1	0.047-1.5	0.125	0.38	0.047–4	0.25	0.75	0.016–3	0.19	0.5	0.032–2	0.19	0.5
Caspofungin																	
C. albicans	117	0.125–1	0.125	0.25	0.016–0.38	0.047	0.094	0.032–1.5	0.094	0.19	0.016–0.38	0.064	0.125	0.016–0.25	0.032	0.064
C. glabrata	28	0.125–0.25	0.25	0.25	0.094–0.38	0.125	0.19	0.094–0.38	0.19	0.25	0.094–0.64	0.125	0.38	0.047–0.25	0.125	0.125
C. krusei	10	0.25–1	0.5	0.5	0.38–0.75	0.5	0.75	0.5–3	0.75	1	0.25–1	0.5	1	0.125–0.75	0.38	0.5
C. parapsilosis	16	0.125–1	0.5	1	0.19–1	0.5	1	0.5–1.5	1	1.5	0.094–0.75	0.38	0.75	0.38–32	0.38	1
C. tropicalis	13	0.125–0.5	0.125	0.4	0.047–0.19	0.064	0.19	0.064–0.38	0.125	0.25	0.064–0.25	0.125	0.19	0.032–0.38	0.094	0.125
Others	14	0.125–0.5	0.25	0.5	0.032–0.5	0.094	0.5	0.064–1.5	0.19	0.5	0.016–1.5	0.125	0.25	0.016–0.5	0.64	0.38
All species	198	0.125–1	0.125	0.5	0.016–1	0.064	0.38	0.032–3	0.125	0.5	0.016–1.5	0.094	0.38	0.016–32	0.047	0.38

CLSI assay was categorized as resistant by the Etest assay. A minor error (mE) occurred when an isolate that was determined to have intermediate resistance by the CLSI assay was categorized as either sensitive or resistant by the Etest assay. The EA and CA were analyzed at 48 h.

The effect of the yeast species on amphotericin B and caspofungin MICs was tested by using analysis of variance (ANOVA) with Tukey *post hoc* tests to control the type I error. Analyses were performed with SPSS 17.0.2 statistical software (SPSS Inc.); all tests were two sided, and a P value of <0.05 was considered statistically significant.

RESULTS

Details of hospitalization wards, specimens, and *Candida* species collected by the different centers are presented in Table 1. Most of the *Candida* strains were isolated in lower respiratory tract (36%) and urine (22%) specimens from patients hospitalized in medical wards. As expected, *Candida albicans* was the predominant species (59%). Finally, MIC results for caspofungin and amphotericin B for the four centers could be analyzed for 198 isolates by using both methods. The MIC values of the three quality controls strains were within the expected value for each experiment and both antifungals with the Etest and the CLSI method.

Etest interlaboratory reproducibility. Caspofungin MIC values at 24 h were (i) lower than 0.25 mg/liter for all the *C. albicans* isolates by both the CLSI and Etest assays in 2 centers and (ii) higher than 0.12 mg/liter for 1 to 10 *C. glabrata* strains by the Etest assay (depending on the center) and at least 0.25 mg/liter for 13 *C. glabrata* strains by the CLSI assay. At 48 h, 10 strains (1 *C. albicans*, 2 *C. glabrata*, and 7 *C. krusei* strains) were categorized as being resistant to caspofungin by at least one method in at least one center. At 48 h, caspofungin MIC_{50} and MIC_{90} values ranged from 0.047 to 0.125 mg/liter and from 0.38 to 0.75 mg/liter, respectively, whatever the species (Table 2). For all centers, caspofungin MIC_{90} values were $\leq$1 mg/liter for all species except for *Candida parapsilosis*, where the caspofungin MIC_{50} and MIC_{90} ranged from 0.5 to 1 mg/liter and from 0.75 to 1.5 mg/liter, respectively. The overall Etest interlaboratory reproducibility was highly significant ($P < 10^{-4}$) for caspofungin, with ICCs of 0.80 (95% CI, 0.761 to 0.842) and 0.81 (95% CI, 0.771 to 0.846) at 24 and 48 h, respectively. Noticeably, 24 or 48 h of incubation did not significantly impact the reproducibility of Etest caspofungin MICs. The 2-by-2 center EA of caspofungin MICs ranged from 95.48% to 98.99%, with a mean EA of 97.06%.

Amphotericin MIC values were >1 mg/liter in at least one center for 3 and 15 *Candida* isolates at 24 h and 48 h, respectively. MICs of >1 mg/liter were reported by more than one center for 4 of these 15 isolates; for the 11 remaining isolates, this MIC of >1 mg/liter was a single-center finding. At 48 h, amphotericin MIC_{50} and MIC_{90} values ranged from 0.125 to 0.25 mg/liter and from 0.38 to 0.75 mg/liter, respectively, whatever the species considered (Table 2). With regard to *C. krusei*, the mean MIC values by center ranged from 0.75 to 1.85 mg/liter. The overall Etest interlaboratory reproducibility of amphotericin B testing was relatively lower than that for caspofungin and higher at 48 h than at 24 h, with ICCs of 0.509 (95% CI, 0.433 to 0.584) and 0.687 (95% CI, 0.630 to 0.740) at 24 and 48 h, respectively. The 2-by-2 center EA of amphotericin MICs ranged from 94.42 to 98.99%, with a mean EA of 97.54%.

Comparison of Etest and CLSI results. The between-technique ICCs were 0.69 (95% CI, 0.63 to 0.74) and 0.62 (95% CI, 0.55 to 0.68) for CAS and AMB, respectively. EAs ranged from 76.5 to 98.5% for CAS and from 90.3 to 97.4% for AMB according to the centers. All isolates were categorized as being susceptible to amphotericin B by both the CLSI and Etest assays at 24 h of incubation whatever the center. Hence, the CA was perfect at 24 h and ranged from 97.5 to 99.5%, with four major errors at 48 h, depending on the center. The discrepancies are detailed in Table 3, with five major errors corresponding to four isolates (three *C. krusei* isolates and one *C. parapsilosis* isolate) that were categorized as sensitive by the CLSI method and resistant by the Etest.

For caspofungin, the CAs between the Etest and CLSI methods were very similar whatever the center and ranged from 88.38 to 88.88%. Seven very major errors (VME) were observed: one *C. albicans* isolate was categorized as being resistant (MIC = 1 mg/liter) by the CLSI assay and sensitive (MIC = 0.047 or 0.094 mg/liter) by the Etest method in the four laboratories (Table 4), and

TABLE 3 Details of amphotericin B MICs of five isolates that displayed one major categorical error in at least one center with the Etest or CLSI method[a]

Species	MIC (mg/liter) determined by:				
	Etest				CLSI method
	Montpellier	Marseille	Nice	Nîmes	
C. krusei	1.5	1.5	1	0.5	1
C. krusei	2	0.75	2	0.38	1
C. parapsilosis	4	0.25	0.19	0.75	0.5
C. krusei	3	0.75	3	2	1

[a] Outlying MICs are underlined.

one *C. krusei* isolate was resistant (MIC = 1 mg/liter) by the CLSI assay and sensitive (MIC = 0.38 mg/liter) by the Etest method in three laboratories. Using the interpretative breakpoints proposed previously by Pfaller (19) for the Etest method, the CA ranged from 87.87 to 91.41%, with three VME (Table 4).

Interspecies MIC differences. Amphotericin B and caspofungin MICs determined by the CLSI method and Etest at 48 h were statistically different with respect to yeast species ($P < 10^{-4}$). Caspofungin MICs could be split into two homogeneous groups by using Tukey *post hoc* tests. The caspofungin MICs were significantly higher for *C. krusei* and *C. parapsilosis* than for the group that included all other yeast species. Amphotericin B MICs could be split into three groups with statistically significantly increasing MIC values: the group (referred to as "others" in Table 2) that included all rarely isolated species with relatively low amphotericin B MICs; the group that included *C. albicans*, *C. glabrata*, *C. kefyr*, *C. parapsilosis*, and *C. tropicalis*; and, lastly, *C. krusei* species for which MICs were significantly higher than those for the two other groups. Indeed, the 10 *C. krusei* isolates studied displayed relatively elevated MICs for amphotericin B, as shown in Table 2.

DISCUSSION

The good interlaboratory reproducibility of the MICs obtained by using Etest yeast antifungal sensitivity testing, as evidenced by our findings, paves the way toward a clinical laboratory-based regional survey of antifungal drug susceptibility. The good interlaboratory reproducibility of Etest susceptibility testing of azoles was established previously by Matar et al. (12). Our findings for amphotericin B Etest susceptibility testing are in line with those reported previously by Pfaller et al. (22). However, this study is the first to report the interlaboratory reproducibility of Etest susceptibility testing of caspofungin. This study's design had both strengths and limitations. On the one hand, the isolates studied were representative of the routine activity in clinical laboratories of teaching hospitals; on the other hand, the CA analysis was poorly informative, because most of the strains studied had "wild-type" MICs and thus were very seldom classified as resistant. The Etest MICs were also highly correlated to those obtained with the CLSI assay. For the Etest method, we also demonstrated that whatever the breakpoints considered, the CAs between both methods were very good, and the VME observed in both cases corresponded to the same isolates, one *C. albicans* isolate and one *C. krusei* isolate. This excellent correlation between the CLSI and the Etest assays for caspofungin *in vitro* susceptibility testing was in keeping with that reported previously by others (18). However, this correlation was overall lower for amphotericin B than for caspofungin, in line with data reported previously by Fleck et al. (7), who found 89% and 98% EAs for amphotericin B and caspofungin, respectively. With a different study design and comparing the Etest to the EUCAST broth dilution assay, Dannaoui et al. (4) previously found a 75%

TABLE 4 Details of categorical agreement errors for caspofungin testing by Etest[e] in each center compared to the CLSI reference method, using new interpretative breakpoints depending on species[f]

Testing site	Categorical error[g]	No. of isolates with error					Total no. of errors	Categorical agreement (%)
		C. albicans ($n = 116$)	*C. glabrata* ($n = 28$)	*C. krusei* ($n = 10$)	*C. tropicalis* ($n = 13$)	*C. parapsilosis* and others ($n = 31$)		
Montpellier	mE	0	15	5[b]	1[d]	0	22	88.88
	ME	0	0	0	0	0		
	VME	1[a]	0	0	0	0		
Marseille	mE	0	15	5[b]	1[d]	0	23	88.38
	ME	0	0	0	0	0		
	VME	1[a]	0	1[c]	0	0		
Nice	mE	0	15	5[b]	1[d]	0	23	88.38
	ME	0	0	0	0	0		
	VME	1[a]	0	1[c]	0	0		
Nîmes	mE	0	15	5[b]	1[d]	0	23	88.38
	ME	0	0	0	0	0		
	VME	1[a]	0	1[c]	0	0		

[a] The same *C. albicans* isolate in the 4 centers.
[b] The same *C. krusei* isolates in the 4 centers.
[c] The same *C. krusei* isolate in 3 centers.
[d] The same *C. tropicalis* isolate in the 4 centers.
[e] Considered breakpoint of 2 mg/liter.
[f] See reference 19.
[g] mE, minor error; ME, major error; VME, very major error.

EA for both drugs and 77% and 88% CAs for amphotericin B and caspofungin, respectively.

Unsurprisingly, we found elevated MICs of caspofungin for *C. parapsilosis*, since it is known that the usually significantly higher MICs of caspofungin are the consequence of a recently identified functional point mutation in the Fks1 gene of this species (8). Our finding of significantly elevated caspofungin MICs for *C. krusei* was also in keeping with previous reports by others (17, 19). Noteworthy, the MIC_{50} of the 10 *C. krusei* strains studied encompassed the newly revised CLSI clinical breakpoints defining caspofungin resistance at an MIC of >0.5 mg/liter (19). Furthermore, *C. krusei* displayed significantly higher MICs of amphotericin B, as evidenced by the results of the Etest but not the CLSI assay. The discrepancy between the results of these two assays is in keeping with the fact that amphotericin B resistance is more easily detected by the Etest than by broth microdilution techniques (11, 21). The emergence of a multidrug resistance profile of *C. krusei* was recently reported (17). Fortunately, this species remains relatively infrequently isolated in the clinical setting (9, 13, 15).

In conclusion, our findings for amphotericin B and caspofungin indicate that Etest susceptibility testing results are reproducible between laboratories and correlate well with the results of the reference broth microdilution CLSI technique. The Etest assay is well suited to the routine clinical laboratory and can thus be used in this setting to monitor the *in vitro* susceptibilities of clinical yeast isolates. Altogether, these findings strongly support the relevance of implementing hospital laboratory-based regional monitoring of yeast epidemiology and Etest-based antifungal drug susceptibility.

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