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ORIGINAL ARTICLE

ANTIFUNGAL SUSCEPTIBILITY TESTING AND GENOTYPING CHARACTERIZATION OF *Cryptococcus neoformans* AND *gattii* ISOLATES FROM HIV-INFECTED PATIENTS OF *RIBEIRÃO PRETO, SÃO PAULO,* BRAZIL

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SUMMARY

Cryptococcosis is a leading invasive fungal infection in immunocompromised patients**.** Considering the high prevalence and severity of these infections in immunocompromised patients attended at HC-FMRP-USP, the present research aimed to characterize the clinical isolates of *Cryptococcus* strains by biochemical and molecular methods and evaluate antifungal susceptibility of clinical isolates. Fifty isolates from 32 HIV-positive patients were obtained at HC-FMRP-USP. Most of the isolates (78.1%) were identified as *C. neoformans,* and 100% of *C. neoformans* and *C. gattii* strains were susceptible to amphotericin B, ketoconazole and fluconazole. All isolates were classified as serotype A (*grubbii* variety) by PCR and most of them were characterized in mating type MATα. PCR analysis of specific M13 microsatellite sequence revealed that VNI type was predominant among *C. neoformans,* while VGII was predominant among *C. gattii*. The strains did not show a significant resistance to the antifungals tested, and Canavanine-Glycine-Bromthymol Blue Agar (CGB) proved to be a reliable test presenting a good correlation with the molecular characterization. *C. neoformans* isolated from disseminated infections in the same patient showed molecular identity when different anatomical sites were compared; besides, the studied strains did not present a significant increase in resistance to antifungal agents. In addition, the homogeneity of the molecular types and detection of the mating types suggested a low possibility of crossing among the strains.

KEYWORDS: *Cryptococcus neoformans*; Serotyping; Genotyping; Fingerprinting; Antifungal agents; Mating type; HIV-patients.

INTRODUCTION

Cryptococcus sp is a saprophyte encapsulated yeast that exhibits single or multiple asynchronous buds, and has five serotypes, A, B, C, D and AD. It is subdivided into two varieties known as *C. grubii* (serotype A) and *C. neoformans* (serotypes D and AD), while the serotypes B and C are grouped into *C. gattii* species[1-3]. However, there is a new nomenclature classifying *C. neoformans* as the strains considered var. *grubii* (serotype A) and *C. deneoformans* as the var. *neoformans* (serotype D). In addition, *C. gattii* will comprise five species: *C.gattii, C. bacillisporus, C. deuterogattii, C. tetragattii and C. decagattii*[4,5].

Representatives of this genus grow at 37 °C, hydrolyze starch, produce urease and do not ferment lactose[6]. Cryptococcal infections occur worldwide in undefined endemic areas. However, the environmental serotype distribution shows some differences, presenting *C. gattii* as the prevalent species in tropical and subtropical areas[7-9].

Routinely, *C. neoformans* and *C. gatti* cultures are distinguished in CGB-Agar, an enriched medium, which inhibits *C. neoformans* growth and favors *C. gattii* development. The color change from yellow-green to blue-cobalt indicates serotypes B and C[10].

Another important identification method for this species is the sexual mating type analysis. The mating *locus* MAT of *C. neoformans* serotype A and D is unique. It is characterized by different regions with very similar structures and functions, which can be also found in other fungi. MAT *locus* has an average size of 100 Kb, comprising more than 20 genes,

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some being present in both alleles MATa and MATα, and other genes present in only one of these alleles[11-15]. Although diploid or aneuploidy strains are rare, they have already been isolated in nature[16,17].

In *C. gatii*, it is only possible to determine the sexual type using oligonucleotides flanking the genes MFα1 and MFa2. On the other hand, the serotype B/C is identified by PCR using a primer pair specific for *C. gattii* superoxide dismutase gene[18-19].

Serotypes A and D, mating type α, are the most common variety causing infections in humans. Although studies with serotypes A, D, AD, B and C have not demonstrated differences in the susceptibility to antifungal agents, the higher incidence of serotypes A, D and AD in HIV-infected patients have suggested a pattern behavior that is demonstrated by clinical and epidemiological studies[20-23].

C. neoformans is the major cause of cryptococcosis affecting millions of people worldwide. In patients with the acquired immunodeficiency syndrome (AIDS) or immunosuppressive conditions, such as organ transplantation or submitted to chemotherapy treatment, cryptococcosis represents the most common fungal infection. During the disease, various strategies are employed to treat these infections, including amphotericin B alone or in combination with 5-flucytosine and azoles, as fluconazole, itraconazole or voriconazole, which are the standard reference drugs nowadays[24,25]. Considering the high frequency of cryptococcosis in HIV-infected patients attended at the University Hospital of *Ribeirão Preto* Medical School, University of Sao Paulo (HC-FMRP-USP), the present investigation aimed to characterize the clinical isolates of *Cryptococcus* strains by biochemical and molecular methods. The molecular typing, serotyping and mating type identification by molecular biology techniques were used to identify and distinguish the isolates. In addition, the strains were characterized regarding the sensitivity profile to the antifungal agents, amphotericin B, ketoconazole, itraconazole, fluconazole and 5-fluorocytosine.

MATERIAL AND METHODS

Cryptococcus isolates

Fifty isolates collected from 32 patients at HC-FMRP-USP and belonging to the *Cryptococcus* collection from the Mycology Laboratory - Department of Cell and Molecular Biology, and Pathogenic Bioagents (FMRP-USP) were selected for phenotypic and genotypic studies. The strains were maintained in Sabouraud Dextrose Agar medium (SDA) at room temperature. The strains were previously identified as belonging to the genus *Cryptococcus* using the classical identification tests such as capsule observation using Indian ink stain and biochemical tests, such as urea degradation and sugars assimilation analysis[26].

The isolates were divided into three groups as follow: Group I – strains isolated from different patients; Group II – strains isolated from the same patient at the same period, in different anatomical sites; and Group III – strains isolated from the same patient at different periods.

Biochemical characterization of *Cryptococcus*

The isolates were subcultured in CGB-Agar, pH 5.6, in which the fungi culture presents the typical greenish yellow color according to Kwon-Chung *et al.*[10]. Isolates were incubated at 37 °C for 48 hours and the reading was performed according to the growth characteristic in this medium: the maintenance of the original medium color is an indication of *C. neoformans* (serotypes A, D, and AD), while the change to blue-cobalt shade characterizes *C.gattii* (serotypes B and C). Simultaneously, *Cryptococcus* reference strains were used as experimental controls: A (CDC 9759), B (ATCC 32269), C (ATCC 24066), and D (ATCC 28958).

DNA isolation

The genomic DNA extraction method was based on the methodology described by Bolano *et al.*[27].

Determination of mating type and serotype

The amplification reactions for sexual type and serotype characterization were performed with Taq DNA Polymerase (Fermentas, Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer's instructions in a PTC-200 thermal cycler (MJ Research, GMI Inc., Ramsey, Minnesota, USA), and it was stablish 32 cycles for the PCR. Table 1 presents the primer sequences, annealing temperatures and PCR product sizes according to each pair of pairs[28,17]. The amplification products were submitted to electrophoresis on 1% agarose gels in 1X TAE (Tris-Acetate-EDTA) at 75 volts for 2 hours. Gels were stained with ethidium bromide, visualized under UV light and the images were captured by the Alpha-Innotech Image System (Alpha-Innotech Corp., San Leandro, CA, USA)[29].

PCR-fingerprinting

The molecular typing of *Cryptococcus* isolates was performed according to the methodology described by Meyer[30-32], which is based on random amplification of DNA fragments generated by primers recognizing specific minisatellite sequences, allowing a molecular classification defined as VNI, VNII, and VNIII VNIV to *C. neoformans,* and VGI, VGII, and VGIII to VGIV to *C. gattii.* The primers were M13 (5'-GAGGGTGGCGGTTCT-3') and $(GACA)_4$[31]. All reactions were performed in a final volume of 50 µL containing 100 ng of genomic DNA, 10 pmol of each primer, 2 mM of $MgCl_2$, 2.5 U of Taq DNA polymerase, 1x PCR Buffer and 10 mM of each dNTP. Amplification conditions were: initial denaturation step at 94 °C for 5 minutes, followed by 35 cycles: denaturation at 94 °C for 1 minute, annealing at 49 °C for 1 minute, extension at 72 °C for 1 minute, followed by a final extension at 72 °C for 10 minutes. The generated amplification products were submitted to electrophoresis on 1% agarose gels as previously described, and the interpretation of results was based on the number and size of the amplification products.

Antifungal susceptibility testing

The susceptibility of *C. neoformans*, *C. gattii* and the control strains to antifungal agents was determined by the microdilution plate method, with some modifications based on the protocol recommended by the National Committee for Clinical Laboratory Standards, document (CLSI M27-A2). The drugs used here were amphotericin B (Fungizon, Bristol-Myers-Squibb, Brazil), ketoconazole (Janssen Cilag, Brazil), itraconazole (Janssen Cilag, Brazil), fluconazole (Pfizer, Brazil) and 5-fluorocytosine (Roche, Brazil). The stock solution of

Table 1
Identification of primers, primer sequences, annealing temperature, molecular weight of amplification products to determine mating type and serotype and references

Primer name	Sequences "Forward and Reverse"	Annealing T°C	Product size (bp)	Reference
STE20aA	5'-TCCACTGGCAACCCTGCGGAG-3' 5'ATCAGAGACAGAGAAGCAAGAC-3'	55	865	[28]
SXI2aD	5'-GGTCCGCACTTGGGTAAGTG -3' 5'-GGCGTAGACGGACGAGCTC -3'	61	1420	Lin; Heitman, Personal Contact
SXI1αA	5'-GCTTGGCGTACGCTGTGG -3' 5'-GGCGTCGCTTGGTACGGGT -3'	55	1150	Lin; Heitman, Personal Contact
STE20αD	5'-GATTATCTCAGCAGCCACG-3' 5'-AAATCGGCTACGGCACGTC-3'	61	443	[28]
SOD1 B/C	5'-GATCCTCACGCCATTACG-3' 5'-GAATGATGCGCTTAGTTGGA-3'	52	1000	[20]
MFα1 – universal	5'-CTTCACTGCCATCTTCACCA-3' 5'-GACACAAAGGGTCATGCCA-3'	57.5	101	[17]
MFa2–B/C	5'-ACACCGCCTGTTACAATGGAC-3' 5'-CAGCGTTTGAAGATGGACTTT-3'	55	213	[18]

each drug was prepared with sterilized water for amphotericin B, fluconazole and 5-fluorocytosine, and DMSO (dimethyl sulfoxide) for ketoconazole and itraconazole, divided in aliquots in sterilized microcentrifuge tubes and stored at -80 ^{o}C until used. Two serial dilutions of each antifungal agent were prepared with RPMI 1640 medium (Sigma Chemical Co., Saint Louis, Missouri, USA) with L-glutamine and without sodium bicarbonate, and buffered to pH 7.0 with 0.165 M MOPS (morpholinopropanesulfonic acid). The final concentrations ranged from 0.0312 to 16 µg/mL for amphotericin B; 0.0312 to 16 µg/mL for itraconazole; 0.0625 to 32 µg/mL ketoconazole; 0.25 to 128 µg/mL for 5-fluocytosine and 0.25 to 128 µg/mL fluconazole. The microdilution assay was performed in 96-well microdilution plates. Results were visually evaluated after 72 hours of incubation for *C. neoformans* isolates and 48 hours for *Candida parapsilosis* standard sample. In order to validate the test, the standard sample used in all reactions showed minimal inhibitory concentrations (MIC) for each antifungal, within known ranges. The MIC determined for ketoconazole, itraconazole, fluconazole and 5-fluorocytosine corresponded to the concentration that inhibited more than 50% of the fungal growth, compared with the positive control, and for amphotericin B the MIC was considered the concentration showing 100% of growth inhibition. Isolates were considered susceptible or resistant to the tested antifungal agents in accordance to breakpoint values defined by Clinical and Laboratory Standards Institute (CLSI), 2002[33,34].

RESULTS

The strains collected from 32 HIV-positive patients comprised 50 isolates, from which 78.1% were phenotipically identified as *C. neoformans* and 21.9% corresponded to *C. gattii* species according to biochemical tests. Serotype and sexual type (mating type) characterizations were simultaneously performed by PCR, showing that every sample from group I was classified as MaTα type. From the seven isolates biochemically identified as *C. gatti*, only one sample (number 46) was positive for the MFa2 primer pair, which is specific for *C. gattii* sexual type "a". The amplifications for MFα1 and MFa2 showed that this sample is diploid α/a, serotype B/C. The remaining *C. gattii* isolates were compatible with serotype B/C which are sexual type α. The presence of bands for the gene SXI1Aα in the tested isolates, compared with bands for the genes STE20Dα, STE20Aa and SXI2Da in the positive controls showed that all isolates in group I were classified as serotype A and mating type α, which is characteristic of *C. neoformans* variety *grubbi* (Fig. 1).

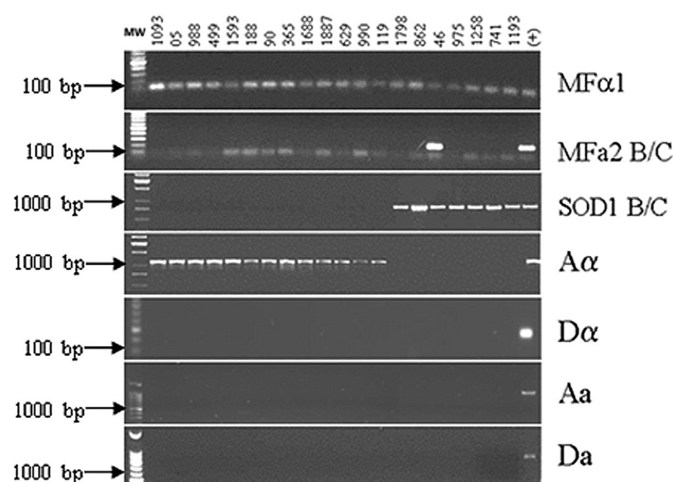

Fig. 1 - Group I *Cryptococcus* isolates. Agarose gel of PCR reactions used for determination of sexual type and serotype. PCR products were obtained using specific primer pairs (MFα1, MFa2 B/C, SOD1 B/C, Aα, Dα, Aa and Da). Molecular weight markers are shown on the left side; the lane on the right side indicates the (+) control of each reaction. The remaining lanes represent the studied isolates, identified at the top of the figure by numbers.

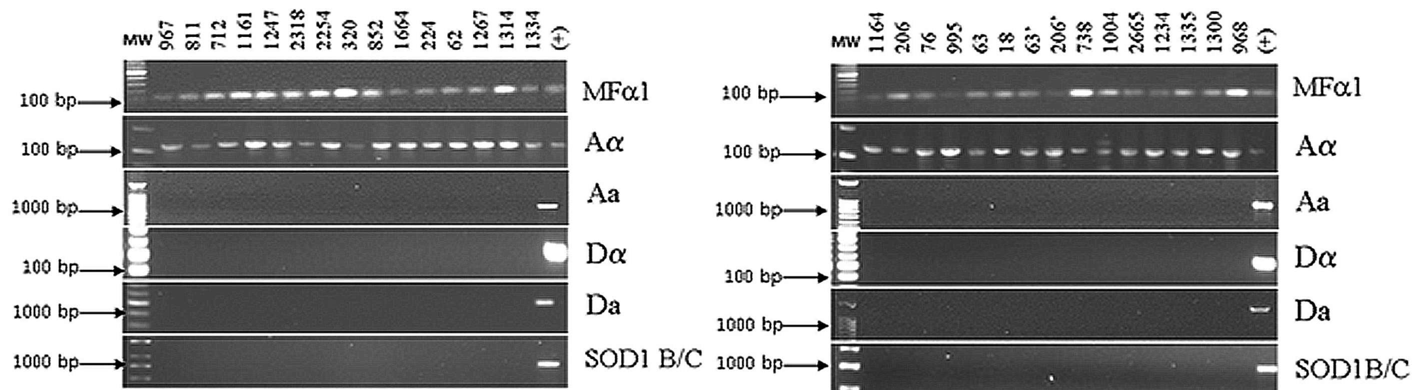

Fig. 2 - Agarose gels showing sexual types and serotypes of *Cryptococcus* isolates. 2A (on the left) corresponds to Group II samples and 2B (on the right) to Group III samples. PCR products were obtained using specific primers (MFα1, MFa2 B/C, SOD1 B/C, Aα, Dα, Aa and Da). The first lane on the left side shows the molecular weight markers; the right lane on the right side shows the (+) control of each reaction. The remaining lanes represent the studied isolates, identified at the top of the figure by numbers.

Using the same primers mentioned above, for the sexual typing and serotyping characterization, identical behavior was observed for the Groups II (strains isolated from the same patient in different anatomical sites) and III (strains isolated from the same patient at different periods of observation), all the isolates were characterized as MATα serotype A variety *C. grubbi.* Indeed, amplification with the SOD1/BC primers was not observed, corroborating the expectations of our previous characterization using the CGB method (Fig. 2).

The technique with the specific microsatellite sequence M13, allowed the molecular typing of all the strains and revealed that the VNI molecular type predominated, occurring in 55% of the *C. neoformans* strains. Concerning *C. gattii* strains, the VGII molecular type was the most frequent, appearing in 71% of the strains (Table 2). Moreover, for almost all the patients that presented a new disease episode, even after one year without symptoms, the molecular typing results suggested an endogenous reactivation, due to the persistency of the original strain.

The *in vitro* sensitivity tests performed by microdilution assays showed that from all the studied groups, 100% of *C. neoformans* and *C. gattii* strains were susceptible to amphotericin B, ketoconazole and fluconazole. *C. neoformans* strains presented a dose-dependent sensitivity profile to the antifungal 5-fluorocytosine. A high frequency of MIC values for itraconazole was also observed, corresponding to a susceptible dose-dependent and resistance pattern (Table 3 and 4).

DISCUSSION

Cryptococcus is a cosmopolitan fungus, isolated from soil, animals and bird excrement. The mycosis caused by this fungus, cryptococcosis, is one of the main diseases affecting especially immunocompromised individuals. In this study, the species characterization of isolates from HIV-infected patients living in the city of *Ribeirão Preto*, state of *São Paulo*, Brazil, was performed by analyses of fungal growth in CGB-Agar and molecular characterization. There was a 100% agreement between these two methods for the species identification, showing that the CGB biochemical test is reliable and offers advantages because it is not expensive, is little laborious and provides fast results [17,21,35,36].

It was also observed that among the 32 patients, the majority of the isolates (78.1%) were identified as *C. neoformans*, and 21.9% were infected with *C. gattii*. In several studies in HIV patients, the isolation rate of *C. neoformans* is around 90%. Regarding *C. gattii,* overall rates of isolation from clinical material are around 11%, which is higher in immunocompetent patients than in patients with HIV. However, there is no definite explanation for this fact[37-44]. The high *C. gatti* rate found in this work can be explained by the proximity with its natural reservoirs or by the fact that HC-FMRP-USP is a reference center for the treatment of this pandemic infection, which may concentrate more exotic cases of cryptococcosis.

Table 2

Isolates of *Cryptococcus* species (*C. neoformans* or *C. gattii*) distributed according to the group (I, II, III), frequencies and molecular type

Group	n (%)	*C. neoformans* (%)	Molecular type (%)	*C. gattii* (%)	Molecular type (%)
I	20 (62.5)	13 (65.0)	VNI = 10 (77.0) VNII = 3 (23.0)	7 (35.0)	VGII = 5 (71.4) VGIII = 2 (28.6)
II	15 (21.9)	15 (100.0)	VNI = 13 (86.7) VNII = 2 (13.3)	0 (0)	0 (0)
III	15 (15.6)	15 (100.0)	VNI = 1 (6.7) VNII = 14 (93.3)	0 (0)	0 (0)
Total	50 (100.0)	43 (78.1)	VNI = 24 (55.8) VNII = 19 (44.2)	7 (21.9)	VGII = 5 (71.4) VGIII = 2 (28.6)

Table 3
Frequency of *Cryptococcus* isolates identified as *C. neoformans* or *C. gattii*, distributed in groups I/II or III, with respect to their susceptibility to the three antifungal agents* (susceptible, intermediate and resistant)

Antifungal		Susceptible			SDD			Resistant	
		C. neoformans	*C. gattii*		*C. neoformans*	*C. gattii*		*C. neoformans*	*C. gattii*
Itraconazole	GI/II	2 (20%)	2 (29%)	GI/II	18 (80%)	5 (71%)	GI/II	0	0
	GIII	3 (20%)	-	GIII	9 (60%)	-	GIII	3 (20%)	-
Fluconazole	GI/II	20 (100%)	7 (100%)	GI/II	0	0	GI/II	0	0
	GIII	14 (93%)	-	GIII	1 (7%)	-	GIII	0	-
5-Fluocytosine	GI/II	17 (75%)	7 (100%)	GI/II	3 (15%)	0	GI/II	0	0
	GIII	13 (87%)	-	GIII	2 (13%)	-	GIII	0	-

GI/II = group I and II; GIII = group III; SDD = susceptible-dose-dependent; - = group III does not present any *C. gattii* isolates. * All the isolates were susceptible to Amphotericin B and Fluconazole.

Table 4
Susceptibility test of the samples to five antifungals, and distribution according to the group (I/II and III), breakpoint concentrations, MIC range, MIC 50 and MIC 90

Antifungal	Groups	Breakpoint concentrations	MIC range	MIC 50	MIC 90
Amphotericin B	GI/II	1.0	0.0156-8.0	0.125	0.5
	GIII			0.25	0.5
Ketoconazole	GI/II	4.0	0.0312-16.0	0.125	0.25
	GIII			0.25	0.5
Itraconazole	GI/II	0.125	0.0156-8.0	0.25	0.5
	GIII			1.0	1.0
Fluconazole	GI/II	8.0	0.125-64.0	2.0	2.0
	GIII			2.0	4.0
5-Fluorcytosine	GI/II	4.0	0.125-64.0	2.0	4.0
	GIII			2.0	4.0

GI/II = groups I and II; GIII = group III. Values are presented in μg/mL. NCCLS MIC breakpoint concentrations.

C. gattii was isolated only among patients from Group I, demonstrated by the positive amplification products of SOD1 sequence B/C. All of the groups (I, II and III) showed sexual type MATα prevalence (MFα1 gene recognition), which is consistent with reports of the literature for clinical isolates[35,45]. Meanwhile, in this study, only one *C. gattii* isolate (number 46) was identified and was also recognized by specific primers for the gene MFa1-B/C, resulting in a 213 bp amplification product, thus characterizing the sample as a diploid (MATa / α). Reports on this behavior are rare, especially for serotypes B and C, since the occurrence of diploidy is preferably checked in the case of serotypes A, D and AD isolates (AαDa or AaDα)[2,16].

All the *C. neoformans* isolates (Groups I, II and III) were characterized as belonging to serotype A, MATα, a characteristic frequently observed in the literature [45,46]. For serotype A *(C. neoformans* var. *grubii*), Lengeler[16] and Keller[47] suggested that MATa is becoming extinct, because these isolates are usually non fertile or because they belong to an ecological niche that has not yet been found. In the present study, the serotype D was not identified in any of the studied groups, regarding MATa or MATα.

It is known that the mating type condition can influence the fungus virulence since there is a predominance of MATα cells in these patients. Furthermore, these cells are more pathogenic in the murine model, although the MATα type is also common in nature[48]. Because of the mating gene *locus* that is not the same among MATa, MATα alleles and serotypes, the molecular characterization is a safer identification method and requires the use of various combinations of primers to determine the serotype, as well as the corresponding species and sexual type. Therefore, this methodology is important since it can indicate differences in virulence and shows differences in the antifungal medical treatment responses. Besides, the knowledge of these variations is essential to understand the infection source, recurrence cases or reinfection and the host immune response[49, 50].

In this study, the molecular typing was performed using RAPD-PCR with the specific microsatellite sequences M13 and $(GACA)_4$ according to the definition and molecular types standardization previously established by Meyer *et al.*[31]. Two *C. neoformans* molecular types were observed for the M13 specific primer, being the molecular type VNI the predominant, and for *C. gattii* two molecular types were detected, being the molecular

type VGII the most frequent. It is important to emphasize that the *C. neoformans* serotype A (var. *grubii*) identification was confirmed, since the VNI and VNII molecular types correspond precisely to this strain, corroborating previous data[51,52]. It was possible to observe that with this analysis, four patients had recurrent episodes of cryptococcosis, and one patient was re-infected by a new *C. neoformans* [53-60].

The MIC values observed for isolates from Groups I and II, regardless of the species, was consistent with the values reported in the literature, excepting for the values obtained for itraconazole[7,40,61]. No difference was observed between the MIC values for *C. neoformans and C. gattii* regarding the five antifungal agents tested. They were all in agreement with MIC values reported by Sorrel[39], but in disagreement with those reported by Fernandes[40] that observed higher MIC values for *C. gattii*. For group III (patients with recurrent episodes of the disease) a discrepancy of MIC values was observed only for itraconazole, but for the other four drugs, MICs values indicated that 90% of the isolates had the MIC sensitivity profile defined for these drugs.

Amphotericin B alone or in combination with 5-fluorocytosine is the regimen of choice for the treatment of cryptococcal meningitis. This drug alone or the combination is used in the early stages of therapy and is associated with high toxicity. Fluconazole is primarily indicated for secondary prophylaxis of this disease, aiming to reduce the risk of infection recurrence in patients that still maintain the status of immunosuppression[41,42]. Ketoconazole and itraconazole are not first-line drugs for the treatment of this disease in Brazil, however, they were included in this research only to investigate the strains behavior regarding these antifungals and to evaluate possible cross-resistance among azoles.

Based on the results obtained in this study, it was possible to conclude that the strains isolated from HIV-patients living in the city of *Ribeirão Preto*, a region that did not present a significant increase in resistance to the antifungal agents used in the clinical practice, and also that the biochemical test CGB-Agar is reliable given the good correlation between this method and the molecular characterization. Furthermore, the homogeneity of the molecular and mating types detected in these strains indicate a low probability of crossing among strains.

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AUTHOR CONTRIBUTIONS

Design and development of the study: Thais P. Figueiredo; Rosymar C. de Lucas. Analysis of the results: Rodrigo A. Cazzaniga and Carolina N. França. Development of the manuscript: Fernando Segato, Rafael Taglialegna & Claudia M. L. Maffei.

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