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***IN VITRO* SUSCEPTIBILITY OF *Cryptococcus*  
*neoformans* OF CLINICAL ISOLATES FROM AIDS  
PATIENTS: COMPARATIVE STUDY OF ETEST AND  
BROTH MACRODILUTION METHODS**

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**ABSTRACT**

A comparative study of Etest and broth macrodilution methods for susceptibility testing to fluconazole, ketoconazole, itraconazole and amphotericin B was conducted for sixty strains of *C. neoformans* isolated from Aids patients. The MICs obtained by these two methods were read after 72 hours of incubation for broth macrodilution and 48 hours for Etest. The rate agreement was obtained between the broth macrodilution and Etest methods using differences with 2 doubling dilutions. Our data showed that there was a good correlation between the MICs obtained by both methods. The correlation between these two methods tested was 96.6% (58/60), 90.0% (54/60), 93.3% (56/60) and 100% (60/60) for fluconazole, ketoconazole, itraconazole, and amphotericin B, respectively. These results indicate that Etest could be considered useful for antifungal sensitivity evaluation of yeasts in clinical laboratories.

**KEYWORDS:** *Cryptococcus neoformans*. Antifungal agents. *In vitro* susceptibility.

**INTRODUCTION**

The increased prevalence of fungal infections and the introduction of new antifungal agents have intensified the need for useful antifungal susceptibility methods (18). Methods for testing the antifungal susceptibility

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of *C. neoformans* could become important tools in the selection and monitoring of an appropriate antifungal drug for the treatment of cryptococcal infections.

A broth macrodilution technique for yeast susceptibility has been accepted after several collaborative studies, serving to provide a standard basis from which other methods have been developed (8, 16, 15). However this method is cumbersome and time consuming. Several alternative methods have been proposed. One of these, the Etest, is commercially available and has been introduced mainly due to its simplicity and good correlation with the reference method (6, 5). In this study we compared broth macrodilution technique and Etest method for testing fluconazole, ketoconazole, itraconazole and amphotericin B against 60 clinical isolates of *Cryptococcus neoformans*.

## MATERIALS AND METHODS

### Isolates

A total of 60 *C. neoformans* strains were obtained from cerebrospinal fluid specimens from Aids patients at the Hospital de Doenças Tropicais de Goiânia, Goiás, Brasil from October 1999 through April 2001. All isolates were identified as *C. neoformans* by a positive DOPA agar response, positive urease test and ability to grow at 37°C (10).

Each isolate was tested by the broth macrodilution (reference method) and by Etest methods. The organisms tested included one American Type Culture Collection *C. krusei* ATCC 6258, used as quality control for susceptibility tests.

### Susceptibility testing in broth macrodilution method

The following antifungal agents were used, fluconazole (Pfizer, Inc., New York, N.Y), ketoconazole, itraconazole (Janssen Pharmaceuticals), and amphotericin B (E.R. Squibb & Sons).

Broth macrodilution was performed according to the NCCLS M27-A guidelines (9). RPMI-1640 medium with L-glutamine was prepared according to the manufacturer instructions. After reconstitution the medium was supplemented with glucose 2% and buffered to pH 7.0 with 3- N morpholinepropanesulfonic acid (MOPS) to a final concentration of 165 M. Drug dilution was prepared at 10 times the strength of the final drug concentration by additive drug dilution schemes for minimizing systematic pipetting errors.

The stock yeast inoculum suspensions were adjusted to  $1 \times 10^6$  to  $5 \times 10^6$  CFU/mL by the spectrophotometric method. Briefly, the inoculum was

prepared by picking five colonies of 1 mm in diameter from 48 hours old cultures and suspending the colonies in 5 mL of sterile saline (0.85%). The resulting suspension was adjusted to a cell density of a 0.5 McFarland standard with the aid of a spectrophotometer at a wavelength of 530 nm. The adjusted stock suspension was diluted 1:100 in the medium followed by a 1:20 dilution of the stock suspension with RPMI 1.640 in sufficient volume to directly inoculate in each MIC tube with 0.9 mL. Inoculum sizes were confirmed with the final higher inoculum for all the strains tested by enumeration of the CFU/mL on subcultures on *sabouraud* dextrose agar. Yeast inocula (0.9 mL) were added to the 10X drug dilutions in tubes, bringing the drug dilutions to the final test concentrations: 0.002 to 32  $\mu\text{g/mL}$  for ketoconazole, itraconazole and amphotericin and 0.016 to 256  $\mu\text{g/mL}$  for fluconazole. The incubation was at 35°C for 72 hours. 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 (12, 17).

#### Susceptibility testing in the Etest method

The Etest® strips were provided by manufacturer (AB BIODISK, Solna, Sweden). The concentration gradient strips for ketoconazole, itraconazole and amphotericin B ranged from 0.002 to 32  $\mu\text{g/mL}$  and for fluconazole from 0.016 to 256  $\mu\text{g/mL}$ . RPMI agar for the agar diffusion Etest was prepared the same way as the RPMI broth and supplemented with 18 g of glucose and 15 g of Bacto agar per liter and buffered at pH 7.0 with phosphate buffer for azoles agents and MOPS for amphotericin B. Petri plates containing 60 mL of medium were inoculated by using a nontoxic swab dipped in a cell suspension adjusted to the turbidity of a 1.0 McFarland standard. The agar surface was allowed to dry, and the Etest® strips were placed onto the inoculated agar. The plates were incubated at 35°C and the MICs were read at 48 hours.

#### Analysis of results

A total of 976 MICs for the ATCC isolates and the 60 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  $\mu\text{g/mL}$ ) was converted to the next highest (64 and 512  $\mu\text{g/mL}$ ) concentration, and the low off-scale (MICs < 0.002 and < 0.016  $\mu\text{g/mL}$ ) was left unchanged. Differences

among MIC endpoints of no more than 2 dilutions were used to calculate the percent agreement.

## RESULTS

The data collected by the two methods are reported as MIC ranges and the MICs required to inhibit 50 and 90% of 60 *C. neoformans* isolates to fluconazole, ketoconazole, itraconazole and amphotericin B. The MICs of all drugs except amphotericin B covered a broad range.

In 80% of the tests, the MIC endpoints obtained with the broth macrodilution and the Etest were identical or comparable (differing by no more than 2 twofold dilutions). Discrepancies between the results of the Etest and the broth macrodilution methods were uncommon, but they occurred for three of the four drugs studied. For itraconazole and ketoconazole MICs generated by Etest method were higher than broth reference method, but for fluconazole MICs generated by reference method were higher. The highest MIC values were found for fluconazole (range of 2-256) and the lowest for ketoconazole (range of 0.016-0.75). The percentages of agreement were 96.6% for fluconazole, 90.0% for ketoconazole, 93.3% for itraconazole and 100% for amphotericin B. Table 1 summarizes MIC ranges of 60 isolates of *C. neoformans* and agreement between Etest and macrodilution methods for four drugs tested.

The fluconazole, itraconazole, ketoconazole and amphotericin B MICs *C. krusei* ATCC 6.258 isolate were 256, 0.25, 0.5 and 0.5 by reference method and 256, 0.38, 0.38 and 0.25 by Etest method respectively.

Table 1. MIC results of antifungal susceptibility of 60 clinical isolates of *C. neoformans* and agreement rate between Etest and NCCLS methods

Antifungal	Etest ( $\mu\text{g/mL}$ )			Macrodilution ( $\mu\text{g/mL}$ )			% Agreement rate
	Range	MIC <sub>50</sub>	MI C <sub>90</sub>	Range	MIC <sub>50</sub>	MIC <sub>90</sub>	
Fluconazole	2 - 256	12	256	2 - 256	12	256	96.6
Ketoconazole	0.016-0.75	0.094	0.38	0.016-0.75	0.094	0.38	90.0
Itraconazole	0.047 - 1.5	0.25	0.38	0.047-0.75	0.19	0.38	93.3
Amphotericin B	0.25 - 1.0	0.5	0.75	0.25 - 1.0	0.5	0.75	100

## DISCUSSION

In this study, we compared the MICs of fluconazole, itraconazole, ketoconazole and amphotericin B by broth macrodilution and Etest techniques against 60 clinical isolates of *C. neoformans*. The Etest has been introduced as a mean of producing an accurate quantitative MIC result by using an agar diffusion format. Our analysis of the antifungal susceptibility tests showed that the overall agreements between Etest and reference

methods were 96.6%, 90%, 93.3% and 100% for fluconazole, ketoconazole, itraconazole and amphotericin B, respectively. Similar experiences have already been reported by others authors. Colombo et al. (6) obtained a similar range by both methods for azoles. Sewel et al. (17) demonstrated an agreement of approximately 90% between both methods in *Candida* species for fluconazole. However, Aller et al. (2000) (1) found that essential agreement for Etest and the broth microdilution method was lower than that obtained in our study. The broth microdilution method represents an adaptation of the reference method and shows identical results (3).

In our study, itraconazole and ketoconazole presented the majority of MIC disagreements between reference and Etest methods. For fluconazole the MICs by Etest were lower than the reference. Warnock et al. (19) showed that the Etest MICs results for flucytosine, fluconazole and itraconazole were higher than those obtained with reference. Odds (1980) (13) has reported that absolute azole MICs generated by agar based techniques tend to be lower than those produced by broth assays.

Our results seem to indicate susceptibility of *C. neoformans* strains to amphotericin B, ketoconazole and itraconazole. Similar data was found by Alves et al. (2) who demonstrated that *C. neoformans in vitro* was extremely susceptible to these drugs. For fluconazole, our results seem to indicate *in vitro* resistance in four *C. neoformans* isolates, with MIC of 256 µg/mL. This drug resistance has been showed in the majority of cases of meningitis in Aids patients after prophylaxis treatments with fluconazole (4). However, some aspects about susceptibility tests deserve attention. The narrow MIC ranges of amphotericin B have been pointed out to be a consequence of RPMI 1640 medium which may not be a good culture medium to warrant good *C. neoformans* growth and thus, could be hindering the detection of resistance (14). Reports about amphotericin B resistant *C. neoformans* are scarce (9, 11).

However studies that establish the value of MICs as predictors of the drug resistance are scarce. The establishment of interpretative breakpoints for *C. neoformans*, have not yet been proposed buy the NCCLS (NCCLS M27 A, 1997). Pharmacodynamic studies of antifungal agents will be important for establishing these breakpoints.

The simplicity and familiarity of the Etest methodology to personnel in most clinical microbiology laboratories makes it a potentially useful method for testing the drugs susceptibility of yeasts.

Suscetibilidade *in vitro* de *Cryptococcus neoformans* isolados de pacientes com Aids: estudo comparativo do Etest e métodos de macrodiluição

Sessenta isolados de *Cryptococcus neoformans* obtidos de pacientes com Aids foram estudados através dos métodos de macrodiluição em caldo e Etest, para análise comparativa da concentração inibitória mínima (CIM) para itraconazol, cetoconazol, fluconazol e anfotericina B. A leitura da CIM foi feita após incubação das leveduras por 72 horas para o método de diluição em caldo e por 48 horas para o Etest. A comparação da CIM, considerando-se uma diferença de duas diluições, mostrou que os dois métodos apresentaram excelente correlação, havendo um acordo de 96,6% (58/60), 90% (54/60), 93,3% (56/60) e 100% (60/60) para fluconazol, cetoconazol, itraconazol e anfotericina B respectivamente. Os resultados obtidos mostraram que o método de Etest é semelhante ao método de referência (diluição em caldo), podendo ser utilizado para avaliar a suscetibilidade de leveduras em laboratórios clínicos.

UNITERMOS: *Cryptococcus neoformans*. Suscetibilidade *in vitro*. Agentes antifúngicos.

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