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Original

Fluconazole and amphotericin B susceptibility testing of *Cryptococcus neoformans*: Results of minimal inhibitory concentrations against 265 isolates from HIV-positive patients before and after two or more months of antifungal therapy

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ABSTRACT

Cryptococcosis, a fatal disease without appropriate treatment, is still one of the major opportunistic mycoses in AIDS patients in Argentina despite the availability of high active anti-retroviral therapy (HAART).

Over the last decade, drugs employed in the treatment of disseminated cryptococcosis at Infectious Diseases Hospital "F. J. Muñiz" included amphotericin B (AMB) followed by fluconazole (FCZ), due to the fact that flucytosine was not available in Argentina during this period. A considerable number of patients did not negativize cultures after 2–3 weeks of treatment as it was expected, and in some of them the isolation of *Cryptococcus neoformans* in different samples was still possible after 2 or more months of adequate therapy and even in cases with clinical improvement.

The aim of this study was to establish the susceptibility profile of *C. neoformans* clinical isolates to those antifungals and to investigate whether there were any changes after at least 2 months of treatment. A total of 265 strains were studied (116 obtained from patients at diagnosis and 149 corresponding to the same individuals 2 months or more after receiving therapy). Susceptibility patterns before treatment to AMB showed MICs $\leq 1 \mu\text{g/ml}$ for all the strains, and no increase was seen after treatment.

All the strains were susceptible to FCZ (MIC $\leq 8 \mu\text{g/ml}$) at diagnosis; but in a group with relapses or those who did not negativize cultures, one isolate became resistant after therapy (MIC $\geq 64 \mu\text{g/ml}$) and other four showed dose-dependent susceptibility (MIC 16–32 $\mu\text{g/ml}$). There was no relation between these results and clinical outcome as it was pointed out in other publications.

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Resultados de las pruebas de sensibilidad a fluconazol y anfotericina B de cepas de *Cryptococcus neoformans* aisladas de pacientes VIH-positivos antes y después de dos o más meses de tratamiento antifúngico

RESUMEN

La frecuencia de la criptococosis en los pacientes con sida sigue siendo alta en Argentina, a pesar de contar con terapia antirretroviral de alta eficacia. Sin tratamiento, esta micosis es habitualmente fatal.

En la última década, el esquema terapéutico empleado para la criptococosis en el Hospital de Infecciosas "F. J. Muñiz" ha consistido en la administración de anfotericina B (AMB) seguida por fluconazol (FCZ), ya que la 5-fluorocitosina no se comercializa en Argentina. En un número considerable de pacientes no se negativizan los cultivos en 2–3 semanas de iniciada la terapia antifúngica, tal como sería deseable, y es posible aislar *Cryptococcus neoformans* en diferentes muestras clínicas aún después de dos ó más meses de tratamiento, inclusive en casos que mejoran clínicamente.

El objetivo de este estudio fue evaluar el perfil de sensibilidad de aislamientos de *C. neoformans* obtenidos de 116 pacientes a las dos drogas mencionadas y compararlos con los de 149 aislamientos de los mismos enfermos después de, al menos, dos meses de tratamiento.

Pudimos comprobar que la concentración inhibitoria mínima (CIM) de AMB fue $\leq 1 \mu\text{g/ml}$ para los 265 aislamientos antes y después del tratamiento.

La CIM de FCZ antes de iniciada la terapia fue $\leq 8 \mu\text{g/ml}$ en todos los casos. Solamente un aislamiento de un enfermo que presentó una recidiva mostró resistencia in vitro a esta droga (CIM $\geq 64 \mu\text{g/ml}$), y otros

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cuatro mostraron sensibilidad dosis dependiente (CIM 16–32 µg/ml): tres de ellos de pacientes con recidivas y el cuarto de un enfermo que continuó con cultivos positivos durante largo tiempo. Estos valores no tuvieron correlación con la evolución de la micosis, como ya ha sido señalado en otras publicaciones.

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Cryptococcosis is still one of the major opportunistic mycoses in HIV-infected patients in Argentine despite the fact that high active anti-retroviral therapy (HAART) is available in most public hospitals.

This is a fatal disease if it is not treated, but even in cases of appropriate therapy the mortality rate ranges from 17%¹⁰ to nearly 30%,¹⁹ especially during the first 3 weeks after initiation of antifungal treatment.¹

Most of the observed recurrences during the maintenance period are due to the persistence of the original strain more than re-infection with a different isolate as it was suggested in previous studies.¹⁰ There are not significant variations in serial MICs for strains isolated from patients with persistent cryptococcosis but occasionally fluconazole (FCZ)-resistant isolates do appear.²⁶

Chronic use of azoles as prophylaxis or treatment could exert selection pressure in favor of azole-resistant strains and is one of the reasons for in vitro susceptibility monitoring of these drugs.^{8,25} Many reports suggest that emergency of resistance against amphotericin B (AMB), flucytosine (5-FC), fluconazole or itraconazole (ITZ) is very unlikely; however, an increase ranging from 2.5% to 14% was registered between 2001 and 2002 in a Hospital in Cambodia.^{6,15,21}

During the last decade, in the Mycology Unit of the Infectious Diseases Hospital “F.J. Muñiz”, an average of 100 new cases/year of cryptococcosis associated with AIDS were diagnosed and treated. As 5-FC was not available in our country, the standard treatment for this mycosis was AMB 0.7 mg/kg daily during 3 weeks followed by FCZ 800 mg/day up to 10–12 weeks. As secondary prophylaxis FCZ at a daily dose of 200 mg was used.¹⁹

The aim of this study was to determine the susceptibility profile of *Cryptococcus neoformans* in this group of patients in our Institution against AMB and FCZ and also to evaluate whether there were any changes in MIC levels in those patients who continued with positive cultures after 2 or more months of treatment.

Materials and methods

Patients

A total of 116 patients (99 males and 17 females), with a mean age of 31.4 years, with cryptococcosis associated with AIDS diagnosed at the Mycology Unit between 1999 and 2005 were included.

All of them had at least one positive culture 2 or more months after the standard antifungal treatment mentioned above was

initiated. In 14 cases *C. neoformans* was isolated on several occasions (without intermediate negative cultures) and other 11 had relapses (positive culture after at least two previous negative cultures).

Microorganisms

A total of 265 *C. neoformans* strains were studied; they were recovered from CSF ($n = 226$) or blood ($n = 34$) but yeasts from other locations were also obtained (one skin biopsy, one bronchoalveolar lavage, three urine samples). In this study 116 isolates cultured at diagnosis and 149 obtained after more than 2 months receiving therapy were included. The number and distribution of strains corresponding to patients who had suffered relapses or those cases that continued with positive cultures for longer periods are shown in Table 1.

The isolates were identified by standard procedures in our laboratory^{2,5}: positive India ink, urease activity, phenol-oxidase in sunflower seed agar, growth at 37 °C. Differentiation from *Cryptococcus gattii* was made in glycine-canavanine-bromothimol blue (GCB) and glycine-cicloheximide-phenol red (Salkin-agar) media.^{13,23}

All the strains were maintained in distilled water at room temperature up to the moment of test performance. First they were recovered on sunflower agar to assess viability and purity, and then sub-cultured on Sabouraud-dextrose agar during 48 h at 35 °C.

Antifungal susceptibility tests

Broth micro-dilution technique according to CLSI (formerly NCCLS) guidance (M27-A2 document)¹⁸ was performed to determine minimal inhibitory concentration (MIC) of fluconazole and amphotericin B. RPMI (Gibco[®]) buffered with 0.165 M morpholine-propane sulphonic acid (MOPS) to pH 7.0 and with 2% dextrose was used. The final inoculum size for all isolates and control strains was $0.5\text{--}2.5 \times 10^3$ CFU/ml. The final concentrations of drugs ranged between 0.03 and 16 µg/ml for AMB (Sigma[®], USA) and between 0.25 and 64 µg/ml for FCZ (provided by Pfizer UK). Microplates (U bottom) were incubated at 35 °C for 72 h and MICs were determined visually as the concentration of drug that produced a prominent decrease in turbidity compared with growth control for FCZ and a complete inhibition of growth for AMB. As quality controls for this technique, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used.¹⁸

Since no interpretive breakpoints have been determined for *C. neoformans*,^{4,14} we used those considered in document M27-A2

Table 1

Number and distribution of the 265 *Cryptococcus neoformans* isolates studied from 116 patients with positive cultures for more than 2 months.

Group of patients	No. of patients	No. of isolates before treatment/patient	No. of isolates after treatment/patient	Total number of isolates
Patients with one isolate after 2 months	91	1	1	182
Patients with several positive cultures	11	1	2	33
	1	1	3	4
	1	1	4	5
	1	1	5	6
Patients with relapses	9	1	2	27
	2	1	3	8
Total no. of isolates		116	149	265

for *Candida* as we used the methodology corresponding to that guideline.¹⁸ In brief, breakpoints for FCZ were: MIC \leq 8 μ g/ml: susceptible (S), MIC = 16–32 μ g/ml: susceptible dose-dependent (S-DD) and MIC \geq 64 μ g/ml: resistant (R); and for AMB, MIC \leq 1 μ g/ml indicated that the isolate was not resistant to this antifungal.

Results

The results of susceptibility tests of the isolates at diagnosis and after therapy of the 116 patients are presented in Table 2. MIC₅₀ and MIC₉₀ of both drugs before and after treatment were the same, and no significant differences were seen in geometric mean values.

AMB showed MICs \leq 1 μ g/ml for all the 265 isolates studied.

Before therapy, strains of the 116 patients were susceptible to FCZ (MIC \leq 8 μ g/ml) and there was no increase in MIC values of this antifungal in the 91 patients with only one positive culture after 2 months of treatment.

In 11 individuals who suffered relapses, MIC of FCZ remained at the same values in seven, in three strains it became S-DD (MIC = 16 μ g/ml in two and MIC = 32 μ g/ml in the third), and in one patient (MIC = 1 μ g/ml before therapy) *C. neoformans* isolate turned out resistant (MIC \geq 64 μ g/ml).

On the other hand, in 14 patients who maintained positive cultures for longer periods, the MIC of FCZ did not change except in one, in which the isolate became S-DD (MIC at diagnosis was 4 μ g/ml, and 16 μ g/ml in the last determination done).

Discussion

There were several reports showing that *C. neoformans* is susceptible in vitro to AMB, FCZ, itraconazole, voriconazole, albaconazole and flucytosine^{9,12,17} but resistant isolates could appear when azoles like FCZ are used for a long period; nevertheless, clones with a high level of resistance appear only with 0.7–4.6% of frequency.^{7,8,20,26} Defects in Δ ^{8,7} isomerase are associated with resistance and has been found in a case of cryptococcosis²⁵ and also heteroresistance has been shown in *C. neoformans* (approximately 4.7%),^{14,26} the same as in *Candida albicans* exposed to azoles.¹⁶ Another possible mechanism is lack of affinity to Erg11p of this yeast.^{8,14,25}

At our institution, cryptococcosis affects approximately 10% of AIDS patients admitted to the hospital, and some of them continue having positive cultures for long periods despite treatment. One of the possible explanations could be that the

Table 2

Results of susceptibility testing to amphotericin B and fluconazole of *Cryptococcus neoformans* isolates from 116 patients with cryptococcosis associated with AIDS at diagnosis and after at least 2 months of treatment.

	Amphotericin B (μ g/ml)		Fluconazole (μ g/ml)	
	At diagnosis	After treatment	At diagnosis	After treatment
No. of isolates	116	149	116	149
Range	0.03–1	0.03–1	0.25–16	0.25–64
Geometric mean	0.18	0.20	2.50	2.55
MIC ₅₀ ^a	0.25	0.25	4	4
MIC ₉₀ ^b	0.50	0.50	8	8

^a MIC₅₀: Minimal inhibitory concentration for at least 50% of isolates.

^b MIC₉₀: Minimal inhibitory concentration for 90% of isolates.

initial treatment was with AMB alone, as 5-FC was not available during the last decade in our country. This delay in negativization does not seem to be caused by an increased resistance to drugs, as it is shown in this study where we found that all strains seemed to be susceptible to AMB (MICs \leq 1 μ g/ml) before and after treatment and remained stable even in case of relapses. MIC values were similar to those found in other publications.^{9–11} All the tests were performed in RPMI but some strains were also tested in AM3 (data not shown) and no differences were found.

On the other hand, in only five patients with relapses or with positive cultures for long periods, strains turned S-DD in four, and one isolate became resistant to FCZ (MIC \geq 64 μ g/ml). Some authors consider that timed-kill curves are more efficient in the demonstration of tolerant strains to some antifungals.²² However, in a multicenter study carried out from 1990 to 2004 on 1811 isolates from different countries, resistance observed was less than 1%.⁷

As it is pointed out in other research papers, it is difficult to predict outcome in this group of patients with cryptococcosis based on in vitro susceptibility tests.^{3,10} The facts that no interpretive breakpoints have been determined for *C. neoformans* and still there is no agreement between CLSI and EUCAST in this matter make the methods in use at the moment insufficient to accurately predict clinical evolution.²⁴

The outcome in this disease depends on many factors, especially intracranial pressure, other concomitant opportunistic infections, drugs interactions, adherence to therapy, immune recovery (increase in CD4+ T-cell counts), viral load, and capsular polysaccharide level, among others.¹⁹

It could be demonstrated that there were no resistant strains among patients suffering from cryptococcosis associated with AIDS in our institution, and susceptibility tests and timed-kill curves should be reserved for those with clinical failure. Besides, in case of relapses, it should be investigated whether the increase in resistance is due to selection pressure or whether it is a new strain, using pulsed field gel electrophoresis or other molecular techniques.⁸

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