

***In vitro* SUSCEPTIBILITY TO ANTIFUNGAL AGENTS OF CLINICAL AND ENVIRONMENTAL *Cryptococcus neoformans* ISOLATED IN SOUTHERN OF BRAZIL**

Sydney Hartz ALVES(1), Loiva T. OLIVEIRA(2), Jane M. COSTA(2), Irina LUBECK(3), Agnes Kiesling CASALI(4) & Marilene Henning VAINSTEIN(4)

SUMMARY

The purpose of the present study was to compare the susceptibility to four antifungal agents of 69 *Cryptococcus neoformans* strains isolated from AIDS patients with that of 13 *C. neoformans* strains isolated from the environment. Based on the NCCLS M27-A methodology the Minimal Inhibitory Concentrations (MICs) obtained for amphotericin B, itraconazole and ketoconazole were very similar for clinical and environmental isolates. Clinical isolates were less susceptible to fluconazole than environmental isolates. The significance of these findings and aspects concerning the importance, role and difficulties of *C. neoformans* susceptibility testing are also discussed.

KEYWORDS: *Cryptococcus neoformans*; Susceptibility; Antifungal agents

INTRODUCTION

Cryptococcus neoformans is an opportunistic yeast recognized as the etiological agent of human cryptococcosis. This widespread organism was noted to occur in immunosuppressed hosts especially those with AIDS, for whom disseminated disease is being increasingly reported²⁵.

Cryptococcus neoformans is rarely isolated from healthy individuals and does not appear to be a common human commensal. The sporadic nature of human cryptococcosis, the extreme rarity of documented human-to-human transmission events^{3,12} and the high prevalence of *C. neoformans* in the environment indicate that human infection is acquired from environmental sources⁵. It was assumed that inhalation of infectious particles from avian excreta, the main natural source of environmental strains of the var. *neoformans*, is the major route for human infection⁵.

Based on the strong relationship between environmental and clinical *C. neoformans* strains⁵ and the few comparative studies carried out on antifungal susceptibilities^{9,10}, the purpose of this study was to evaluate the susceptibility of 82 *C. neoformans* strains isolated in the state of Rio Grande do Sul, Brazil.

MATERIAL AND METHODS

***Cryptococcus neoformans* strains:** a total of 82 strains were studied. Forty-eight isolates were obtained from cerebrospinal fluid (43), blood (3) and sputum (2) from 38 AIDS patients at the Hospital Universitário de Santa Maria, Santa Maria (RS, Brazil) from January 1996 through December 2000. Thirty-eight strains were obtained from patients during

the initial diagnosis of cryptococcal infection and ten strains were obtained after the patients had been treated with amphotericin B or fluconazole. Other twenty-one clinical samples were kindly provided by Central Laboratory of Instituto de Pesquisas Biológicas da Secretaria da Saúde e Meio Ambiente in Rio Grande do Sul State – LACEN (fourteen) and by Hospital São Lucas (seven), from Porto Alegre. Environmental samples (thirteen) were from pigeon excreta originated from two different cities in the Rio Grande do Sul State, Porto Alegre and Santa Cruz do Sul, located in the South region of Brazil and separated apart by a distance of 150 km. For *C. neoformans* isolation, 1.0 g of weathered pigeon excreta was added to 10 ml sterilized saline solution with both chloramphenicol and ampicillin at 150 mg ml⁻¹ each. After filtration through sterilized gauze, aliquots of 10⁻¹ and 10⁻² dilutions were inoculated onto birdseed agar plates and incubated at 37 °C up to 21 days. All strains were identified as *C. neoformans* by a positive Niger seed agar response, positive urease test, ability to grow at 37 °C and negative nitrogen test. The profiles of carbon compound assimilation were also determined⁵. Each isolate was identified as *C. neoformans* var *neoformans* by the canavanine-glycine-bromothymol-blue-agar method described by KWON-CHUNG *et al.*¹⁸.

Antifungal agents: the antifungal agents used were amphotericin B (Sigma), itraconazole and ketoconazole (Jansen Pharmaceutica), and fluconazole (Pfizer).

Susceptibility testing: we used the macrodilution technique¹⁹. RPMI 1640 medium (American Biorganics Inc.) containing L-glutamine was prepared according to manufacturer instructions. After reconstitution the medium was supplemented with glucose to obtain a final

(1) Prof. Adjunto Microbiologia. Departamento Análises Clínicas e Toxicológicas; Laboratório de Pesquisas Micológicas (LAPEMI). Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brasil.

(2) Hospital Universitário de Santa Maria, Santa Maria, RS, Brasil.

(3) Acadêmica do Curso de Veterinária, Bolsista do PBIC (CNPq), UFSM, Santa Maria, RS, Brasil.

(4) Programa de Pós-graduação em Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brasil.

Correspondence to: Sydney Hartz Alves, Departamento de Análises Clínicas e Toxicológicas, CCS, Universidade Federal de Santa Maria. 97119-000 Santa Maria, RS, Brazil. E-mail: hartzsa@fatecnet.ufsm.br

concentration of 2%, and buffered to pH 7.0 with 3-(N-morpholino)propanesulfonic acid (MOPS; Sigma) to a final concentration of 165 mM. The procedures of inoculum preparation and incubation were those of the M27-A methodology¹⁹. The minimal inhibitory concentration (MIC) of amphotericin B was defined as the lowest concentration of drug which resulted in complete inhibition of visible growth. The MICs of azoles were defined as the lowest concentration of drug which resulted in an 80% reduction of fungal growth compared to control. The assays were read 48 h after inoculation. The data are reported as MIC ranges and MICs at which 50% and 90% of the isolates were inhibited. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control for the susceptibility tests.

RESULTS

Table 1 summarizes the *in vitro* susceptibilities of 69 clinical and 13 environmental isolates.

Table 1

In vitro susceptibility of *C. neoformans* strains to amphotericin B and azoles

Antifungal agents and strains group ^b	MIC (µg/ml) ^a		
	Range	50%	90%
Amphotericin B			
Clinical	0.0625-0.5	0.125	0.25
Environmental	0.0625-0.5	0.25	0.5
Fluconazole			
Clinical	0.125- 16	2.0	8.0
Environmental	0.125- 16	0.5	4.0
Itraconazole			
Clinical	0.031-0.25	0.125	0.25
Environmental	0.031-0.25	0.125	0.125
Ketoconazole			
Clinical	0.031-0.5	0.125	0.125
Environmental	0.031-0.25	0.0625	0.125

^a 50% and 90%, MICs at which 50 and 90% of the strains, were inhibited, respectively; ^b Clinical isolates (n = 69); environmental isolates (n = 13).

Amphotericin B showed similar MIC ranges against the strains from both sources but MIC 50% and MIC 90% were slightly more elevated for the environmental strains.

Fluconazole showed a broad MIC range, varying from 0.125 to 16 µg/ml for clinical and environmental isolates. The MICs 50% and 90% were more elevated for clinical than environmental isolates.

For itraconazole the MICs were within narrow ranges (0.031-0.25 µg/ml) for clinical and environmental isolates and the MIC 50% was the same for all strains. The MIC 90% for clinical isolates was more elevated than environmental isolates. Ketoconazole showed narrow MIC ranges from 0.031 µg/ml to 0.5 µg/ml. The MIC 50% for clinical isolates was more elevated than environmental isolates, but the MIC 90% was similar for clinical and environmental strains.

DISCUSSION

The concern about *C. neoformans* susceptibility is not recent⁴; however considering the newly standardized testing, the results currently obtained are becoming more appreciated because they allow inter-laboratory comparisons. Our purpose was to compare clinical and environmental isolates because the emergence of resistance is an apparently rare phenomenon that encompasses development after long treatment with antifungals (secondary resistance) or primary resistance without former exposure to these agents^{8,23}.

Our results are closely similar to those reported by other authors^{7,9,12,14,27} and seem to indicate susceptibility of *C. neoformans* strains to antifungal agents. 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^{8,11,23}. Reports about amphotericin B-resistant *C. neoformans* are scarce^{15,16,17,24} even during the AIDS era. Based on the M27-A methodology, our MIC ranges obtained for amphotericin B allow us to conclude that the clinical and environmental isolates studied are susceptible to the drug, as also reported by others^{7,9,14}.

Fluconazole has been shown to be an effective alternative to amphotericin B in the treatment of cryptococcal meningitis and is the most commonly used antifungal agent in maintenance therapy for this disease²⁵. The majority of cases of meningitis due to *C. neoformans* resistant to fluconazole have been reported in AIDS patients after long treatments or prophylaxis with fluconazole^{1,2,21,22}. Furthermore, a fluconazole resistant strain isolated from an immunocompetent patient without exposure to this triazole has been reported, which alerts that environmental strains can be primarily resistant to fluconazole²⁰. So, cryptococcal susceptibility to fluconazole could be an important predictor of treatment success and MICs can be useful to monitor the possible development of resistance during therapy and to identify primary resistance^{8,28}. Our results based on MIC 50% and MIC 90% show that clinical isolates were less susceptible to fluconazole than environmental isolates even though MIC ≤ 16 µg/ml was observed in both groups of strains. However, the overall susceptibility of the southern Brazilian *C. neoformans* isolates to fluconazole was very similar to that reported for American isolates and in another Brazilian study^{9,27,28}. FRANZOT & HAMDAN⁹ found that 100% of isolates from Minas Gerais (a central Brazilian state) were inhibited by ≤ 16 µg/ml of fluconazole, as also observed in the present investigation.

C. neoformans is extremely susceptible to itraconazole *in vitro*²⁶; our data are similar to those reported by several other investigators who used the same methodology^{7,9}. It seems clear that *C. neoformans* resistance to itraconazole is too rare and cross-resistance with fluconazole does not occur^{1,6,21,22}. However, IWATA *et al.*¹³ obtained an itraconazole-resistant *C. neoformans* strain after exposure to N-nitro-nitrosoguanidine. The mechanism of action of itraconazole which blocks the lanosterol 14 α-demethylase and the NADPH-dependent-3-ketosteroid reductase in *C. neoformans* may have implications both for its potent antifungal activity and for its reduced development of cross-resistance compared to other azoles⁵. We did not observe differences in itraconazole MICs

50% between clinical and environmental isolates and our results are similar to those reported by others^{5,9}.

Ketoconazole is not indicated for the treatment of cryptococcal meningoencephalitis but was included in the present study in order to evaluate a possible cross-resistance among azoles. The ketoconazole MICs observed were as low as that obtained for itraconazole and the interpretation is the same. The 3 *C. neoformans* strains that showed fluconazole MICs = 16 µg/ml did not demonstrate elevated MICs for itraconazole and ketoconazole.

Finally, we found that clinical and environmental *C. neoformans* strains showed a similar pattern of susceptibility to amphotericin B, itraconazole and ketoconazole. The clinical isolates were less susceptible to fluconazole than the environmental isolates, possibly as a consequence of therapy with this agent. As postulated by others, during therapy of cryptococcosis the susceptibility tests of recurrent isolates may monitor the development of secondary resistance, because if susceptibility does not predict successful therapy, resistance should often predict therapeutic failure^{1,2,6,8,15,16,19,21,22,23,24,28}.

RESUMO

Suscetibilidade *in vitro* a antifúngicos, de amostras clínicas e ambientais de *Cryptococcus neoformans* isoladas no sul do Brasil

Comparou-se a suscetibilidade de 69 amostras de *C. neoformans* isoladas de pacientes com SIDA com 13 amostras de *C. neoformans* isoladas do meio ambiente, frente a quatro agentes antifúngicos. Com base na metodologia preconizada pelo NCCLS (M27-A) as concentrações inibitórias mínimas (CIMs) obtidas para a anfotericina B, itraconazol e cetoconazol foram muito semelhantes nos dois grupos estudados. Todavia, frente ao fluconazol, os isolados clínicos evidenciaram menor sensibilidade do que os provenientes do meio ambiente. Alguns aspectos envolvendo a importância e dificuldades dos testes de suscetibilidade com *Cryptococcus neoformans* são também discutidos pelos autores.

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Received: 15 February 2001

Accepted: 22 August 2001