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EVALUATION OF ETEST AND MACRODILUTION BROTH METHOD FOR ANTIFUNGAL SUSCEPTIBILITY TESTING OF *Candida* sp STRAINS ISOLATED FROM ORAL CAVITIES OF AIDS PATIENTS

Maria do Rosário R. SILVA(1), Márcio R. COSTA(1), André T.B. MIRANDA(1), Orionalda de F.L.FERNANDES(1), Carolina R. COSTA(1) & Claudete R. de PAULA(2)

SUMMARY

A comparison of the Etest and the reference broth macrodilution susceptibility test for fluconazole, ketoconazole, itraconazole and amphotericin B was performed with 59 of *Candida* species isolated from the oral cavities of AIDS patients. The Etest method was performed according to the manufacturer's instructions, and the reference method was performed according to National Committee for Clinical Laboratory Standards document M27-A guidelines. Our data showed that there was a good correlation between the MICs obtained by the Etest and broth dilution methods. When only the MIC results at ± 2 dilutions for both methods were considered, the agreement rates were 90.4% for itraconazole, ketoconazole and amphotericin B and 84.6% for fluconazole of the *C. albicans* tested. In contrast, to the reference method, the Etest method classified as susceptible three fluconazole-resistant isolates and one itraconazole-resistant isolate, representing four very major errors. These results indicate that Etest could be considered useful for antifungal sensitivity evaluation of yeasts in clinical laboratories.

KEYWORDS: Etest; Broth macrodilution; Antifungal susceptibility tests.

INTRODUCTION

Unlike antibacterial assays, standardized methods for testing antifungal agents are not generally available in most microbiological laboratories. Owing to the increased fungal infection rates in immunocompromised patients, including those with AIDS and recipients of solid organ transplants and cytotoxic chemotherapy, have created new challenges in the area of antimicrobial drug therapy, much effort has been undertaken to obtain a standardized rapid and reliable routine method for *in vitro* susceptibility testing of antifungal substances^{5,6,9,16}. So, the publication in 1992 by the National Committee for Clinical Laboratory Standards (NCCLS)¹⁰ of the proposed broth macrodilution reference for yeast, NCCLS M27-P document, which was moved to the tentative level, or M27-T, in 1995¹¹ and the NCCLS M-27 A in 1997¹², documents are an important landmark in the field of testing antifungal susceptibilities. This method provides guidelines for testing the antifungal susceptibilities of *Candida* spp and *Cryptococcus neoformans* and shows interpretative breakpoints in susceptible, dose dependent and resistant for *Candida* species against itraconazole, fluconazole and flucytosine (NCCLS, 1997)¹².

The Etest (AB Biodisk, Solna, Sweden) is now well established as an excellent method for *in vitro* testing of antimicrobial agents against numerous microbial pathogens and the adaptation to *in vitro* susceptibility

testing of fungi has also met with reasonable success^{1,2,8,18,20}. This method is based on the diffusion of a continuous concentration gradient of the antifungal agent tested from a plastic strip into an agar medium. Since these strips contain a predefined continuous grade of a drug, it is possible to obtain reproducible quantitative MIC readings^{13,18}.

In the present study, we compared a broth macrodilution assay, performed in accordance with NCCLS guidelines, and Etest method by determining the MICs of four antifungal agents for 59 clinical isolates of yeasts pathogenic isolated from patients with AIDS.

MATERIALS AND METHODS

Organisms: Fungal isolates were obtained from 86 swabs of the oral mucosa from AIDS patients during the initial diagnosis of candidiasis infections who were attended in the Hospital de Doenças Tropicais de Goiânia (HDT) from January to December 2000. Swabs were streaked for isolation onto Sabouraud dextrose agar (SDA) and the yeasts isolated were identified by standard methods⁷. Fifty nine isolates belonged to the genus *Candida*; of these 88.1% (52/59) were identified as *C. albicans*, 6.7% (4/59) as *C. tropicalis* and 5% (3/59) as *C. krusei*. Each isolated was stored at ambient temperature until testing was performed, when were subcultured on SDA for 18-24 hours at 35 °C to ensure their purity and viability. These isolates were selected for susceptibility testing.

(1) Instituto de Patologia Tropical e Saúde Pública da Universidade Federal de Goiás, Goiânia, GO, Brasil.

(2) Instituto de Ciências Biomédicas da Universidade de São Paulo, SP, Brasil.

Correspondence to: Maria do Rosário Rodrigues Silva, Rua 15 n° 108, apto. 700, Setor Oeste. 74140-030 Goiânia, GO, Brazil.; E-mail: rosario@iptsp.ufg.br

The test organism included American Type Culture Collection *C. albicans* (ATCC 10231) used as control in susceptibility tests.

All the isolates were tested by reference broth macrodilution method and by the Etest. The antifungal agents evaluated were amphotericin B (E.R. Squibb & Sons), ketoconazole and itraconazole (Janssen Pharmaceuticals) and fluconazole (Pfizer, Inc., New York, N.Y). All the tests were run in duplicate.

Broth macrodilution method: The broth macrodilution method was performed by following the guidelines in NCCLS document M27-A¹². It was performed in RPMI 1640 supplemented with 0.3 g of glutamine/L, buffered with 34.6 g morpholinepropanesulfonic acid (MOPS) and adjusted pH 7.0, contained 2 g of glucose. Fluconazole, ketoconazole, itraconazole and amphotericin B were obtained as reagent-grade powders from their respective manufacturers. Amphotericin B, ketoconazole and itraconazole were dissolved in dimethyl sulfoxide at a concentration of 320 µg/mL, and fluconazole was dissolved in 0.85% saline at a concentration of 2560 µg/mL. Stock solution were stored at -70 °C until used. The stock yeast inoculum suspensions were adjusted to 1X 10⁶ to 5X 10⁶ CFU/mL by the spectrophotometric method. Briefly, an inoculum suspension was prepared for each isolate from 24 hours old cultures grown at 35 °C and the turbidities of the resulting yeast suspension were measured with spectrophotometer at 530 nm and were adjusted to the percent transmission that matched a 0.5 Mc Farland standard. The working suspension was made by a 1:100 dilution followed by a 1:20 dilution in RPMI 1640 broth medium in sufficient volume to directly inoculate each MIC tube with 0.9 mL. Yeast inocula (0.9 mL) were added to each tube containing 0.1 mL antifungal drug solution. The range final concentration was from 0.002 to 32 µg/mL for amphotericin B, ketoconazole and itraconazole and 0.016 to 256 µg/mL to fluconazole. The tubes were incubated at 35 °C and were inspected after 48 hours of incubation. Drug-free and yeast-free controls were included. For the azoles, itraconazole, ketoconazole and fluconazole, the MIC was established as the lowest antifungal concentration that inhibited 80% of the control growth (0.2 mL of growth control plus 0.8 mL of uninoculated RPMI). For amphotericin B, endpoints were determined visually by recording the lowest concentration of the agent that prevented the appearance of visible growth^{3,12}.

Agar diffusion Etest: The Etest antifungal gradient strips were provided by the manufacturer (AB BIODISK, Solna, Sweden). RPMI agar for the agar diffusion Etest was prepared the same way as the RPMI broth and supplemented with 18 g of glucose/L and 15 g of Bacto agar/L. The concentration gradient for itraconazole, ketoconazole and amphotericin B ranged from 0.002 to 32 µg/mL and the fluconazole ranged from 0.016 to 256 µg/mL. The strips were stored at -20 °C until use. Each solidified medium was inoculated by dipping a sterile swab into the cell suspension (5X 10⁵ cells/mL) and streaking it across in the surface of the agar in three directions. The plates were allowed to dry for at least 15 min before the Etest strip was applied to the surface. The plates were incubated at 35 °C and MICs were determined following incubation time of 24 h. Determination of MIC endpoints by the Etest was the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip.

Analysis of results: A total of 960 MICs for ATCC isolates and the 59 clinical yeast isolates were obtained and analyzed. Both on-scale and

off-scale results were included in the analysis. The high off-scale MIC (>32 and >256 µg/mL) were converted to the next highest (64 and 512 µg/mL) concentration and the low off-scale (MICs < 0.002 and < 0.016 µg/mL) were left unchanged. Discrepancies among MIC endpoints of no more than 2 dilutions were used to calculate the percent agreement.

According to the NCCLS¹² criteria, isolates for fluconazole which MICs are ≤ 8 µg/mL are classified as susceptible, while those for which MICs are ≥ 64 µg/mL are classified as resistant and isolates for which MICs are 16 to 32 µg/mL are termed susceptible dose dependent (S-DD). Isolates for which itraconazole MICs are ≤ 0.125 µg/mL are classified as susceptible, while those for which MICs are 0.25 to 0.5 µg/mL are classified as S-DD, and those for which MICs are ≥ 1 µg/mL are classified as resistant.

To permit comparison between the results of the two methods, the NCCLS breakpoints were applied to both tests for these agents. Major errors were defined as results in which the reference method result was susceptible and the Etest method result was resistant, while very major errors were defined as results in which the reference method result was resistant and the Etest method result was susceptible. Minor errors were defined as variations in results from resistant to S-DD or S-DD to susceptible between the two methods⁶.

RESULTS

Table 1 summarizes the *in vitro* susceptibility (MICs) of 52 *C. albicans*; 04 *C. tropicalis*; 03 *C. krusei* isolates for itraconazole, fluconazole, ketoconazole and amphotericin B by the two methods. The data are reported as the concentrations of antifungals agents necessary to inhibit 50% (MIC₅₀) and 90% (MIC₉₀) of the isolates tested. Itraconazole, ketoconazole and fluconazole MICs showed a broad range for all the species. Amphotericin B MICs showed a very narrow range for the species tested.

Agreement between the macrodilution method and the Etest method was good for all antifungal agents toward *C. albicans*. In contrast, the agreement among the methods was lowest for itraconazole toward *C. krusei* (66.7%) and fluconazole, ketoconazole and amphotericin B toward *C. tropicalis* (75%). With regard to fluconazole, the Etest often produced inhibition zones with difuses edges. For 25% of the *C. tropicalis* isolates growth of microcolonies with decreasing intensity just inside the border of the inhibition zone was observed. The percent of agreement between the broth macrodilution and Etest methods of the 59 isolates to amphotericin B, fluconazole, ketoconazole and itraconazole is showed in table 1. A complete agreement of Etest MIC with reference broth macrodilution method MIC was verified when the four antifungal agents were tested against *C. albicans* ATCC 10231.

The itraconazole and fluconazole susceptibility data are reported in Table 2. This table shows the number of isolates (for each specie) classified as susceptible (S), susceptible dose dependent (S-DD), and resistant (R) based on the results of the two methods tested.

Table 3 details the susceptibility interpretation discrepancies between the two methods for the two drugs with NCCLS- defined breakpoints (fluconazole and itraconazole). Very major errors, in which the reference method classified an isolate as resistant and the Etest method classified

Table 1
Antifungal susceptibilities of 59 clinical isolates of yeasts: MIC results, agreement rate between Etest and NCCLS methods

Organism	Antifungals	Broth macrodilution (µg/mL)			Etest (µg/mL)			% agreement
		Range	MIC ₅₀	MIC ₉₀	range	MIC ₅₀	MIC ₉₀	
<i>C. albicans</i> n = 52	Itraconazole	0.002->32	0.064	0.50	0.002->32	0.094	0.50	90.4
	Ketoconazole	0.064->32	0.25	>32	0.064->32	0.25	>32	90.4
	Fluconazole	0.19->256	4.0	>256	0.016->256	3.0	>256	84.6
	Amphotericin B	0.125-2.0	0.50	1.50	0.094-3.0	1.0	1.50	90.4
<i>C. tropicalis</i> n = 04	Itraconazole	0.016-0.38	0.064	0.38	0.032-0.38	0.064	0.38	100
	Ketoconazole	0.25->32	1.0	>32	0.125->32	0.38	>32	75
	Fluconazole	0.125->256	1.0	>256	0.25->256	3.0	>256	75
	Amphotericin B	0.032-0.50	0.50	0.50	0.064-1.50	1.0	1.50	75
<i>C. krusei</i> n = 03	Itraconazole	0.064->32	1.50	>32	0.047->32	0.064	>32	66.7
	Ketoconazole	0.125->32	>32	>32	0.125->32	>32	>32	100
	Fluconazole	4.0->256	>256	>256	4.0->256	>256	>256	100
	Amphotericin B	0.25-0.75	0.50	0.75	0.25-1.0	0.50	1.0	100

Table 2
Itraconazole and Fluconazole susceptibility test results obtained with each of the two methods

Species	Classified as	Antifungals agents/methods			
		Itraconazole		Fluconazole	
		Macrodilution	Etest	Macrodilution	Etest
<i>C. albicans</i> (n = 52)	S	37	36	42	46
	S-DD	10	11	1	0
	R	5	5	9	6
<i>C. tropicalis</i> (n = 04)	S	2	3	2	2
	S-DD	2	1	0	0
	R	0	0	2	2
<i>C. krusei</i> (n = 03)	S	1	2	NA ^a	NA
	S-DD	0	0	NA	NA
	R	2	1	NA	NA

^aNA, not applicable - Reference MIC breakpoints have not been applied to *C. krusei*.

Table 3
Discrepancies between test method for antifungal agents with NCCLS-defined MIC breakpoints for all organisms tested

Organisms/ antifungal agents	No. of discrepant results ^a					
	Minor ^b				Major ^c	Very major ^d
	S vs S-DD ^e	S-DD vs S	S-DD vs R	R vs S-DD	S vs R	R vs S
<i>C. albicans</i> (52)	Itraconazole	3	2	0	0	0
	Fluconazole	0	1	0	0	3
<i>C. tropicalis</i> (04)	Itraconazole	0	1	0	0	0
	Fluconazole	0	0	0	0	0
<i>C. krusei</i> (03)	Itraconazole	0	0	0	0	1
	Fluconazole	0	0	0	0	0

^aS, susceptible; S-DD, Susceptible-dose dependent; R, resistant; ^bMinor difference between method were those which changed the susceptibility category from susceptible to S-DD, S-DD to susceptible, S-DD to resistant, or resistant to S-DD; ^cMajor differences were those in which the reference method classified isolates as susceptible and the Etest method classified isolates as resistant; ^dVery major differences were those in which the reference method classified isolates as resistant and the Etest method classified isolates as susceptible; ^eSusceptibility results by the NCCLS method versus those by the Etest method

it as susceptible occurred for fluconazole with three *C.albicans* isolates and for itraconazole with one *C. krusei* isolate. The greatest percentage of minor errors was noted for itraconazole (six of the seven instances) and the one error was observed for fluconazole against *C. albicans*. Overall, the results indicated that there were only 04 (3.4%) very major errors and 07 (5.9 %) minor errors between methods for all organisms tested against fluconazole and itraconazole.

DISCUSSION

Although the NCCLS broth macrodilution methods (M27-A document)¹² improved the level of interlaboratory agreement of antifungal MIC endpoint, these procedures are not convenient and efficient testing tools for the clinical laboratory. Several tests that are alternatives to the NCCLS methodology has been developed and evaluated. Among them, the Etest is the commercial method that has been investigated for the testing of pathogenic yeasts. The Etest method has the advantage of being simple and easy for use^{1,19}. However literature's data comparing MICs obtained by Etest with those obtained by macrodilution or microdilution have shown variable results depending of the antifungal agent and the species tested, as well as on the medium used for testing^{1,3,19}. RPMI 1640 medium with 2% glucose used in our study is the medium recommended for use in the susceptibility testing of yeasts and indicates that medium improves the reproductibility of the MICs as determined by the Etest^{3,17}. Previous comparison by independent laboratories between Etest and broth macrodilution methods, have showed good agreement rates for the azoles and flucytosine, when is used the RPMI 1640^{3,24}.

In this study we compared the MICs of fluconazole, itraconazole, ketoconazole and amphotericin B by Etest and broth macrodilution techniques against 59 isolates of *Candida* obtained from patients with AIDS during the initial diagnosis of candidiasis infection. In general, our data showed that there was a good correlation between the MICs obtained by the Etest and broth dilution methods, verifying that both methods showed high MICs values for fluconazole and ketoconazole in *Candida* isolates. The highest MICs were observed to fluconazole, mainly for *C. krusei* with MIC₅₀ and MIC₉₀ > 32 µg/mL. This specie is considered inherently resistant to fluconazole¹⁵. Our results are in accordance with others that showed high MIC values for azoles in patients with AIDS²². The strains of *Candida* are generally sensitive to ketoconazole and itraconazole²³, however in our study MIC₉₀ of ketoconazole for all the species tested were > 32 µg/mL and for itraconazole MIC₉₀ was > 32 µg/mL for *C. krusei*. These characteristics can be explained by the phenomenon of cross-resistance observed among the family of azole compound⁴. Besides, the selection of resistant isolates to azoles would be explained by the long term of azoles in the prophylaxis therapy for patients with AIDS²³. When the MIC results at ± 2 dilutions for both methods were considered, the agreement rates were 90.4% for itraconazole, ketoconazole and amphotericin B and 84.6% for fluconazole of the *C. albicans* tested. However, higher discrepancies were noted for *C. krusei* and *C. tropicalis* (Table 1). The determination of the endpoints because the usual partial growth inhibition (trailing) observed with azoles was difficult, representing a significant factor in the variability of MIC results for these drugs. As expected, this was a problem with significant number of isolates. The growth of microcolonies inside all of the

inhibition zone around the Etest strips was judged in this study to be the most difficult reading pattern.

It is well known that with exception of fluconazole, itraconazole and flucytosine breakpoint concentration have not been defined for other antifungals by the NCCLS. In addition, for fluconazole, these interpretative breakpoints are not aplicable to *C. krusei*, because this specie is assumed to be intrinsically resistant to fluconazole. An analysis of the discrepancies between test methods for fluconazole and itraconazole with NCCLS defined MIC breakpoints showed that the greatest number of very major errors occurred with *C. albicans* isolates tested against fluconazole and with *C. krusei* for itraconazole. These discrepancies in MIC results between the two 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-GERMAIN²¹ emphasized that trailing growth occur mainly with *C. albicans* and *C. tropicalis*. POSTERARO *et al.*¹⁴ related that the Etest failed to provide conclusive data on the fluconazole susceptibility of certain isolates of *C. parapsilosis*.

In summary, we obtained a good agreement between the Etest and the broth dilution methods for the drugs susceptibility testing of *Candida* when endpoints determinations were recorded after 24 hours of incubation for the Etest and 48 hours of incubation for the broth macrodilution.

In conclusion, considering the high concordance of the results of both test systems, Etest could be considered useful for antifungal sensivity evaluation of yeasts in clinical laboratories.

RESUMO

Avaliação dos métodos de suscetibilidade antifúngica: Etest e macrodiluição em caldo para leveduras do gênero *Candida* isoladas da cavidade bucal de pacientes com AIDS

Foram comparados dois testes de suscetibilidade *in vitro*, macrodiluição em caldo e Etest através da determinação das concentrações inibitórias mínimas de fluconazol, cetoconazol, itraconazol e anfotericina B para 59 isolados de *Candida* obtidos da cavidade bucal de pacientes com AIDS. O método de Etest foi feito de acordo com as instruções do fabricante e o método de macrodiluição em caldo foi realizado seguindo as normas do NCCLS (National Committee for Clinical Laboratory Standards), documento M27-A, considerado método de referência. Nossos dados mostraram uma boa correlação entre os valores de concentração inibitória mínima encontrados para o Etest e o método de macrodiluição em caldo. Foi verificada concordância entre os resultados de CIM, de 90,4% para itraconazol, cetoconazol e anfotericina B e de 84,6% para fluconazol, quando se analisou isolados de *C. albicans*. A comparação de breakpoints mostrou que havia quatro grandes discrepâncias de resultados entre os dois métodos, sendo que pelo método de referência três isolados de *Candida albicans* classificados como resistentes ao fluconazol e um de *C. krusei* considerado resistente ao itraconazol foram sensíveis ao Etest. Estes resultados mostraram que o Etest é de grande valor nos testes de suscetibilidade antifúngica para leveduras do gênero *Candida*.

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