ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1996, p. 822–824 Vol. 40, No. 3
0066-4804/96/$04.00+0
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In Vitro Susceptibilities of Clinical and Environmental Isolates of *Cryptococcus neoformans* to Five Antifungal Drugs

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Received 31 August 1995/Returned for modification 26 October 1995/Accepted 11 December 1995

A total of 53 *Cryptococcus neoformans* strains, including clinical and environmental Brazilian isolates, were tested for their susceptibilities to amphotericin B, 5-flucytosine, ketoconazole, fluconazole, and itraconazole. The tests were performed according to the National Committee of Clinical Laboratory Standards recommendations (document M27-P). In general, there was a remarkable homogeneity of results for all strains, and comparable MICs were found for environmental and clinical isolates. This paper represents the first contribution in which susceptibility data for Brazilian *C. neoformans* isolates are provided.

Cryptococcus neoformans is an opportunistic fungal pathogen responsible for severe meningoencephalitis in approximately 6 to 8% of AIDS patients (24). Despite the availability of newer antifungal drugs, therapeutic difficulties have been encountered during treatment of the acute phases of cryptococcosis in patients with AIDS, and lifelong therapy is necessary to suppress the infection (12, 17).

Few studies on the antifungal susceptibilities of clinical and environmental strains of *C. neoformans* have been performed (3, 9), especially in Brazil, where little is known about the occurrence and characterization of the yeast (15, 22). The purpose of the current investigation was to assess the in vitro susceptibilities of clinical and environmental isolates of *C. neoformans* to five antifungal drugs by using the reference macrodilution method proposed by the National Committee for Clinical Laboratory Standards (NCCLS) (18).

***C. neoformans* strains.** A total of 53 strains were included in this study. Thirty-six clinical isolates were obtained from cerebrospinal fluid specimens from AIDS patients (33 patients) and non-AIDS patients (2 renal transplant recipients and 1 patient with no recognizable underlying condition). All except three isolates were obtained from patients during the initial diagnosis (not relapse) of cryptococcal infection. The recurrent isolates were obtained after the patients had been treated with amphotericin B and 5-flucytosine. Fifteen environmental isolates were obtained from pigeon droppings sampled in Belo Horizonte, Minas Gerais, Brazil. To recover *C. neoformans* from pigeon excreta, 44 random samples were collected from different sites in Belo Horizonte, and after appropriate dilution, they were inoculated on antibiotic-biphenyl-supplemented Niger seed agar plates (5). Each positive site was represented by a single isolate. The other two strains, GSU836C (*C. neoformans* var. *gattii* ATCC 34883) and GSUF3502A (*Filobasidiella neoformans* var. *neoformans* ATCC 34874), were obtained from laboratory collections at Georgia State University, Atlanta. All clinical and environmental isolates were identified as *C. neoformans* by a positive Niger seed agar response, positive urease test, ability to grow at 37°C, and negative nitrate test (13). They were all further characterized by evaluating their profiles of assimilation of 22 carbon compounds (13). Each isolate was identified as *C. neoformans* var. *neoformans* by using the canavanine-glycine-bromothymol blue agar method described by Kwon-Chung et al. (14). The strains were maintained at 4°C on Sabouraud dextrose agar slants (Difco).

Susceptibility testing. The antifungal agents fluconazole (provided by Pfizer, São Paulo, Brazil), ketoconazole and itraconazole (provided by Janssen Pharmaceutica, São Paulo, Brazil), and amphotericin B and 5-flucytosine (purchased from Sigma Chemical Co., St. Louis, Mo.) were used.

The standard method for antifungal susceptibility testing proposed by the NCCLS (proposed standard M27-P) was used for in vitro susceptibility tests (18). Briefly, isolates were tested by a broth macrodilution technique with RPMI 1640 medium containing L-glutamine but no sodium bicarbonate (Sigma) and buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS) (Sigma). An inoculum of 10^3 cells per ml was prepared by a spectrophotometric method, and incubation was at 35°C for 72 h. Final drug concentrations ranged from 64 to 0.03 μg/ml for azoles and 5-flucytosine and from 16 to 0.06 μg/ml for amphotericin B. Following incubation, the growth in each tube was scored as follows (7): 0, optically clear; 1+, slightly hazy; 2+, prominent reduction in turbidity compared with that of the drug-free control (80% inhibition end point); 3+, slight reduction in turbidity compared with that of the drug-free control; and 4+, no reduction in turbidity compared with that of the drug-free control. Fluconazole, ketoconazole, itraconazole, and 5-flucytosine MICs were defined as the lowest drug concentration which resulted in a visual turbidity less than or equal to 80% inhibition compared with that produced by the growth control. Amphotericin B MICs were defined as the lowest drug concentration at which there was an absence of growth.

The MIC ranges and the MICs required to inhibit 50 and 90% of the isolates of the five drugs are summarized in Table 1. Flucytosine and fluconazole MICs showed a broad range for clinical and environmental isolates, whereas those of amphotericin B, ketoconazole, and itraconazole were within narrower ranges. Most of the *C. neoformans* isolates showed uniform patterns of susceptibility to the antifungal agents tested. The MICs for the control *C. neoformans* var. *gattii* and *F. neoformans* var. *neoformans* strains tested concomitantly were within the expected range (8).

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TABLE 1. In vitro activities of five antifungal agents against 53 *C. neoformans* isolates

Agent and strain group	No. of strains tested	MIC (μg/ml)[a]		
		Range	50%	90%
Amphotericin B				
Environmental	15	0.125–1	0.50	1
Clinical	36	0.25–1	0.50	1
Control	2	0.25		
5-Flucytosine				
Environmental	15	1–8	2	8
Clinical	36	0.25–8	2	8
Control	2	8		
Ketoconazole				
Environmental	15	≤0.03–0.25	0.125	0.25
Clinical	36	≤0.03–0.25	0.125	0.25
Control	2	0.125–0.25		
Itraconazole				
Environmental	15	0.06–0.25	0.125	0.25
Clinical	36	≤0.03–0.25	0.125	0.25
Control	2	≤0.03–0.06		
Fluconazole				
Environmental	15	2–8	2	4
Clinical	36	0.5–16	4	8
Control	2	1–4		

[a] 50% and 90%, MICs at which 50 and 90% of the strains, respectively, are inhibited.

The in vitro susceptibilities of different *C. neoformans* strains to amphotericin B, 5-flucytosine, ketoconazole, fluconazole, and itraconazole have been studied by a number of investigators (4, 12, 16, 17, 19). However, direct comparisons of MIC data between studies are complicated by variability in test methods and the lack of standardized approaches (11, 20, 21, 23). The proposed reference macrodilution method of the NCCLS (M27-P) (18) provides a standardized guideline for in vitro testing of yeast isolates with acceptable (85 to 90%) intra- and interlaboratory reproducibilities (23). Moreover, the use of this methodology facilitates comparison of MICs between reports (21).

Although the macrodilution method reported by the NCCLS document has been proposed only for flucytosine, amphotericin B, ketoconazole, and fluconazole, we applied the same technique for use with itraconazole.

Our results on the in vitro activities of amphotericin B, 5-flucytosine, ketoconazole, fluconazole, and itraconazole against *C. neoformans* are similar to those published earlier (2, 10). Among the azoles, fluconazole showed lower activity than either ketoconazole or itraconazole. In fact, previous reports have already showed the low activity of fluconazole against *C. neoformans* isolates, even though the drug has proven to be more active in vivo (16, 17). According to those authors, the good therapeutic results obtained with fluconazole are largely attributable to high concentrations in cerebrospinal fluid.

Some previous reports showed that RPMI 1640 medium, which is suggested as part of the M27-P methodology, does not support suitable growth of *C. neoformans* isolates (6, 12). However, in our study, we found that this medium provided adequate growth of the 53 cryptococcal strains tested. This finding agrees with that reported by Anaisse et al. (1), who found that RPMI 1640 is a suitable medium for testing cryptococci.

As shown in Table 1, no significant differences in antifungal susceptibilities between environmental and clinical strains were observed. We believe that this is the first study in which in vitro susceptibility data for environmental and clinical *C. neoformans* isolates obtained from Brazil have been investigated. However, our findings are in good agreement with those reported by Bava and Negroni (3) and Fromtling et al. (9), who found no difference in the in vitro antifungal susceptibilities of environmental and clinical *C. neoformans* strains obtained from Argentina and Puerto Rico, respectively. Thus, the origin of the strain does not seem to influence its susceptibility to antifungal drugs.

The antifungal susceptibility data reported here provide a new basis for comparing the susceptibility profiles of Brazilian *C. neoformans* isolates by using test conditions in conformity with the NCCLS guidelines. Moreover, these data may serve as a reference point for other in vitro comparative studies using the same methodology. Further studies are necessary to establish the relationship between the in vitro test results and the clinical outcome of the cryptococcal infection.

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