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 fluconazole 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 $\leq 1 \text{ mg/ml}$ for all the strains, and no increase was seen after treatment.

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

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Cryptococcosis is still one of the major opportunistic mycoses in HIV-infected patients in Argentina 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% to nearly 30%, 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 emergence 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 laboratory5,6: positive India ink, urease activity, phenoloxidase in sunflower seed agar, growth at 37 °C. Differentiation from Cryptococcus gattii was made in glycine–canavanine–brothiromil 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 0.5–2.5 × 10^5 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,4,16 we used those considered in document M27-A2

<table>
<thead>
<tr>
<th>Group of patients</th>
<th>No. of patients</th>
<th>No. of isolates before treatment/patient</th>
<th>No. of isolates after treatment/patient</th>
<th>Total number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with one isolate after 2 months</td>
<td>91</td>
<td>1</td>
<td>1</td>
<td>182</td>
</tr>
<tr>
<td>Patients with several positive cultures</td>
<td>11</td>
<td>1</td>
<td>2</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Patients with relapses</td>
<td>9</td>
<td>1</td>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Total no. of isolates</td>
<td>116</td>
<td>149</td>
<td>265</td>
<td></td>
</tr>
</tbody>
</table>
for Candida as we used the methodology corresponding to that guideline. In brief, breakpoints for FCZ were: MIC<sub>s</sub> ≤ 8 μg/ml: susceptible (S); MIC = 16–32 μg/ml: susceptible dose-dependent (S-DD) and MIC≥64 μg/ml: resistant (R); and for AMB, MIC≤1 μg/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. MIC<sub>50</sub> and MIC<sub>90</sub> of both drugs before and after treatment were the same, and no significant differences were seen in geometric mean values.

AMB showed MIC<sub>50</sub> ≤ 1 μg/ml for all the 265 isolates studied. Before therapy, strains of the 116 patients were susceptible to FCZ (MIC<sub>≤</sub>8 μg/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 μg/ml in two and MIC = 32 μg/ml in the third), and in one patient (MIC = 1 μg/ml before therapy) C. neoformans isolate turned out resistant (MIC≥64 μg/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 μg/ml and 16 μg/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, but resistant isolates could be found in vitro to AMB, FCZ, itraconazole, voriconazole, (S-DD) and MIC<br>1. Alexander B, Pfaller M. Contemporary tools for the diagnosis and management of invasive mycoses. Clin Infect Dis. 2006;43:515–27.
11. Ecker M, Lamrous D, Rossignol S, Milam M, Battegay M, Hattenberg T, et al. 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.
12. 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.

**Table 2**

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>At diagnosis</th>
<th>After treatment</th>
<th>At diagnosis</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>0.03–1</td>
<td>0.03–1</td>
<td>0.25–15</td>
<td>0.25–64</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>0.18</td>
<td>0.20</td>
<td>2.50</td>
<td>2.55</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.25</td>
<td>0.25</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>0.50</td>
<td>0.50</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> MIC<sub>50</sub>: Minimal inhibitory concentration for at least 50% of isolates.

<sup>b</sup> MIC<sub>90</sub>: Minimal inhibitory concentration for 90% of isolates.


