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Cryptococcus neoformans and *C. gattii* isolates from both HIV-infected and uninfected patients: antifungal susceptibility and outcome of cryptococcal disease

Erika Nascimento[1], Lucia Helena Vitali[1], Marcia Regina von Zeska Kress[2], Roberto Martinez[1]

ABSTRACT

One of the factors causing treatment failure in cryptococcosis is the resistance of *Cryptococcus* spp. to antifungal drugs, which has motivated the susceptibility assessment of isolates from patients with cryptococcosis, different clinical conditions and infections outcomes. Clinical isolates of *Cryptococcus* spp. from three different groups of patients were studied in the present investigation: 19 HIV-positive patients with relapsing and/or refractory meningitis (Group 1), 30 HIV-positive patients who experienced a single and limited episode of cryptococcosis (Group 2), and 19 HIV-negative patients with cryptococcosis (Group 3). Eighty *C. neoformans* var. *grubii* isolates and 7 *C. gattii* isolates were studied. The minimum inhibitory concentration (MIC) of amphotericin B, azole drugs and flucytosine was determined for *Cryptococcus* spp. by broth microdilution test and E-test. The MIC50 and MIC90 were 0.25 and 0.50 µg/mL for amphotericin B, 4.0 and 8.0 µg /mL for fluconazole, 0.06 and 0.25 µg/mL for itraconazole, 0.25 and 0.50 µg/mL for voriconazole, and 8.0 and 16.0 µg/mL for flucytosine, respectively. Amphotericin B and itraconazole showed higher MICs for *C. neoformans* var. *grubii* and *C. gattii*, respectively. The MICs of fluconazole and itraconazole obtained with the E-test were higher than those obtained with broth microdilution. Isolates from non-HIV coinfected were less sensitive to the azoles. There was no difference in the susceptibility of *C. neoformans* var. *grubii* isolates from patients with a favorable or unfavorable outcome or along the episodes of relapsing and/or refractory meningitis.

KEYWORDS: Cryptococcal meningitis. Relapsing and refractory cryptococcosis. *Cryptococcus* spp. Antifungal susceptibility. HIV.

INTRODUCTION

Cryptococcosis is the second most frequent systemic fungal infection involving HIV-positive patients. It occurs also in other immunosuppression conditions and in immunocompetent individuals[1]. The lethality of cryptococcosis is high even with the use of antifungal drugs. In refractory and/or relapsing cases, neurological sequelae frequently occur in survivors. *Cryptococcus neoformans* var. *grubii* and *C. gattii* are commonly isolated from patients and the most common manifestation is cryptococcal meningitis, occurring in 90% of the cases[2]. *Cryptococcus* spp. usually shows susceptibility to amphotericin B, azole drugs and flucytosine[3,4]. Amphotericin B in combination with flucytosine is recommended for the treatment of cryptococcal meningitis, followed by maintenance therapy with fluconazole[2]. At present, the resistance of *Cryptococcus* spp. to amphotericin B, fluconazole and flucytosine is less than 1%, but non-susceptible strains have arisen all over the world, suggesting

[1]Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto, Departamento de Clínica Médica, Ribeirão Preto, São Paulo, Brazil

[2]Universidade de São Paulo, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Ribeirão Preto, São Paulo, Brazil

Correspondence to: Erika Nascimento Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto, Departamento de Clínica Médica, Av. Bandeirantes, 3900, Monte Alegre, CEP 14040-900, Ribeirão Preto, SP, Brazil

E-mail: erika.nascimento@gmail.com

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progression to a future broader resistance[5,6]. The lack of susceptibility of *Cryptococcus* spp. to these antifungal agents may be one of the critical factors determining an adverse outcome in patients with cryptococcosis[7]. The assessment of antifungal *Cryptococcus* spp. susceptibility is recommended in order to plan patient treatment and also to monitor the tendency of resistance to drugs used in clinical practice.

In the present study, previously genotyped isolates[8] of *C. neoformans* var. *grubii* and *C. gattii* were used in order to assess antifungal drugs susceptibility by comparing the broth microdilution test with the E-test among the *Cryptococcus* species isolated from HIV-negative and HIV-positive, the latter group according to the outcome.

MATERIALS AND METHODS

Clinical isolates

A total of 87 *Cryptococcus* spp. isolates from 68 patients with cryptococcal meningitis were evaluated. The patients were diagnosed and treated at the University Hospital, *Ribeirão Preto Medical School, University of São Paulo*, *Ribeirão Preto*, SP, Brazil, from 2000 to 2011. For the comparison of antifungal susceptibility of the isolates, the patients were divided into three groups. Group 1 consisted of the isolates from 19 immunocompromised HIV-positive patients with relapsing and/or refractory cryptococcosis (a total of 38 isolates, 19 of which corresponded to the initial episode of cryptococcosis and 19 to the refractory or relapse period). The refractory or relapse period was defined as the reisolation of *Cryptococcus* spp. from cerebrospinal fluid (CSF) after a period of negative cultures or as the persistence of isolation of *Cryptococcus* spp. from CSF after two months of antifungal therapy. Group 2 consisted of 30 isolates (CSF collected before antifungal treatment) from 30 immunocompromised HIV-positive patients who had experienced a single and limited episode of cryptococcosis. Group 3 consisted of 19 *Cryptococcus* spp. isolates from apparently immunocompetent (11 cases) or immunocompromised HIV-negative patients (8 cases) (in all patients, the CSF was collected before antifungal treatment). The eventual change of *C. neoformans* var. *grubii* susceptibility was checked in refractory or relapsing cases (Group 1) by comparing the first isolate obtained during the pretreatment phase to the last isolate obtained from each patient after treatment failure (2 to 87 month interval between the isolates). The lethality of cryptococcosis was analyzed according to *C. neoformans* susceptibility in 23 patients with AIDS (Groups 1 and 2) treated with amphotericin B for at least one month followed by maintenance therapy with fluconazole. For the outcome analysis, we considered the deaths attributed to complications of cryptococcosis, including bacterial infections that occurred during the induction and consolidation phase and late deaths in cases of relapsing and/or refractory cryptococcal infection.

Microorganisms and growth conditions

Cryptococcus spp. isolates were previously identified by standard Clinical Mycology methods and also using the automated system Vitek® 1 or Vitek® 2 (bioMérieux, France) and by using molecular methods[8]. The yeasts were maintained in the laboratory by periodic culture in Sabouraud Dextrose Agar (SDA) medium at 25 °C. A total of 87 *Cryptococcus* spp. isolates were studied, -80 *C. neoformans* var. *grubii* isolates were distributed among group 1 (n= 38), group 2 (n=29), and group 3 (n=13). *C. gattii* (n=7) were identified in group 2 (n=1) and group 3 (n=9).

Antifungal susceptibility testing

Broth microdilution

The susceptibility of *Cryptococcus* spp. isolates to the antifungal drugs was determined by the broth microdilution method according to the M27-A2 protocol (Clinical and Laboratory Standards Institute)[9]. Roswell Park Memorial Institute (RPMI) medium 1640 (Sigma Aldrich Chemical Company, St Louis, MO) containing L-glutamine buffer with 2% glucose added and buffered with morpholino propanesulfonic acid (MOPS) and 1 M NaOH, pH 7.0, was added to sterile 96-well flat-bottom plates (TPP, Switzerland). The yeast inoculum was prepared in RPMI culture medium to a final concentration of 2.0×10^3 CFU mL^{-1}, as recommended by the M27-A2[9] document.

The antifungal drugs tested were: amphotericin B, fluconazole, itraconazole, voriconazole, and flucytosine (Sigma Aldrich Chemical Corporation). Amphotericin B, itraconazole and voriconazole were diluted in dimethyl sulfoxide (Sigma Aldrich Chemical Corporation), while fluconazole and flucytosine were dissolved in sterile distilled water. The antifungal drugs were diluted in RPMI medium and serial dilutions were carried out in order to obtain final concentrations of 0.125-64 μg mL^{-1} for flucytosine, 0.125- 64 μg mL^{-1} for fluconazole and 0.03-16 μg mL^{-1} for amphotericin B, itraconazole and voriconazole.

The 96-well plates were incubated at 37 °C for 48 h and the optical density of each well was measured with a microplate reader (Multiskan MS) adjusted to a wavelength of 492 nm. The minimum inhibitory concentration (MIC) was considered as the lowest concentration capable of inhibiting fungal growth by ≥ 80% for amphotericin B and by ≥ 50% for the other antifungal drugs in relation

to the positive control (CLSI, 2002)[9]. The instrumental reading was confirmed by visual reading. *C. neoformans* ATCC 90112 and *Candida parapsilosis* ATCC 22019 were included in all tests as positive controls. The MIC values for all drugs were interpreted according to the CLSI M27-A2 protocol. The susceptibility of each *Cryptococcus* spp. isolate was determined in duplicate.

E-test

E-test was performed according to the manufacturer's instructions (AB Biodisk – Solna, Sweden). The antifungal drugs tested were itraconazole, fluconazole, voriconazole and amphotericin B. The medium used was RPMI 1640 containing 1.5% agar, supplemented with 2% glucose and buffered with MOPS to pH 7. The yeast inoculum was the same used for the broth microdilution. E-test gradient strips were placed on the surface of a previously inoculated plate by seeding the inoculum on the surface of the agar plate and left to dry for 15 min. The E-test strips had the following gradient of antifungal concentration: 0.002-32.0 µg mL^{-1} for amphotericin B, itraconazole and voriconazole and 0.016-256 µg mL^{-1} for fluconazole. The plates were incubated at 37 °C for 48 h. MIC readings were obtained at the point of intersection between the ellipse of growth inhibition and the E-test strip.

Statistical analysis

The Excel 2007 for Windows software (Microsoft Corp., USA) was used to determine the MIC50 and MIC90 values and the geometric mean. The data for groups 1, 2 and 3 were compared by the Kruskal-Wallis test and the Dunn's post-hoc test. The methods (broth microdilution and E-test), the data for the species (*C. neoformans* var. *grubii* and *C. gattii*) and the data for patient outcome (cure and death) were compared by the Mann-Whitney test using the GraphPad Prism 6 software (San Diego, CA). The level of significance was set at $p< 0.05$ in all analysis.

Ethics considerations

The study was approved by the Research Ethics Committee of the University Hospital, *Ribeirão Preto Medical School*, *University of São Paulo* (Protocol HCRP n° 12247/2010).

RESULTS

Comparison of broth microdilution and E-test methods

Four antifungal drugs were tested by broth microdilution method and E-test against 61 clinical isolates of *C. neoformans* var. *grubii* belonging to groups 1, 2 and 3. E-test MICs were higher for fluconazole and itraconazole. In contrast, the MICs for amphotericin B and voriconazole were higher in broth microdilution method ($p< 0.0001$) (Table 1).

In vitro susceptibility of *C. neoformans* var. *grubii* and *C. gattii* comparison

Five antifungal drugs were tested by the broth microdilution method in 61 clinical isolates of *C. neoformans* var. *grubii* and in 7 isolates of *C. gattii*. *C. neoformans* var. *grubii* was less susceptible to amphotericin B ($P = 0.0004$), while *C. gattii* was less susceptible to itraconazole ($P = 0.0114$) (Table 2).

Comparison of *in vitro* susceptibility of *C. neoformans* var. *grubii* according to the patient's group

A significant MIC difference was detected for itraconazole ($P = 0.0186$) and voriconazole ($p = 0.0022$) on *in vitro* susceptibility test. The Dunn's post-test showed that this significant difference was between Group 1 and Group 3 for itraconazole and between Group 2 and Group 3 for voriconazole (Figure 1).

MIC of the antifungal drugs and outcome

C. neoformans var. *grubii* showed similar susceptibility to amphotericin B ($p = 0.2227$) and to fluconazole ($p = 0.1729$) in patients with AIDS (Group 1 and 2) whose outcome was death (n=6) or progression to cure (n = 17) (Figure 1). *C. neoformans* var. *grubii* isolated at the beginning of the treatment and after treatment failure in patients with AIDS and refractory/relapsing cryptococcosis (group 1) have shown no differences in susceptibility to the five antifungal drugs tested (Table 3).

DISCUSSION

The most relevant result obtained in the present study was that there was no association between progression to relapsing/refractory cryptococcosis in patients with AIDS and the MICs of *C. neoformans* for antifungal drugs. Study limitations were the reduced number of *C. gattii*-infected patients and the small number of cases included in the analysis of the impact of antifungal MIC distribution with respect to the cure vs. death outcome.

As previously observed[10], *C. gattii* was less susceptible to azole drugs than *C. neoformans* var. *grubii*. For this

Table 1 - Comparative assessment of the antifungal MICs obtained by the broth microdilution method and by the E-test for *C. neoformans* var. *grubii* isolates (61 isolates)

Antifungal Drug		Susceptibility test		
		Broth Microdilution	E-test	*p* value***
Amphotericin B	MIC Range (µg/mL)	0.13 - 0.50	0.047 - 0.50	< 0.0001
	MIC 50 (µg/mL)*	0.25	0.19	
	MIC 90 (µg/mL)*	0.50	0.38	
	G.M (µg/mL)**	0.30	0.20	
Fluconazole	MIC Range (µg/mL)	1.0 - 16.0	0.125 - 32.0	< 0.0001
	MIC 50 (µg/mL)	4.0	12.0	
	MIC 90 (µg/mL)	8.0	16.0	
	G.M (µg/mL)	4.67	12.60	
Itraconazole	MIC Range (µg/mL)	0.03 - 1.0	0.016 - 2.0	< 0.0001
	MIC 50 (µg/mL)	0.06	0.38	
	MIC 90 (µg/mL)	0.25	0.75	
	G.M (mg/L) (µg/mL)	0.13	0.44	
Voriconazole	MIC Range (µg/mL)	0.03 - 0.50	0.016 - 0.75	< 0.0001
	MIC 50 (µg/mL)	0.25	0.094	
	MIC 90 (µg/mL)	0.50	0.25	
	G.M (µg/mL)	0.27	0.14	

* MIC50 and MIC90. the concentration capable of inhibiting the growth of 50% and 90% of the isolates, respectively.**GM: geometric mean, ****p* value, statistic.

Table 2 - Comparison of the *in vitro* antifungal susceptibility of *C. neoformans* var. *grubii* (n= 61) and *C. gattii* (n = 7) by the broth microdilution methods

Antifungal Drug		Broth Microdilution		
		C. neoformans *var.*grubii	*C. gattii*	*p* value***
Amphotericin B	MIC Range (µg/mL)	0.13 - 0.50	0.06 - 0.50	0.0004
	MIC 50 (µg/mL)*	0.25	0.06	
	MIC 90 (µg/mL)*	0.50	0.13	
	G.M (µg/mL)**	0.30	0.14	
Fluconazole	MIC Range (µg/mL)	1.0 - 16.0	2.0 - 16.0	0.1139
	MIC 50 (µg/mL)	4.0	8.0	
	MIC 90 (µg/mL)	8.0	8.0	
	G.M (µg/mL)	4.67	7.14	
Itraconazole	MIC Range (µg/mL)	0.03 - 1.0	0.13 - 0.25	0.0114
	MIC 50 (µg/mL)	0.06	0.13	
	MIC 90 (µg/mL)	0.25	0.25	
	G.M (µg/mL)	0.13	0.16	
Voriconazole	MIC Range (µg/mL)	0.03 - 0.50	0.25 - 0.50	0.0758
	MIC 50 (µg/mL)	0.25	0.50	
	MIC 90 (µg/mL)	0.50	0.50	
	G.M (µg/mL)	0.27	0.39	
Flucytosine	MIC Range (µg/mL)	4.0 - 16.0	4.0 - 16.0	0.7630
	MIC 50 (µg/mL)	8.0	8.0	
	MIC 90 (µg/mL)	16.0	16.0	
	G.M (µg/mL)	8.19	7.42	

* MIC50 and MIC90 are the concentrations capable of inhibiting the growth of 50% and 90% of the isolates, respectively, **GM: geometric means, ****p value,* statistic.

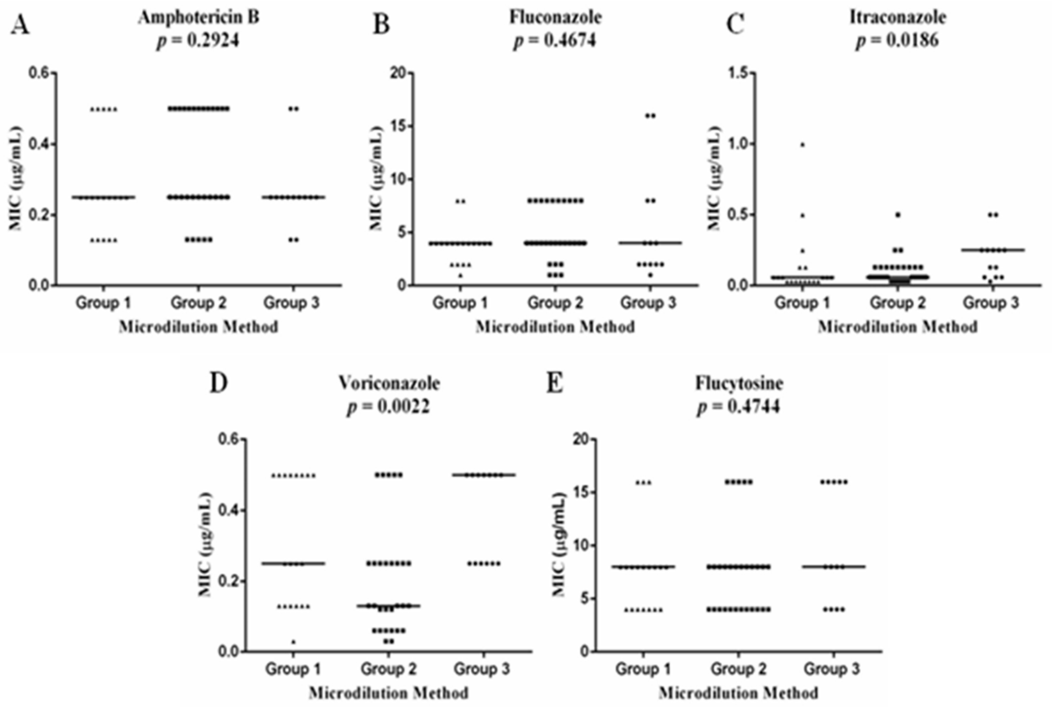

Figure 1 - Comparison of the *in vitro* susceptibility of *C. neoformans* var. *grubii* isolates from group 1 (n=19), 2 (n=29) and 3 (n=13) patients to the antifungal agents amphotericin B (A), fluconazole (B), itraconazole (C), voriconazole (D) and flucytosine (E) by the broth microdilution method. The bars show the mean of the minimum inhibitory concentrations. The *p*-values obtained are represented in the graphs

Table 3 - Antifungal susceptibility of *C. neoformans* var. *grubii* isolates obtained before treatment and during the period refractory to treatment or during relapse of cryptococcosis from 19 patients with AIDS

Antifungal Drug		GROUP 1		
		Pretreatment	Relapse and/or refractory period	*p* value***
Amphotericin B	MIC Range (µg/mL)	0.13 - 0.50	0.13 - 0.50	0.8791
	MIC 50 (µg/mL)*	0.25	0.25	
	MIC 90 (µg/mL)*	0.50	0.50	
	G.M (µg/mL)**	0.28	0.27	
Fluconazole	MIC Range (µg/mL)	1.0 - 8.0	0.13 - 8.0	0.6290
	MIC 50 (µg/mL)	4.0	4.0	
	MIC 90 (µg/mL)	4.0	8.0	
	G.M (µg/mL)	3.84	3.64	
Itraconazole	MIC Range (µg/mL)	0.03 - 1.0	0.03 - 0.13	0.1973
	MIC 50 (µg/mL)	0.06	0.06	
	MIC 90 (µg/mL)	0.25	0.13	
	G.M (µg/mL)	0.13	0.08	
Voriconazole	MIC Range (µg/mL)	0.03 - 0.50	0.25 - 0.50	0.6102
	MIC 50 (µg/mL)	0.25	0.25	
	MIC 90 (µg/mL)	0.50	0.50	
	G.M (µg/mL)	0.30	0.27	
Flucytosine	MIC Range (µg/mL)	4.0 - 16.0	4.0 - 16.0	0.4888
	MIC 50 (µg/mL)	8.0	8.0	
	MIC 90 (µg/mL)	16.0	8.0	
	G.M (µg/mL)	7.78	6.63	

* MIC50 and MIC90. the concentrations capable of inhibiting the growth of 50% and 90% of the isolates, respectively, **GM: geometric means, ****p value,* statistic.

reason and because of the small number of *C. gattii* isolates, only the MICs obtained for *C. neoformans* var. *grubii* were related to other clinical and laboratory data. When comparing the methods, higher MIC values for fluconazole and itraconazole were obtained with the E-test than with the CLSI-broth microdilution standardized test[9]. Discordance for azoles has been detected in other studies and has been known since the first determinations of the fluconazole MIC for *C. neoformans* with the E-test[11,12]. More recently, high concordance was observed between the two methods regarding the MIC of azole drugs[5]. Regarding amphotericin B, the MIC values can be lower when using the E-test than when using broth microdilution. Thus, E-test can better discriminate the susceptibility or resistance to these drugs among *C. neoformans* strains[13].

The MIC distribution of the five antifungal drugs tested against *C. neoformans* was similar to that detected for isolates from AIDS patients from other regions[14] and was also comparable to the susceptibility of isolates with the VNI genotype[3]. Regarding the epidemiological cutoff values (ECV) determined for the five antifungal drugs[15,16], the *C. neoformans* var. *grubii* and *C. gattii* isolates evaluated here had antifungal MICs up to the respective ECV or immediately above it. No elevated MIC values that might characterize any of the isolates as resistant were detected. Thus, the isolates tested here in general were susceptible to drugs of clinical use, including amphotericin B, fluconazole and flucytosine. Similar results have been previously reported, showing only a small percentage of strains with reduced susceptibility, particularly to fluconazole and flucytosine[7,17]. The obtained data support the recommendation to perform the *Cryptococcus* spp. antifungal susceptibility test only in cases of isolates from relapsing/refractory cryptococcosis[2]. Comparison of *C. neoformans* infection in patients with and without AIDS revealed higher MICs for itraconazole and voriconazole in patients not infected with HIV. *Cryptococcus* isolated from Shangai patients with AIDS showed lower susceptibility to fluconazole than isolates from patients without AIDS[18]. Another study revealed that *Cryptococcus* isolates from non-HIV patients were less susceptible to flucytosine than isolates from AIDS patients[19]. The lack of specification of the isolate genotype and the heterogeneity of patients without AIDS make it difficult to re–conclude on eventual differences between isolates from patients with AIDS and without HIV infection regarding susceptibility to antifungal drugs.

Separate analysis of AIDS cases did not show a difference in susceptibility between *Cryptococcus* isolates from patients with relapsing/refractory cryptococcosis and from patients with a good response to antifungal therapy. The MICs of *Cryptococcus* isolates obtained from the same patient before and after treatment failure were also similar, showing that reduced susceptibility to antifungal drugs is not an important factor for the progression of cryptococcosis to relapse and/or to a refractory state. The same observation was reported for isolates from South American patients[20] and those from Vietnam[21]. Conversely, in two other case series, the relapse of cryptococcosis was associated with lower susceptibility to fluconazole[22,23]. *Cryptococcus* spp. isolated from patients may show heteroresistance when exposed *in vivo* to azole drugs[24].

The lethality of cryptococcosis in patients coinfected with HIV and treated with amphotericin and fluconazole was not related to the MIC of these drugs for the pretreatment isolates. The same conclusion was reported in studies on patients from other geographic areas[13,20,25]. Paradoxically, in Taiwanese patients, a MIC for fluconazole above 8 μg $mL^{-1}$was associated with the cure of cryptococcosis[26].

In conclusion, *C. neoformans* and *C. gattii* isolated in the present study proved, in general, to be susceptible to the drugs used in antifungal therapy. The MIC distribution of these drugs was not related to the progression of the infection to the condition of relapsing/refractory meningoencephalitis or to death due to fungal infection.

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