Departamento de Ciências Biológicas[1], Universidade Federal de Alfena (UNIFAL-MG), Alfenas-MG, Instituto Adolfo Lutz[2], São Paulo – SP, Instituto de Pesquisa e Desenvolvimento da Universidade do Vale do Paraíba[3], São José dos Campos, SP, Brazil

Correlation between CLSI, EUCAST and Etest methodologies for amphotericin B and fluconazole antifungal susceptibility testing of *Candida* spp. clinical isolates

A. L. R. Claudino[1], R. F. Peixoto Jr.[1], M. S. C. Melhem[2], M. W. Szeszs[2], J. P. Lyon[3], J. K. Chavasco[1], M. C. Franco[1]

Received September 13, 2007, accepted October 31, 2007

Dr. Juliana Pereira Lyon, Instituto de Pesquisa e Desenvolvimento da Universidade do Vale do Paraíba, São José dos Campos-SP, Brasil. Av. Shishima Hifumi, 2911 Zip Code: 12244-000 — Bairro Urbanova, São José dos Campos — SP
julianalyon@yahoo.com.br

Pharmazie 63: 286–289 (2008) *doi: 10.1691/ph.2008.7741*

This study analyzed the correlation between the results obtained through two microdilution methods: Clinical and Laboratory Standard Institute (CLSI) (M27-A2) and European Committee on Antibiotic Susceptibility Testing (EUCAST) (document E. Dis. 7.1) and an agar base method Etest for determining minimmun inhibitory concentration (MIC) for amphotericin B and fluconazole against 30 clinical isolates of *Candida* spp. The agreement between Etest, CLSI and EUCAST MICs within $\pm 2 \log_2$ dilutions was higher for amphotericin B than for fluconazole However, Pearson correlation demonstrated a greater agreement for fluconazole. The categorical agreement between MICs provided by the Etest/CLSI and Etest/EUCAST methodologies was high for both amphotericin B (100%) and fluconazole ($\geq$ 96,66%). This study demonstrated the adequacy of Etest method using Mueller Hinton agar to evaluate amphotericin B and fluconazole susceptibility of clinical isolates of *Candida* spp.

1. Introduction

Many studies have analyzed the correlation among the CLSI methodology for antifungal susceptibility testing and various commercially available systems (Sewell et al. 1994; Wanger et al. 1995; Eldere et al. 1996; Arendrup et al. 2001; Barry et al. 2002; Pfaller et al. 2004; Fleck et al. 2007), including some suitable methods for *Candida* spp. However, few studies (Chrysanthou and Cuenca-Estrela 2002; Dias et al. 2006) have compared the EUCAST procedure with commercial systems. Etest has been investigated as a suitable antifungal susceptibility testing in several studies (Pfaller et al. 1998; Peyron et al. 2001; Pfaller et al. 2003). Earlier studies demonstrated that Mueller-Hinton agar (MHA) can be employed for determining MICs for *Candida* by Etest method (Pfaller et al. 2003). Furthermore, Barry et al. (2002) affirm that MHA supplemented with 2% glucose and methylene blue (0.5 μg/ml) (MH-GMB) supported the growth of essentially all species of *Candida* and was superior to RPMI agar with 2% glucose for fluconazole disk diffusion testing. The Clinical and Laboratory Standards Institute (CLSI) has adopted MH-GMB as the choice medium for disk diffusion testing of *Candida* spp. (NCCLS 2002a).
Pfaller et al. (2003) showed that Etest method may identify *C. glabrata* isolates that express resistance to fluconazole but no agar-based method can differentiate fluconazole susceptible strains from fluconazole susceptible dose-dependent isolates. Nevertheless, a disadvantage of diffusion tests is the difficulty associated with endpoint interpretation, due to the growth of micro-colonies in the inhibition zone, leading to lower reproducibility when the test is performed by several technicians; for this reason the broth microdilution method is recommend for accurate antifungal susceptibility testing (Arendrup et al. 2001).
The aim of this study was to compare MICs obtained by the proposed CLSI reference method M27-A2 (NCCLS 2002b) and AFST-EUCAST (Cuenca-Estrela et al. 2003) broth microdilution method with those obtained by the Etest agar-based method for determining the *in vitro* susceptibilities of *Candida* species to amphotericin B and fluconazole, the two most widely used antifungal agents.

2. Investigations and results

The susceptibility profile of 30 *Candida* spp. isolates to amphotericin B and fluconazole as determined by the three methods are summarized in Table 1. In general, MICs obtained by all methods appear to be similar. However, in spite of one fluconazole-resistant isolate, fluconazole MIC at which 90% of the isolates tested was inhibited (MIC90) were in the "S" category (MIC $\leq$1.0 μg/ml) by all methodologies studied. The amphotericin B results were consistent with those reported previously (Cuenca-Estrella et al. 2003).
The comparison of MICs obtained by Etest with CLSI and AFST-EUCAST methodologies resulted in a considerable Pearson correlation index varying from 0.51 to 1.0.

Table 1: Amphotericin B and fluconazole Etest, AFST-EUCAST and CLSI MIC data for *Candida* spp isolates and Pearson correlation coefficient among MIC values obtained by each method

Antifungal agent	Test method	MIC (μg ml^{-1})[a]			Pearson Correlation Coefficient (r) Etest[b]
		Range	50%	90%	
Amphotericin B	EUCAST	0.015–0.25	0.06	0.12	0.77
	CLSI	0.015–0.25	0.15	0.12	0.51
	Etest	0.006–0.5	0.047	0.016	–
Fluconazole	EUCAST	0.12–≥64	0.25	1.0	0.98
	CLSI	0.12–≥64	0.012	0,5	1.0
	Etest	0.38–48	0.25	1.0	–

[a] 50 and 90% MICs that inhibited 50 and 90% of the isolates tested, respectively
[b] Person correlation coefficient (The correlation coefficient can range from −1 to +1, with −1 indicating a perfect negative correlation, +1 indicating a perfect positive correlation, and 0 indicating no correlation at all) among Etest with two standards methods CLSI. $P < 0.005$

The correlation among Etest/CLSI and Etest/EUCAST methodologies to fluconazole shows higher (0.98 and 1.0) than amphotericin B (0.51 and 0.77) (Table 1). However, the overall level of agreement (Table 2) among the results (Etest MICs within $\pm 2 \log_2$ dilutions of CLSI and $\pm 2 \log_2$ dilutions of AFST-EUCAST) was higher for amphotericin B (Etest/CLSI 93.33% and Etest/EUCAST 90%) than for fluconazole (Etest/CLSI 66.66% and Etest/EUCAST 86,66%).

The *in vitro* interpretive antifungal susceptibility classification of the *Candida* spp. isolates as "S", "S-DD" or "I" and "R" is summarized in Table 3. MICs for amphotericin B and fluconazole tended to be clustered mainly within the limits of the "S" category. Both antifungal agents showed high category agreement (Table 3).

Wanger et al. (1995) and Law et al. (1997) have demonstrated that the Etest provided a sensitive method for detection of amphotericin B-resistant strains of *Candida*. According to the results of the three methods, no putative resistant isolate to amphotericin B was observed.

Two fluconazole-resistant isolates were observed by the AFST-EUCAST method and one by CLSI and Etest methods. One of the classifications for fluconazole yielded by alternative method Etest represented 'minor' discrepancies with respect to the AFST-EUCAST method (Table 3).

3. Discussion

The present study evaluated the concordance among the CLSI and AFST-EUCAST reference method and Etest commercial techniques. The CLSI M27-A2 (formerly NCCLS) reference procedure has proved to be a reliable and reproducible method for susceptibility testing of yeast since its introduction in 1997 (NCCLS 2002b). However, despite the considerable effort required by the development and evaluation of this procedure, the CLSI method still has some unresolved limitations, and these include the trailing growth phenomenon seen in tests with azole antifungal agents, the unreliable detection of resistance to amphotericin B, the subjective visual determination of MIC endpoint, and the need for an extended turnaround time to obtain the MIC (Pfaller et al. 1990). Reports have indicated that the Etest provides better discrimination between amphotericin B susceptible and resistant *Candida* isolates than does the CLSI reference method and have suggested that MICs obtained by this test are more predictive of treatment outcome (Arendrup et al. 2001; Peyron et al. 2001). In this study no isolate resistant to amphotericin B was observed with the three methods employed.

We adopted breakpoints according to Cuenca-Estrella et al. (2005). Fluconazole and amphotericin B MICs ob-

Table 2: Distribution of differences in MICs for 30 *Candida* isolates and percentage agreement within 3 dilutions for the Etest and two microdiluiton methods

Methods	Antifungal agent	No. isolates with Etest MICs different from the microdilution methods							$\pm 2 \log_2$ dilution (%)[a]
		>+2	+2	+1	0	−1	−2	>−2	
Etest/CLSI	Amphotericin B	1	8	8	3	7	2	1	93.33
	Fluconazole	10	15	1	3	1	–	–	66.66
Etest/EUCAST	Amphotericin B	1	2	2	6	15	2	2	90.00
	Fluconazole	4	13	8	2	2	1	–	86.66

[a] Percentage of agreement between the results is defined as the proportion of Etest MIC results that were within $\pm 2 \log_2$ dilution of the broth microdilution MIC results

Table 3: Comparison of Etest, Eucast and CLSI categorical agreement and error rates

Comparison	Antifungal agent	% Categorical agreement[a]	No. of samples by interpretative category[b]			No. of errors/total no. of samples tested (%)[c]		
			S	S-DD or I	R	Minor	Major	Very major
Etest vs	Amphotericin B	100.00	30	0	0	0	0	0
EUCAST	Fluconazole	96.66	28	0	2	1	0	0
Etest vs	Amphotericin B	100.00	30	0	0	0	0	0
CLSI	Fluconazole	100.00	28	1	1	0	0	0

[a] Agreement rates reflect the percentage of isolates classified in the same category by the proposed CLSI and AFST-EUCAST method and the alternative Etest method.
[b] No. of isolates classified in the given categories, see Methods for definitions.
[c] Percentage of the results representing minor, major or very major discrepancies with respect to those of the CLSI and AFST-EUCAST method, see methods for definitions.

tained by Etest were lower than the CLSI and AFST-EUCAST MICs. This finding is in agreement with previous reports Sewell et al. 1994; Eldere et al. 1996). For Etest determinations and for reading of the microdilution method at 24 h, application of interpretative breakpoints for fluconazole of $\leq$2 μg/ml for S, 4–8 μg/ml for SDD and $\geq$16 μg/ml for R provide a more correct classification of fluconazole susceptibility according to the CLSI reference method. Others have found Etest endpoints and those obtained by the reference method to be within the same range (Pfaller et al. 1998). This illustrates the necessity of including control strains and to confirm the performance of diffusion tests by reference tests, since inter-laboratory variations occur despite strict application of the manufacturer's guidelines.

It was recently shown that the agreement between the AFST-EUCAST and the CLSI method was very good for amphotericin B and fluconazole susceptibility testing (Cuenca-Estrella et al. 2005). In the present study, good agreement between all the methods was achieved for two antifungal agents, amphotericin B and fluconazole (Tables 2 and 3). Moreover, Person correlation coefficients were statistically significant ($P < 0.05$), indicating a good reproducibility and correlation among MICs obtained by the CLSI and EUCAST standard method and the proposed Etest.

The Etest MICs were generally higher than the MICs determined by the microdilution broth methods. The Etest MICs were generally one dilution higher at 24 h than at 48 h, in contrast to the CLSI and EUCAST methods in which the 24 h MIC was usually lower. This phenomenon is explained by the fact that, for most *Candida* spp, there was little or no distinction between the markedly decreased growth zone and the no-growth zone at 24 h in Etest procedures. In addition, the differences may relate to the use of glucose supplemented Mueller-Hinton agar for Etest Pfaller et al. 1998). This medium was specifically recommended for use in routine agar-based antifungal susceptibility testing (NCCLS 2002a). However, this phenomenon did not affect the results of the fluconazole-resistant strain (*C. tropicalis*), resulting in similar results by the Etest and microdilution methods. We confirm that the agar-based methods yielded correct identification of isolates with resistance to fluconazole (Pfaller et al. 2003).

The range of fluconazole MICs for all isolates was 0.12–64.0 μg/ml; however, a MIC of 1.0 μg/ml or lower was seen in 93.33% of all isolates. Higher MICs were only found in *C. parapsilosis* (1) and *C. glabrata* (1), similar to those of previous studies comparing the Etest and CLSI methods of susceptibility for fluconazole, to which 90.4% of species were susceptible (Fleck et al. 2007).

A better Pearson correlation was observed between Etest and CLSI endpoint MICs to fluconazole (Table 1). Categorical agreements were high for all methods. However, there was lower agreement to fluconazole (66.66 and 86.66) than amphotericin B (90.0 and 93.33). These discrepancies in MIC results between the methods were likely due to the difficulties of consistently reading visual MIC endpoints for isolates which produce trailing growth in the presence of azole antifungal agents. Recently, ST-Germain emphasized that trailing growth occur mainly with *C. albicans* and *C. tropicalis* (St Germain 2001). Posteraro et al. (2000) reported that the Etest failed to provide conclusive data on the fluconazole susceptibility of certain isolates of *C. parapsilosis.* However, this study showed that despite the trailing phenomenon, Etest results were in accordance with CLSI and AFST-EUCAST.

In conclusion, the agreement results among CLSI, AFST-EUCAST and Etest methodology were lower to fluconazole than to amphotericin B. Overall the MICs of amphotericin B and fluconazole obtained by the Etest alternative method exhibited a high agreement and a good correlation with those achieved by the AFST-EUCAST and CLSI methods.

4. Experimental

4.1. Isolates

A total of 30 isolates of *Candida* spp., identified with the API 20C AUX system (Biomerieux Vitek, Hazelwood, MO) were randomly selected, from the Adolfo Lutz Institute strain collection, Brazil. The isolates included 22 *Candida albicans* (73.3%), 3 *C. parapsilosis* (10%), 3 *C. tropicalis* (10%) and 2 *C. glabrata* (6.7%). Samples were stored in brain heart infusion semi-agar supplemented with 25% glycerin at −70 °C and subcultured 24 h prior to testing. Quality control included *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258.

4.2. Inoculum suspension

Yeast inoculum suspensions were prepared as described in CLSI M27-A2. The suspensions were adjusted to match a 0.5 McFarland density standard using a spectrophotometer (a = 0.05 to 0.08; $\lambda = 530$ nm) and resulted in an inoculum containing 1×10^6 to 5×10^6 yeast CFU/ml. This suspension was used to directly inoculate agar plates for the Etest procedure or diluted in RPMI-1640 medium as recommended by CLSI M27-A2 [14] and AFST-EUCAST (Cuenca-Estrela et al. 2003) metodologies.

4.3. Antifungal agents

EUCAST and CLSI microdilution plates containing serial drug dilutions of amphotericin B (Sigma), and fluconazole (Pfizer Central Research) were prepared following CLSI M27-A2 and EUCAST guidelines (NCCLS 2002b; Cuenca-Estrela et al. 2003). Amphotericin B and Fluconazole drug dilution ranged from 0.008 to 16 μg/ml and 0.12 to 128 μg/ml respectively. Amphotericin B and fluconazole Etest strips were obtained from AB Biodisk (Solna, Sweden) with concentration ranging from 0.002 to 32 μg/ml and 0.016 to 256 μg/ml, respectively.

4.4. CLSI broth microdilution method (M27-A2 document)

U-bottom microdilution plates containing 100 μL of the two-fold serial dilutions of the antifungal drugs in standard RPMI 1640 medium (0.2% glucose) were mixtured with 100 μL of inoculum containing 0.5 to 2.5×10^3 CFU/ml. After inoculation, the microdilution plates were incubated at 35 °C, and MICs were determined after 48 h. Reference MICs corresponded to the lowest drug dilution that showed prominent growth inhibition ($\geq$ 80%) to fluconazole and 100% growth inhibition to amphotericin B. The quality control isolates were included in each plate. Interpretive endpoints were based in M27A-2 (NCCLS 2002b).

4.5. EUCAST broth microdilution method

Flat-bottom microdilution plates containing 100 μL of the two-fold serial dilutions of the antifungal drugs in double-strength RPMI 1640 medium (2% glucose) were mixtured with 100 μL of inoculum containing 0.5 to 2.5×10^5 CFU/ml. The microdilution plates were incubated at 35 °C, and MICs were determined after 24 h; MICs were determined with a spectrophotometer at a wavelength of 550 nm. EUCAST MICs corresponded to the lowest drug dilution that showed a reduction of growth of 50% to fluconazole and 90% to amphotericin B (Cuenca-Estrela et al. 2003). Quality control isolates were included in each plate.

4.6. Agar diffusion Etest method

The test was performed on Mueller-Hinton agar plates supplemented with 2% glucose and methylene blue (0.5 μg/ml, Hardy Diagnostics, Santa Maria, CA) according to the CLSI M44-A disc-diffusion method (Pfaller et al. 2004). Each plate was inoculated with sterilized swabs. After drying for 15 min, two Etest strips, one of amphotericin B and one of fluconazole, were applied in the plate. The plates were incubated at 35 °C. In addition, quality control specimen were tested with an amphotericin B and fluconazole Etest strip. Plates were read after at 24 h. Fluconazole MIC and the inhibitory zone diameter were measured at the transition point where growth abruptly decreased as determined by a marked reduction in colony size, number, and density. The MIC for amphotericin B was read in the point in that a zone of inhibition intercepted the growth. All tests were done in duplicate.

Although interpretive breakpoints have not yet been established for amphotericin B, arbitrary values were established according values published by

Cuenca-Estrella et al. (2005). MIC interpretative criteria were: fluconazole susceptible (S) ≤ 2 µg/ml, susceptible dose dependent (S-DD) 4–8 µg/ml, and resistant (R) ≥ 16 µg/ml and amphotericin B, susceptible (S) ≤ 0.25 µg/ml, intermediate (I) 0.50–1.0 µg/ml, and resistant (R) ≥ 2 µg/ml.

4.7. Statistical analysis

Both on-scale and off-scale results were included in the analysis. The low off-scale MICs were left unchanged and the high off-scale MICs were converted to the next highest concentration. The concordance among the CLSI, EUCAST and Etest MICs was evaluated by distinct statistical tests. The concordance was defined as a difference in MICs equal to two two-fold dilutions. The Etest MICs were elevated to the next drug concentration that matched the microdilution scheme to facilitate the comparison among MIC results. A p value < 0.05 was considered statistically significant. Values were considered in agreement when the discrepancies between the methods were no more than 2 $\log_2$ dilutions. For the comparison between Etest and the two broth microdilution methods, the Pearson correlation coefficient designed by r (Fleck et al. (2007) was calculated. r can range from -1 to $+1$, with -1 indicating a perfect negative correlation, $+1$ indicating a perfect positive correlation, and 0 indicating no correlation at all. So, if r is inside the values cited and $p < 0.05$ the groups are significantly correlated. Etest, CLSI and EUCAST MIC90 were also determined. Categorical agreement was defined as the percentage of isolates classified in the same category with the reference procedure and Etest technique. Discrepancies were considered to be great if an isolate classified as resistant by one method was categorized as susceptible by other. Errors were classified as minor when susceptible vs. intermediate, resistant vs. intermediate, intermediate vs. susceptible or intermediate vs. resistant discrepancies were observed. All statistical analysis was performed with the software Analyse-it (version 1.73, Analyse-it Software).

Acknowledgements: This work has been supported by CAPES. We thank Amanda Latercia Tranches Dias for the help with the statistical analysis.

References

Arendrup M, Lundgren B, Jensen IM, Hansen BS, Frimodt-Moller N (2001) Comparison of Etest and a tablet diffusion test with the NCCLS broth microdilution method for fluconazole and amphotericin B susceptibility testing of *Candida* isolates. J Antimicrob Chemother 47: 521–526.

Barry AL, Pfaller MA, Rennie RP, Fuchs PC, Brown SD (2002) Precision and accuracy of fluconazole susceptibility testing by broth microdilution, Etest, and disk diffusion methods. Antimicrob Agents Chemother 46: 1781–1784.

Chryssanthou E, Cuenca-Estrella M (2002) Comparison of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing proposed standard and the E-test with the NCCLS broth microdilution method for voriconazole and caspofungin susceptibility testing of yeast species. J Clin Microbiol 40: 3841–3844.

Cuenca-Estrella MCB, Moore F, Barchiesi J (2003) Multicenter evaluation of the reproducibility of the proposed antifungal susceptibility method for fermentative yeasts of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST). Clin Microbiol Infect 9: 467–474.

Cuenca-Estrella M, Gomez-Lopez A, Mellado E, Rodriguez-Tudela JL (2005) Correlation between the procedure for antifungal susceptibility testing for *Candida* spp. of the European Committee on Antibiotic Susceptibility Testing (EUCAST) and four commercial techniques Clin Microbiol Infect 11: 486–492.

Dias ALTD, Matsumoto FE, Melhem SMC, Silva EG, Auler ME, Siqueira AM, Paula CR (2006) Comparative analysis of Etest and broth microdilution method (AFST-EUCAST) for trends in antifungal drug susceptibility testing of Brazilian *Cryptococcus neoformans* isolates. J Med Microbiol 55: 1693–1699.

Eldere, J van., Joosten L, Verhaeghe V, Surmont I (1996) Fluconazole and amphotericin B antifungal susceptibility testing by National Committee for Clinical Laboratory Standards broth macrodilution method compared with E-test and semiautomated broth microdilution test. J Clin Microbiol 34: 842–847.

Fleck R, Dietz A, Hof H (2007) *In vitro* susceptibility of *Candida* species to five antifungal agents in a German university hospital assessed by the reference broth microdilution method and Etest. J Antimicrob Chemoter 59: 767–771.

Law D, Moore CB and Denning DW (1997) Amphotericin B resistance testing of *Candida* spp.: a comparison of methods. J Antimicrob Chemother 40: 109–112.

National Committee for Clinical Laboratory Standards (2002a) Method for antifungal disk diffusion susceptibility testing in yeasts. Approved guideline M44-A: CLSI, Wayne, Pa.

National Committee for Clinical Laboratory Standards (2002b) Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2: CLSI, Wayne, Pa.

Pfaller MA, Rinald MG, Galgiani JN (1990) Collaborative investigation of variables in susceptibility testing of yeasts. Antimicrob Agents Chemother 34: 1648–1654.

Pfaller MA, Messer SA, Karlsson A, Bolmström A (1998) Evaluation of the Etest Method for Determining Fluconazole Susceptibilities of 402 Clinical Yeast Isolates by Using Three Different Agar Media. J Clin Microbiol 36: 2586–2589.

Pfaller MA, Diekema DJ, Boyken L, Messer SA, Tendolkar S, Hollis RJ (2003) Evaluation of the Etest and disk diffusion methods for determining susceptibilities of 235 bloodstream isolates of *Candida glabrata* to fluconazole and voriconazole. J Clin Microbiol 41: 1875–1880.

Pfaller MA, Boyken L, Messer SA, Tendolkar S, Hollis RJ, Diekema DJ (2004) Evaluation of the Etest method using Mueller-Hinton agar with glucose and methylene blue for determining amphotericin B MICs for 4,936 clinical isolates of *Candida* Species. J Clin Microbiol 42: 4977–4979.

Peyron F, Favel A, Michel-Nguyen A, Gilly M, Regli P, Bolmstrom A. (2001) Improved detection of amphotericin B-resistant isolates of *Candida lusitaniae* by Etest. J Clin Microbiol 39: 339–342.

Posteraro B, Romano L, Sanguinetti M (2000) Commercial system for fluconazole susceptibility testing of yeasts: comparison with the broth microdilution method. Diagn Microbiol Infect Dis 38: 29–36.

Sewell DL, Pfaller MA, Barry AL (1994) Comparison of broth macrodilution, broth microdilution, and Etest antifungal susceptibility tests for fluconazole. J Clin Microbiol 32: 2099–2102.

St-Germain G (2001) Impact of endpoint definition on the outcome of antifungal susceptibility tests with *Candida* species: 24-*versus* 48-h incubation and 50 *versus* 80% reduction in growth. Mycoses 44: 37–45.

Wanger A, Mills K, Nelson PW, Rex JH (1995) Comparison of Etest and National Committee for Clinical Laboratory Standards broth microdilution method for antifungal susceptibility testing: enhanced ability to detect amphotericin B-resistant *Candida* isolates. Antimicrob Agents Chemother 39: 2520–2522.