In vitro antifungal susceptibility of clinical isolates of *Cryptococcus neoformans* var. *neoformans* and *C. neoformans* var. *gattii*

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Summary

One of the differences observed between the two varieties of *Cryptococcus neoformans* is the greater difficulty to achieve an adequate therapeutical response in patients affected by *C. neoformans* var. *gattii*, an observation that has been validated *in vitro* only rarely. The aim of this work was to study the susceptibility patterns of 35 Colombian clinical isolates of *C. neoformans,* 20 of which belonged to the var. *neoformans* and 15 to the var. *gattii.* The minimal inhibitory concentration (MIC) was determined by broth microdilution, according to a modification of the methodology proposed by the National Committee for Clinical Laboratory Standards (NCCLS), using the breakpoints recently suggested by Nguyen *et al.* (Antimicrob Agents Chemother 1998; 42:471-472). The antifungals tested were amphotericin B, fluconazole and itraconazole. Most of the isolates were susceptible to the three antimycotics tested regardless of the variety. Resistance to amphotericin B (MIC=2 µg/ml) was documented in two (10%) *C. neoformans* var. *neoformans* isolates; additionally, five (33%) *C. neoformans* var. *gattii* isolates felt in the category of fluconazole susceptible but dose dependent (MIC 16 µg/ml). In general, all *C. neoformans* var. *gattii* isolates proved susceptible only to the higher concentrations of the antifungals tested. For amphotericin B, seven (47%) isolates of this variety had MICs of 1µg/ml, for fluconazole there were seven (47%) with MICs of 8µg/ml and in the case of itraconazole, 10 isolates (66%) had MICs> 0.03µg/ml. The data showed that although these isolates would be classified as susceptible, they actually require greater concentrations of the antifungals to be inhibited. This finding may well correlate both with the difficulty to attain therapeutic success in patients affected with *C. neoformans* var. *gattii* and with the need for more prolonged treatment courses in such cases.

Key words

Cryptococcus neoformans var. *neoformans, Cryptococcus neoformans* var. *gattii,* Minimal inhibitory concentration (MIC), Resistance, Susceptible, Susceptible dose dependent

Sensibilidad *in vitro* a los antimicóticos de aislamientos clínicos de *Cryptococcus neoformans* var. *neoformans* y *C. neoformans* var. *gattii*

Resumen

Las dos variedades de *Cryptococcus neoformans*, presentan algunas diferencias entre si, siendo una de ellas, la dificultad para el tratamiento exitoso de los pacientes infectados por *C. neoformans* var. *gattii.* Esta observación ha sido raramente validada *in vitro*. En el presente trabajo, se determinó la sensibilidad de 35 aislamientos clínicos del hongo, 20 de *C. neoformans* var. *neoformans* y 15 de *C. neoformans* var. *gattii*, recuperados todos de pacientes colombianos con criptococosis. La concentración mínima inhibitoria (CMI) se determinó con la prueba de microdilución en caldo, de acuerdo con una modificación a los procedimientos del National Committee for Clinical and Laboratory Standards (NCCLS). Los puntos de corte se hicieron siguiendo lo propuesto recientemente por Nguyen *et al.* (Antimicrob Agents Chemother 1998; 42:471-472). Se emplea-

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ron tres antimicóticos, la anfotericina B, el fluconazol y el itraconazol. La mayoría de los aislamientos fueron sensibles a los tres antimicóticos; sin embargo, se encontró que dos (10%) aislamientos de *C. neoformans* var. *neoformans* eran resistentes a la anfotericina B (CMI=2 µg/ml); adicionalmente, cinco (33%) de *C. neoformans* var *gattii* aunque sensibles al fluconazol, lo eran en la categoría de sensibles dosis dependiente (CMI=16 µgml). En general, los aislamientos de esta variedad fueron inhibidos sólo a las concentraciones más altas de los tres antimicóticos; en siete de ellos (47%), la CMI para anfotericina B fue de 1 µg/ml, un número igual de ellos presentó una CMI de 8µg/ml para el fluconazol mientras que 10 (66%) requirieron concentraciones >0,03 µg/ml de itraconazol para ser inhibidos. Estos resultados indican que, *in vitro,* los antimicóticos usados regularmente en el tratamiento de la criptococosis no ejercen sobre *C. neoformans* var. *gattii* una acción inhibitoria tan marcada como era de esperar, hecho que podría reflejarse no sólo en las dificultades para el tratamiento exitoso de los pacientes afectados por esta variedad, sino también en la necesidad de terapias prolongadas.

Palabras clave *Cryptococcus neoformans* var. *neoformans. Cryptococcus neoformans* var. *gattii,* Concentración mínima inhibitoria, Resistente, Sensible, Sensible dosis dependiente

Cryptococcosis is a life-threatening opportunistic fungal infection of wide geographic distribution [1]. *Cryptococcus neoformans*, the etiological agent, is classified in two varieties, var. *neoformans* and var. *gattii* [2]. Clinical disease due to *C. neoformans* var. *neoformans* is found everywhere while clinical disease due to *C. neoformans* var. *gattii* has been reported only in certain regions, mostly in tropical and subtropical areas [3]. In Colombia, from 370 *C. neoformans* clinical isolates studied, 95.1% were *C. neoformans* var *neoformans* and 4.9% *C. neoformans* var. *gattii* [4].

One of the differences observed between the two varieties, is the difficulty to obtain an adequate therapeutical response in patients infected with *C. neoformans* var. *gattii* [5-7]. Lack of response to treatment and resistance *in vitro* to fluconazole has been reported only once [8]. However, validation of this single observation needs further support.

The aim of this work was to explore *in vitro*, possible differences in the antimycotic susceptibility between the two varieties of *C. neoformans* to amphotericin B, fluconazole and itraconazole, which could have some bearings on the inadequate therapeutic responses observed in patients infected by *C. neoformans* var. *gattii*.

MATERIALS AND METHODS

Isolates. Thirty five clinical isolates were studied, 20 *C. neoformans* var. *neoformans* and 15 *C. neoformans* var. *gattii*. The isolates had been previously identified according to standard procedures [9]; the varieties were determined in canavanine-glycine-bromothymol blue agar (CGB) [10]. Isolates were kept by repeated transfers to Sabouraud dextrose agar (BBL, Beckton Dickinson Microbiology Systems, USA).

Strains of *C. neoformans* ATCC 90112 and *Candida krusei* ATCC 6258 were used as controls and were included whenever a test was run.

Antifungals. A modification of the standard method recommended by the NCCLS M27 document [11] was used. The antifungals tested were: amphotericin B (Fungizone, Squibb, USA), fluconazole (Diflucan, Pzifer Pharmaceuticals, USA) and itraconazole (Sporanox, Janssen Research Foundation, Belgium). The antimycotics used were those administered to patients (M. Rinaldi, HSCTU San Antonio, TX, personal communication, l995). Amphotericin B and fluconazole were dissolved in distilled water and itraconazole in polyethylene glycol (PEG) (PM 400, Fisher Scientific Co., USA). For complete dissolution of the latter product, heating and stirring for 1 h at 75 °C was necessary. The antimycotic concentrations ranged from 0.03-16 µg/ml for amphotericin B, 0.125-64 µg/ml for fluconazole and 0.0078-16 µg/ml for itraconazole. Dilutions of the antifungals were made in RPMI-1640 medium (Sigma Chemical Co., USA) that contained L-glutamine but no sodium bicarbonate and were buffered to pH 7.0 with 0.165 M N-morpholino-propanesulfonic acid (MOPS) (Sigma).

Microplates flat bottom, sterile, were covered with 100 µl of the different concentrations of the antifungals and stored in plastic bags at -70 °C up to 6 months before their use [11].

Inocula. All the isolates and control strains were grown on Sabouraud dextrose agar (BBL, Beckton Dickinson) at 35 °C for 48 h. From each culture, five colonies were selected and resuspended in 5 ml of sterile, distilled water. Turbidity was read at 530 nm (Spectronic, Bausch and Lomb, USA) and adjusted at 85% transmittance. The suspensions were diluted at 1:100 in RPMI-1640 medium (Sigma) containing L-glutamine but not sodium bicarbonate and buffered to pH 7.0 with 0.165 M MOPS (Sigma), in order to obtain final concentrations of 1×10^3 to 5×10^3 CFU/ml [11].

Susceptibility testing. The minimal inhibitory concentration (MIC) was determined using the broth microdilution method as recommended by the NCCLS [11]. Each well of a microdilution tray was inoculated with 100 µl of the yeast suspension at a final concentration of 0.5×10^3 CFU/ml to 2.5×10^3 CFU/ml. The microdilution plates were incubated at 35 °C for 72 h.

The MIC for amphotericin B was established as the lower concentration of the antifungal that completely inhibited yeast growth. For the azoles, fluconazole and itraconazole, the MIC was established as the lowest antifungal concentration that inhibited 50% of the control growth.

As the breakpoint susceptibility values have not yet been proposed by the NCCLS for *C. neoformans* , Nguyen *et al.* recently adapted to *Cryptococcus* spp. [12], the fluconazole and itraconazole breakpoint values proposed by this Committee for *Candida* spp. [11], values that were

found to be associated with a greater likelihood of therapeutic failure by others [13]. On the same token, Lozano-Chiu *et al.* [14] recommended that amphotericin MIC´s above 2µg/ml indicates resistance to this polyene. Accordingly, resistance was defined as follows: for amphotericin B ≥ 2 µg/ml [14], for fluconazole ≥ 64 µg/ml [12,13] and for itraconazole ≥ 1 µg/ml [12]; dose depending susceptibility (DDS) for fluconazole was defined as MIC´s of 16-32 µg/ml and for itraconazole, of 0.25-0.5 µg/ml [12,13].

Data analysis. The median of the MICs for the antifungals tested was established. The medians were compared by Student´s t test using the Epi Info software version 6.0 (CDC, Atlanta, USA).

RESULTS

MICs for the control strains agreed with the NCCLS interpretation and were the following: *C. neoformans*, 1 µg/ml for amphotericin B, 4 µg/ml for fluconazole and 0.03 µg/ml for itraconazole. *C. krusei*, 0,5 µg/ml for amphotericin B, 16 µg/ml for fluconazole and 0.125 µg/ml for itraconazole.

From the 35 *C. neoformans* clinical isolates tested, 28 (80%) were susceptible to the three antifungals tested, regardless of the variety. Two of the 20 *C. neoformans* var. *neoformans* (10%) proved resistant to amphotericin B with MICs ≥ 2 µg/ml while five of the 15 (14%) *C. neoformans* var. *gattii* although susceptible to fluconazole, were in the dose dependent range (MICs ≥ 16 µg/ ml).

MIC ranges for the 20 *C. neoformans* var. *neoformans* were between 0.25 to 2 µg/ml for amphotericin B with two resistant isolates, 0.25 to 4 µg/ml for fluconazole, and 0.0078 to 0.06 µg/ml for itraconazole. For the 15 *C. neoformans* var. *gattii*, such ranges were between 0.03 to 1 µg/ml for amphotericin B, 1 to 16 µg/ml for fluconazole, with five isolates in the dose dependent group. For itraconazole, in this variety the corresponding MICs were between 0.0078 and 0.06 µg/ml (Table 1).

Table 1. Minimal Inhibitory concentration (MIC) of the 35 Colombian clinical isolates of *Cryptococcus neoformans.*

	var. *neoformans* (n=20)	var. *gattii* (n=15)
Antifungal (µg/ml)	n (%)	n (%)
Amphotericin B		
0.03	0 (0)	1 (7)
0.06	0 (0)	2 (13)
0.25	1 (5)	0 (0)
0.5	13 (65)	5 (33)
1.0	4 (20)	7 (47)
2.0	2 (10)	0 (0)
Fluconazole		
0.25	1 (5)	0 (0)
0.5	3 (15)	0 (0)
1.0	6 (30)	1 (7)
2.0	8 (40)	1 (7)
4.0	2 (10)	1 (7)
8.0	0 (0)	7 (47)
16.0	0 (0)	5 (32)
Itraconazole		
0.0078	11 (55)	1 (7)
0.015	6 (30)	4 (27)
0.03	2 (10)	6 (40)
0.06	1 (5)	4 (26)

In general, MICs for the 20 *C. neoformans* var. *neoformans* were found to correspond to the lower fluconazole concentration ranges; conversely, the 15 *C. neoformans* var. *gattii* tested were susceptible only to the higher concentrations of both fluconazole and itraconazole. An analysis of the median values showed that in comparison with *C. neoformans* var *neoformans*, the *C. neoformans* var. *gattii* isolates differed significantly for both fluconazole (p=0.03) and itraconazole (p= 0.05) (Table 2).

Table 2. Median of the minimal inhibitory concentrations (MICs, µg/ml) of the 35 *Cryptococcus neoformans* isolates according to variety.

	C. neoformans variety		
Antifungal	*neoformans* n=20	*gattii* n=15	p
Amphotericin B	0.790	0.640	0.47
Fluconazole	1.590	9.530	0.03
Itraconazole	0.015	0.032	0.05

DISCUSSION

In cryptococcosis, amphotericin B is considered the treatment of choice for the initial stages of therapy [15]; consequently, the finding in this study of two (10%) *C. neoformans* var *neoformans* isolates resistant to the polyene, is disturbing. Resistance to amphotericin B has also been informed in an AIDS patient [16] as well as *in vitro* [14], and it has been also possible to generate resistant mutants [17]. However, the number of patients in whom amphotericin B resistance has been demonstrated, continues to be low [13].

As for fluconazole, the drug that now commands maintenance treatment protocols for AIDS patients [18], resistance has began to emerge with *C. neoformans* var. *neoformans* in severely immunocompromised patients undergoing prolonged azole treatment [19-22]; furthermore, isolates with MIC´s values in the dose-dependent range, have been informed in 30% of AIDS patients infected with *C. neoformans* var. *neoformans* [23]. Additionally, a correlation between high MIC´s and lack of response has been demonstrated experimentally in animals [24]. The possibility of acquired resistance in patients subjected to prolonged prophylactic fluconazole treatment, must also be considered [25]. At present, and on the basis of the above findings, high dose fluconazole therapy is being recommended [26].

In spite of the fact that 86% of the isolates tested were susceptible to both fluconazole and itraconazole, there were differences according to the *Cryptococcus* variety. Thus, the *C. neoformans* var *gattii* isolates exhibited significantly higher MIC values than the *C. neoformans* var. *neoformans* and, additionally, among the 15 isolates of the former variety that were tested, five (33%) were classified in the susceptible but dose-dependent group. This category was created to alert the treating physicians on the need to use higher dosages of the azole drugs in *Candida* [22] so as to attain elevated plasma concentrations; in patients infected with *C. neoformans* var. *neoformans* similar criteria have also been adapted [23]. The sensitive but dose dependent category has not been applied to the *C. neoformans* var. *gattii*; however, a recent publication indicates the presence of resistance in an AIDS patient infected with such variety [8].

In this report, the presence of resistant or dose dependent isolates for the three antimycotics tested here, was recorded not in immunocompromised patients as informed elsewhere [8,16,19-21] but in several of the

C. neoformans var. *gattii* infected cases. Nonetheless, not all reports have properly characterized the *C. neoformans* variety infecting the patient [19-21,25].

The presence of amphotericin resistant *C. neoformans* var *neoformans* and of fluconazole dose dependent *C. neoformans* var. *gattii* isolates in this series, as well as the emergence of acquired resistance informed in the literature [8], indicate the importance of determining not only the variety of *C. neoformans* infecting the patient but also of measuring the MIC range of the primary isolates, in order to properly orient treatment [6,12,14,22,24,26,27].

The results of this study tend to suggest that some links may exist between the poor therapeutic response exhibited by patients with cryptococcosis due to *C. neoformans* var. *gattii*, to either fluconazole and itraconazole, and the higher *in vitro* susceptibility values here recorded. It has been known that the latter patients require more prolonged and intense treatment that those infected with *C. neoformans* var. *neoformans* [6,7].

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