# In Vitro Susceptibility Characteristics of *Cryptococcus neoformans* Varieties from AIDS Patients in Goiânia, Brazil

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*Sixty clinical isolates of* Cryptococcus neoformans *from AIDS from Goiânia, state of Goiás, Brazil, were characterized according to varieties, serotypes and tested for antifungal susceptibility. To differentiate the two varieties was used L-canavanine-glycine-bromothymol blue medium and to separate the serotypes was used slide agglutination test with Crypto Check Iatron. The Minimal Inhibitory Concentration (MIC) of fluconazole, itraconazole, and amphotericin B were determined by the National Committee for Clinical Laboratory Standards macrodilution method. Our results identified 56 isolates as* C. neoformans *var.* neoformans *serotype A and 4 isolates as* C. neoformans *var.* gattii *serotype B. MIC values for* C. neoformans *var.* gattii *were higher than* C. neoformans *var.* neoformans. *We verified that none isolate was resistant to itraconazole and to amphotericin B, but one* C. neoformans *var.* neoformans *and three* C. neoformans *var.* gattii *isolates were resistant to fluconazole. The presence of* C. neoformans var. gattii *fluconazole resistant indicates the importance of determining not only the variety of* C. neoformans *infecting the patients but also measuring the MIC of the isolate in order to properly orient treatment.*

Key words: serotypes - antifungal susceptibility - *Cryptococcus neoformans* - Goiânia - Brazil

In the last years fungal infections have emerged as one of the major complications in immunocompromised patients (Pontón & Quindós 1992). Cryptococcosis is the second most important fungal disease in human immunodeficiency virus (HIV)-positive patients, where disseminated infection is commonly fatal (Ito-Kuwa et al. 1994, Pinner et al. 1995, Patterson 1997, Calvo et al. 2001). In Brazil 4.5% of all opportunistic infections in AIDS patients have been reported as being caused by *Cryptococcus neoformans* (Ministério da Saúde 1999).

Epidemiological studies have demonstrated that distribuition of serotypes and varieties are considered to be regionally specific but *C. neoformans* var. *neoformans* (serotypes A and D) are most frequently isolated from AIDS patients throughout the world while *C. neoformans* var. *gattii* causes disease predominantly in normal individuals (Chen et al. 2000). C. *neoformans* var. *gattii* (serotypes B and C) has been prevalent in tropical and subtropical areas and behaves as a primary pathogen, similar in this respect to the other agents of systemic mycosis (Rozembaum & Rios-Gonçalves 1994).

The increased prevalence of fungal infections and the introduction of new antifungal agents have intensified the need evaluation of antifungal susceptibility of *C. neoformans.*

Resistance has begin to emerge with *C. neoformans* var. *neoformans* in severely immunocompromized pati- ents undergoing prolonged azole treatment (Alves et al. 1997, Espinel-Ingroff et al. 1998). Fluconazole is the drug that commands maintenance treatment protocols for AIDS patients (Witt et al. 1996).

In this study, we evaluated 60 clinical isolates of *C. neoformans* colleted from AIDS patients in Goiânia city and analyzed the occurrence of infections caused by the different varieties of *C. neoformans*. Then, we evaluate the in vitro susceptibilities to fluconazole, itraconazole, and amphotericin B.

### MATERIALS AND METHODS

*Isolates* - A total of 60 *C. neoformans* isolates were obtained from AIDS patients with cryptococcal meningitis between October 2001 and April 2002, at Hospital de Doenças Tropicais de Goiânia, state of Goiás. Of these patients 49 (81.7%) were males and 11 (18.3%) were females. The age of the patient ranged between 15 and 56 years. *C. neoformans* isolates were identified by a melanin synthesis on DOPA medium, response positive urease, negative nitrates and ability to grow at 37ºC (Kurtzman & Fell 1998).

*Agar media for physiological tests* - L-canavanine-glycine-bromothymol blue (CGB) agar, described by Kwon-Chung et al. (1982) was used to differentiate the two varietes of *C. neoformans*. Results were read after incubation at 37ºC for 2 to 4 days.

*Slide agglutination test* - Agglutination serotyping was performed with eight factor specific sera Iatron Crypto-Check (Tokio, Japan) and interpreted as described by Ikeda et al. (1982).

*In vitro susceptibility* - Prior to testing, each isolate was grown on Sabouraud agar for 48 h at 35ºC. Suspensions were prepared in 0.85% saline to achieve a 0.5 McFarland standard by spectrophotometric measurement. *Candida krusei* ATCC 6258 was included on each test as quality control strain.

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MICs were determined for the 60 *C. neoformans* isolates according to the procedure of the macrodilution method of antifungal susceptibility testing of the National Committee for Clinical Laboratory Standards (NCCLS M27-A). Fluconazole (Pfizer, New York, US), itraconazole (Janssen Pharmaceuticals, Beerse, Belgium), and amphotericin B (Squibb, Princeton, NJ, US) were obtained as reagent-grade powder. Fluconazole was prepared to obtain two stock solution of 2560 µg/ml and 1920 µg/ml, and the other antifungal drugs were prepared to obtain stock solutions of 3200 µg/ml and 2400 µg/ml. Susceptibility testing was performed in RPMI 1640 medium with L-glutamine and without sodium bicarbonate (Sigma Chemical, St. Louis, MO, US) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) for three antifungal agents. The incubation was at 35ºC for 72 h. The final concentration range was 0.016 to 256 µg/ml (stock solution of 2560 µg/ml) and 0.012 to 192 µg/ml (stock solution of 1920 µg/ml) for fluconazole and 0.002 to 32 µg/ml (stock solution of 3200 µg/ml) and 0.003 to 24 µg/ml (stock solution of 2400 µg/ml) for the other agents. Drug-free and yeast-free controls were included. All susceptibility tests were twice by each antifungal drug.

For the azoles, 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 un-inoculated RPMI). For amphotericin B, endpoints were determined by recording the lowest concentration of the agent that prevented the appearance of visible growth (Espinel-Ingroff et al. 1996, NCCLS 1997). MIC 50 and MIC 90 to fluconazole, itraconazole and amphotericin B were calculated by inhibiting respectively 50% and 90% of the isolates of *C. neoformans*.

Fluconazole, itraconazole and amphotericin B breakpoints values for susceptibility have not yet been proposed by the NCCLS for *C. neoformans*. Nguyen and Yu (1998) recently adapted to *C. neoformans*, the fluconazole and itraconazole values proposed by this Committee for *Candida* spp. values found to be associated with therapeutic failure. In this away, Lozano et al. (1998), recommended that amphotericin MICs above 2 µg/ml indicates resistance to this polyene. Then, we defined resistance for amphotericin B MIC $\geq$ 2 µg/ml, for fluconazole MIC $\geq$ 64 µg/ml and for itraconazole MIC $\geq$ 1 µg/ml.

## RESULTS

According to L-CGB agar, among 60 isolates of *C. neoformans* obtained from AIDS patients, 56 (93.3%) were unable to grow on this medium and were shown to belong to var. *neoformans*, while 4 (6.7%) isolates were able to grow and change the color of the medium from yellow to cobalt blue being characterized as var. *gattii*. Serotyping of this 56 isolates classified all of them as serotype A, and the 4 strains identified of *C. neoformans* var. *gattii* were serotype B.

*Antifungal susceptibility testing* - The MICs of all drugs except amphotericin B covered a broad range. MIC ranges for 56 *C. neoformans* var. *neoformans* were between 2 to 256 µg/ml for fluconazole, 0.047 to 0.75 µg/ml for itraconazole and 0.25 to 1 µg/ml for amphotericin B. MIC ranges for 4 *C. neoformans* var. *gattii* were between 24 to 256 µg/ml for fluconazole; 0.19 to 0.75 µg/ml for itra-conazole and 0.75 to 1 µg/ml for amphotericin B. The fluconazole, itraconazole, and amphotericin B MICs for *C. krusei* ATCC 6258 isolate were 256, 0.5 and 0.5 µg/ml, respectively, while the results obtained by NCCLS (NCCLS 1997) for this same specie are 16-64, 0.12-0.5 and 0.5-2.0 µg/ml, respectively. In general the MICs values for each isolate duplicate were identicals.

All the isolates of *C. neoformans* serotype A were susceptible to amphotericin B and itraconazole, while 98.2% were susceptible to fluconazole. All *C. neoformans* serotype B were susceptible to amphotericin B and itraconazole, but 75% (3/4) were resistant to fluconazole**.**

## DISCUSSION

In this study, 93.3% isolates (56/60) were *C. neo-formans* var. *neoformans*. All these isolates examined were serotype A, consistent with the predominant serotype isolated in most countries. This variety is prevalent among immunosuppressed patients, mainly AIDS patients (Mitchell & Perfect 1995, Steenbergen & Casadevall 2000). In this collection we identified as *C. neoformans* var. *gattii* serotype B four isolates. Human cryptococcosis due to this variety occurs predominantly in non-immuno-compromised individuals (Rozembaum & Rios-Gonçalves 1994). The apparent rarity of *C. neoformans* var. *gattii* infections in AIDS patients is an unexplained observation. Considering that plant species could be reservoirs for *C. neoformans* var. *gattii* (Lazera et al. 1998) and that most HIV-infected individuals reside in urban areas, they might be less exposed to en-vironmental sources of *C. neoformans* var. *gattii* than to sources to variety *neoformans*. The knowledge of the prevalence of serotypes in Goiânia, Brazil has not been determined.

There are no defined breakpoints by NCCLS for the azoles and amphotericin B for *C. neoformans*. For this reason the breakpoints were determined according Nguyen and Yu (1998) and Lozano et al. (1998). They define an isolate as resistant to fluconazole if the MIC was $\geq$ 64 µg/ml, to itraconazole was $\geq$ 1 µg/ml and to amphotericin B was $\geq$ 2 µg/ml. In accordance with these values, in our results all the isolates were susceptible to amphotericin B and itraconazole, and 93.3% were susceptible to fluconazole. In cryptococcosis, amphotericin B is considered the treatment of choice for the initial stages of therapy (White et al. 1994). Resistant isolates to this polyene have been observed in few patients. De Bedout et al. (1999) verified 2 isolates resistant to amphotericin B, but the number of patients whom amphotericin B resistance has been demonstrated, continues to be low. Itraconazole and fluconazole has been also shown to be an effective alternative to amphotericin B in the treatment of cryptococcal meningitis and fluconazole is the most commonly used antifungal agent in maintenance therapy for this disease (Powderly 2000). The results of this study with relation to itraconazole and amphotericin B confirm the observations of Carrilo-Muñoz (1997) that verified all *C. neoformans* isolates were susceptible in vitro to these drugs studied. *C. neoformans* is extremely susceptible in vitro to itraconazole. Our data are similar to those reported

by other investigators who used the same methodology (Alves et al. 2001). However some cases of meningitis due to *C. neoformans* resistant to fluconazole have been reported in AIDS patients (Peetermans et al. 1993, Berg et al. 1998).

In spite of the fact that 93.3% (56/60) of the isolates of *C. neoformans* tested were susceptible to fluconazole, *C. neoformans* var. *gattii* isolates exhibited higher MIC values than *C. neoformans* var. *neoformans*. Additionally, among the 4 isolates of the variety gattii that were tested, 3 were classified as resistant. *C. neoformans* var. *gattii* has been showed more resistant than *C. neoformans* var. *neoformans* by the other authors (Peetermans et al. 1993).

In conclusion, *C. neoformans* serotype A appeared to be most prevalent agent of cryptococcosis in Goiânia. Major differences of in vitro susceptibility were exhibited by the two varieties of *C. neoformans*. *C. neoformans* var. *gattii* isolates were more resistant than *C. neoformans* var. *neoformans* isolates. The presence of *C. neoformans* var. *gattii* isolates fluconazole resistant in this study, indicate the importance of determining not only the variety of *C. neoformans* infecting the patients but also measuring the MIC of the isolate in order to properly orient treatment.

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