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Antifungal susceptibility, serotyping, and genotyping of clinical *Cryptococcus neoformans* isolates collected during 18 years in a single institution in Madrid, Spain

JESÚS GUINEA*,†, FERRY HAGEN‡,§, TERESA PELÁEZ*,†, TEUN BOEKHOUT‡,§, HICHAM TAHOUNE*, MARTA TORRES-NARBONA* & EMILIO BOUZA*,†

**Clinical Microbiology and Infectious Diseases Department, Hospital General Universitario 'Gregorio Marañón', Universidad Complutense de Madrid, Madrid, Spain, †CIBER de Enfermedades Respiratorias (CIBER RES CD06/06/0058), Palma de Mallorca, Spain, and ‡Department of Yeast and Basidiomycete Research, CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands, and §Department of Internal Medicine and Infectious Diseases, University Medical Centre Utrecht, Utrecht, the Netherlands*

We studied the serotypes, mating-types, AFLP genotypes, and antifungal susceptibility of 58 *Cryptococcus neoformans* strains causing 56 episodes of cryptococcosis in 55 patients over an 18-year period in a single institution. The underlying conditions of the patients were classified as HIV infection ($n=48$) or non-HIV-related immunodeficiency ($n=7$). Serotype A ($n=34$; 58.9%) predominated, but serotype AD was involved in 23.2% of episodes. Most of the episodes were caused by mating-type α ($n=41$; 73.2%) or α/**a** strains ($n=12$; 21.5%). The most common genotype was AFLP1 ($n=26$; 44.8%), followed by AFLP3 ($n=21$; 36.2%), and AFLP2 ($n=11$; 19.0%). In two different patients, we showed the coexistence of different serotypes and/or genotypes in the same episode (AFLP1 and 3). The new triazoles voriconazole, posaconazole and isavuconazole showed high and similar antifungal activity (MICs ≤ 0.125 μg/ml). Fluconazole also had good antifungal activity, but two strains from patients with HIV-infections had an MIC of 16 μg/ml (3.4%). However, these two isolates remained very susceptible to the new triazoles (MICs ≤ 0.062 μg/ml). The remaining strains always showed MICs ≤8 μg/ml.

Keywords *Cryptococcus neoformans,* genotyping, serotyping, mating, isavuconazole

Introduction

Cryptococcosis is an opportunistic fungal infection caused by *Cryptococcus neoformans* or *Cryptococcus gattii*. The latter is more prevalent in immunocompetent individuals, while infections by *C. neoformans* mainly affect immunocompromised patients [1,2].

The taxonomy of the *C. neoformans/C. gattii* complex is currently under review, but it includes at least two pathogenic members, i.e., *C. gattii* and *C. neoformans* [3,4]. *C. neoformans* is divided into three varieties: *C. neoformans* var. *grubii* (serotype A; AFLP genotype 1), *C. neoformans* var. *neoformans* (serotype D; AFLP genotype 2), and hybrids of both varieties (serotype AD; AFLP genotype 3) [3,5,6]. *Cryptococcus gattii* strains belong to serotypes B or C (AFLP genotypes 4, 5, 6, and 7). Interspecies hybrids of *C. gattii* × *C. neoformans* var. *neoformans* (serotype BD; AFLP genotype 8) and of *C. gattii* × *C. neoformans* var. *grubii* (serotype AB; AFLP genotype 9) have recently been described [6–8]. Genotyping of *Cryptococcus* spp. strains allows us to determine whether cryptococcosis is caused by a single genotype or represents a co-infection with two or more genotypes.

The recent introduction of new antifungal agents suggests that the antifungal susceptibility profiles of clinical

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Correspondence: Jesús Guinea, PharmD, Servicio de Microbiología Clínica y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañón, C/ Dr. Esquerdo, 46, 28007 Madrid, Spain. Tel: +34 915867163; Fax: +34 915044906; E-mail: jguineaortega@yahoo.es

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C. neoformans strains must be updated. Our study analyzes the agents causing cryptococcosis over the last 18 years in a large teaching institution. We assess the phenotypic and genotypic characteristics and antifungal susceptibility profiles of the strains.

Materials and methods

Episodes, patients, and C. neoformans isolates

From 1990 to 2007, we detected 70 episodes of cryptococcosis in 68 different patients in our hospital. Episodes were considered different when the isolation of *Cryptococcus* spp. was separated by >3 months. We analyzed a subset of 56 episodes (55 patients) for which isolates were available. Cryptococcosis presented as meningitis ($n=24$), fungemia+meningitis ($n=21$), and fungemia alone ($n=11$). The underlying conditions of the patients were classified as HIV infection ($n=48$) or non-HIV-related immunodeficiency ($n=7$). The patients with non–HIV-related immunodeficiency had received solid organ transplants ($n=4$; three renal transplant recipients and one heart transplant recipient) or had other immunosuppressive conditions ($n=3$; one HCV cirrhosis, one rheumatoid arthritis under immunosuppressive therapy with corticosteroids, methotrexate, and infliximab and one systemic lupus erythematosus and alveolar proteinosis).

A total of 95 available *C. neoformans* strains from 55 patients were studied. Samples from patients were taken when clinically indicated. Only one colony from each plate was stored. The number of strains per patient was not homogeneously distributed, i.e., 35 patients had only one strain, nine patients had two strains, six patients had three strains, three patients had four strains and two patients had six strains. The *Cryptococcus* spp. strains were stored at –70ºC in tubes containing sterile distilled water. To ensure viability and purity, each isolate was subcultured on sheep blood agar (Soria Melguizo, Madrid, Spain) before analysis. In patients with multiple isolates available from the same episode, only the first strain of each genotype was selected (58 isolates).

Genotyping, serotyping, and mating-typing

The *Cryptococcus* strains were analyzed for their mating-, sero-, and genotypes, as well as their *in vitro* antifungal susceptibilities. All available strains from each patient were genotyped in order to detect co-existence of different genotypes in a single episode. The mating-types and serotypes of the strains were determined using four different polymerase chain reactions (PCRs) that specifically amplify the *STE20*a and *STE20*α locus of either serotype A and D isolates (9). Amplified fragment length polymorphism (AFLP) fingerprint analysis was carried out to determine the genotypes of all *C. neoformans* isolates [7,8]. The strains CBS8710 (AFLP1/VNI; alphaA), CBS9172 (AFLP1/VNI; **a**A), CBS10511 (AFLP2/VNIV; alphaD), CBS10513 (AFLP2/VNIV; **a**D), CBS10078 (AFLP4/VGI; alphaB), CBS10080 (AFLP3/VNIII; alphaA**a**D), CBS10081 (AFLP5/VGIII; alphaB), CBS10082 (AFLP6/VGII; alphaB), and CBS10101 (AFLP7/VGIV; alphaC) were included as quality control strains for mating-, sero-, and genotyping. A dendrogram was calculated using single linkage clustering in combination with the Pearson correlation using Bionumerics version 4.6.1 (Applied Maths, Sint-Martens-Latem, Belgium).

Antifungal susceptibility testing procedures

A total of 58 strains were available for antifungal susceptibility testing. We used the antifungal agents obtained as reagent-grade powders from their respective suppliers. The panel of five antifungal agents included amphotericin B (Sigma Chemical Co., Madrid, Spain), fluconazole, voriconazole (Pfizer Pharmaceutical Group, New York, New York, USA), isavuconazole (Basilea Pharmaceutica Ltd, Basel, Switzerland), and posaconazole (Schering-Plough Research Institute, Kenilworth, New Jersey, USA). Antifungal activity was determined using the CLSI M27-A3 broth microdilution procedure [10].

The final concentrations for each antifungal agent were as follows; amphotericin B, posaconazole, isavuconazole, and voriconazole, 0.031 μg/ml to 32 μg/ml; and fluconazole, 0.062 μg/ml to 64 μg/ml. No precipitates were observed for concentrations of posaconazole above 8 μg/ml.

Although the CLSI M27-A3 procedure recommends that inocula be prepared after growing strains on Sabouraud dextrose agar or potato dextrose agar, we cultured the *Cryptococcus neoformans* isolates on sheep blood agar for 48–72 h at 35ºC to improve growth. All the inoculated trays were incubated at 35ºC and read macroscopically at 72 h. The MIC endpoint for amphotericin B was defined as the lowest concentration that produced complete inhibition of growth (MIC-0), whereas for the remaining agents it was defined as the lowest concentration at which a significant decrease in turbidity, corresponding to approximately 50% inhibition of growth, was observed (MIC-2) [10].

The same batch of trays was used to assess antifungal susceptibility. Quality control was ensured by testing *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019, and all results were within the recommended CLSI limits. The activity of the five antifungal agents was shown as the MIC_{90}, MIC_{50}, and range of MICs.

Data analysis. We analyzed the correlation between the presence of a specific different mating-type, serotype, or genotype of *C. neoformans*, and the predisposing conditions

of the patients or their sex (Fisher exact test). The activity of the five antifungal agents was shown as the cumulative percentage of isolates in each MIC and geometric mean (GM). For calculation of GM, MICs below the lowest antifungal concentration were considered 0.015 μg/ml (for the new triazoles). We analyzed the differences in the GM of the MICs of the antifungal agents against the strains grouped by mating-type, serotype, and genotype, and predisposing condition (HIV infection or other immunosuppressive conditions). The null hypothesis was rejected in every hypothesis contrast for an alpha error below 0.05.

Results

Mating-, sero-, and genotyping

Tables 1 and 2 and Fig. 1 show the distribution of serotypes, mating-types, and genotypes of the 58 isolates causing the 56 episodes of cryptococcosis. Serotype A ($n=34$; 58.9%) predominated, although serotype AD was involved in 23.2% of the episodes. The mating-type analysis revealed that most of the episodes were caused by α ($n=41$; 73.2%) or α/**a** strains ($n=12$; 21.5%). One episode was caused by a *C. neoformans* isolate that was non-typeable for mating-type and serotype, but was identified based on AFLP genotyping as AFLP1B, corresponding to *C. neoformans* var. *grubii*.

The most widely distributed AFLP genotype was AFLP1 ($n=26$; 46.4%), followed by AFLP3 ($n=21$; 37.5%) and AFLP2 ($n=11$; 19.6%). In 35 episodes (35 patients), only one available isolate was analyzed and no genetic diversity was identified. However, in the remaining 21 episodes (20 patients) we were able to analyze two or more strains per episode, and this allowed us to demonstrate the co-existence of different serotypes and/or genotypes in the same episode in two different patients. One HIV-infected patient had an episode caused by two different *C. neoformans* genotypes (AFLP1 and 3); this was consistent with the results of the mating-type and serotype analyses (two αA strains were isolated from cerebrospinal fluid and blood, and one αA-**a**D strain was isolated from a cerebrospinal fluid sample taken 3 weeks later). Another HIV-infected patient also had an episode caused by two different genotypes (an AFLP1B and an AFLP3 strain, both isolated from cerebrospinal fluid samples taken 15 days apart from each other).

In our hospital, episodes of cryptococcosis were caused by *C. neoformans* var. *grubii* ($n=24$; 42.9%), *C. neoformans* var. *neoformans* ($n=11$; 19.6%), the hybrid *C. neoformans var. grubii* × *C. neoformans* var. *neoformans* ($n=19$; 33.9%), and the co-existence of both *C. neoformans* var. *grubii* and the hybrid *C. neoformans* var. *grubii* × *C. neoformans* var. *neoformans* ($n=2$; 3.6%).

Table 1 Serotype, mating-type, and AFLP genotype of 58 isolates causing the 56 episodes of cryptococcosis.

	Serotype[1]					Mating-type[2]					AFLP genotype[3]			
	A	D	AD	A and AD	Non-typeable	α	α/a	a	α/α	α and α/a	Non-typeable	1	2	3	1 and 3
Episodes($n=56$)	31(55.3%)	11(19.6%)	11(19.6%)	2(3.6%)	1(1.8%)	39(69.6%)	10(17.9%)	3(5.4%)	1(1.8%)	2(3.6%)	1(1.8%)	24(42.8%)	11(19.6%)	19(33.9%)	2(3.6%)

[1]One strain was non-typeable and two episodes were caused by a combination of the A and AD serotypes.
[2]Two episodes were caused by a combination of the α and α/**a** mating-types.
[3]Two episodes were caused by a combination of two different AFLP genotypes: 1+3.

Table 2 Relationship between the different mating-types, serotypes, and genotypes of *Cryptococcus neoformans* and the sex and underlying conditions of the patients.

		Underlying condition[1]			Sex[2]		
	N	HIV(%)	Other(%)	*P*	Female(%)	Male(%)	*P*
Serotype[3]							
A	33	54.9	83.3	0.384	90	51.1	0.034
D	11	21.6	0	0.584	0	23.4	0.183
AD	13	23.5	16.7	1.000	10	25.5	0.426
Mating-type[3,4]							
A	41	70.6	83.3	0.665	90	68.1	0.253
α/a	12	21.6	16.7	1.000	10	23.4	0.671
AFLP genotype							
1, 1A or 1B	26	85.7	39.2	0.038	90.9	34	0.001
2	11	21.6	0	0.327	23.4	0	0.102
3	21	39.2	14.3	0.403	9.1	42.6	0.044

[1]Percentage of patients with HIV infection or other immunodeficiencies infected by each serotype, mating-type and AFLP-type.
[2]Percentage of females or males infected by each serotype, mating-type and AFLP-type.
[3]One strain was non-typeable.
[4]Mating-types a ($n=3$) and **α/α** ($n=1$) were not included in this analysis.

Twenty-two strains belonged to genotype AFLP3, of which 13 were serotype AD by PCR analysis and only the serotype A background could be determined with the remaining nine strains. AD hybrids may become aneuploid, with the result that the full serotype AD background is no longer present in the genomic DNA of these isolates.

Relationship between strain characteristics (mating-type, serotype, and genotype), underlying conditions, and sex

We could not find a specific mating-type or serotype that was more prevalent in HIV-infected patients than in patients with non-HIV-related immunodeficiency. However, we found that AFLP genotypes 1, 1A, and 1B were more prevalent in HIV-infected patients ($P = 0.034$) (Table 2). Serotype A and AFLP genotypes 1, 1A, and 1B were more prevalent in females, whereas AFLP genotype 3 was more prevalent in males ($P < 0.05$) (Table 2).

Antifungal susceptibility testing results

Table 3 summarizes the antifungal susceptibility results of the 58 strains involved in the 56 episodes of cryptococcosis. The new triazoles, i.e., voriconazole, posaconazole, and isavuconazole showed high and similar antifungal activity – all strains had a MIC $\leq$ 0.125 μg/ml. Fluconazole also had good antifungal activity – only two strains from HIV-infected patients had an MIC of 16 μg/ml (3.4%) and both remained highly susceptible to the three new triazoles (MICs $\leq$ 0.062 μg/ml). The remaining strains always showed MICs $\leq$ 8 μg/ml. Only two strains showed an MIC $>$ 1 μg/ml for amphotericin B.

We did not find differences in antifungal susceptibility between strains isolated from HIV-infected patients and non-HIV-infected patients.

We compared the geometric mean of the MICs of each antifungal agent against isolates grouped by serotype, mating type and AFLP genotype. Isolates belonging to serotype A showed significantly higher MICs (0.937 μg/ml) for amphotericin B than those from serotype D (0.818 μg/ml) and serotype AD (0.692 μg/ml) ($P < 0.01$). Isolates belonging to AFLP type 1, 1A, and 1B were less susceptible for amphotericin B (0.981 μg/ml) than AFLP type 3 (0.725 μg/ml) ($P < 0.05$).

No differences in susceptibility to the new triazoles were observed for the different serotypes, mating types, and genotypes of *C. neoformans* studied.

Discussion

Our study evaluated the epidemiology of *C. neoformans* in a single institution over a period of 18 years. Cryptococcosis patients admitted to our hospital were infected mainly by *C. neoformans* var. *grubii* (genotype AFLP1). However, a high proportion of episodes were caused by serotype AD (23.2%) strains. Many clinical *C. neoformans* isolates from Italy, Spain, Portugal, and Greece have been shown to be serotype AD, thus suggesting a high incidence of serotype AD hybrids in southern Europe [11].

AFLP genotyping was employed with all *Cryptococcus* spp. strains recovered from patients to identify their variety within the *C. neoformans* complex, and to confirm the serotyping analysis based on four different PCRs. Serotyping based on PCR alone may lead to incorrect identifications due to the fact that diploid isolates (e.g., serotype AD hybrids) can become aneuploid and lose parts of their genomic content, including regions containing the target for the serotype PCRs. This was observed in some of the *C. neoformans* strains studied and may explain the discrepancy between PCR-based serotyping (58.9% episodes

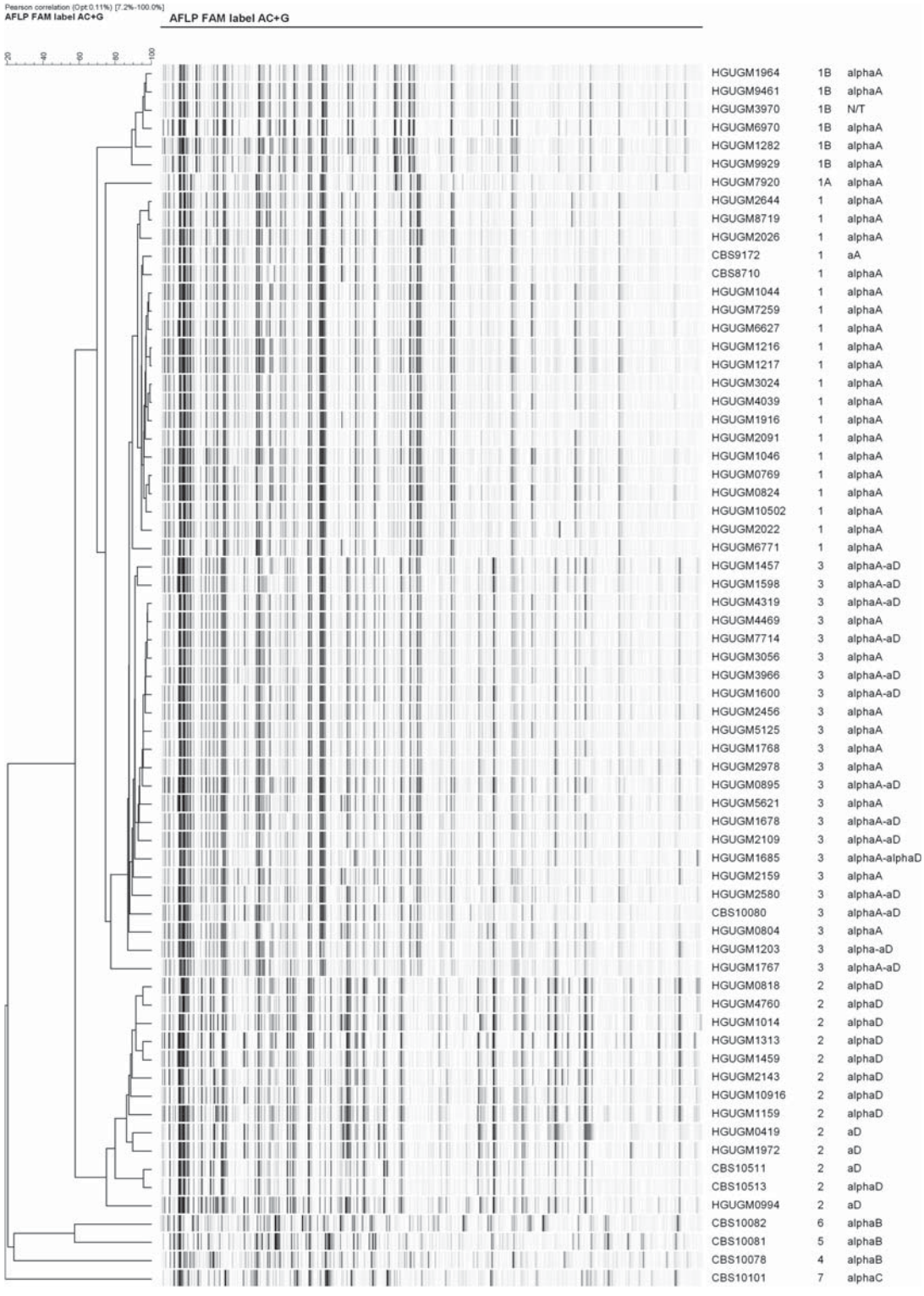

Fig. 1 Results of AFLP genotyping, mating-typing and serotyping of 58 *Cryptococcus neoformans* strains including reference strains.

Table 3 Cumulative percentages of isolates within each MIC (μg/ml) of the five antifungal agents studied.

Antifungal agents	≤0.031	0.062	0.125	0.25	0.5	1	2	4	8	16
Isavuconazole	89.7	96.6	100							
Posaconazole	96.6	100								
Voriconazole	75.4	94.7	100							
Fluconazole					3.45	22.8	52.6	78.9	96.5	100
Amphotericin B					34.5	96.5	100			

caused by serotype A strains and 23.2% of the episodes by serotype AD strains) and AFLP genotyping, i.e., 46.4% of the episodes caused by AFLP1 strains and 37.5% of the episodes by genotype AFLP3 strains.

The presence of two or more different genotypes of *C. neoformans* causing a single episode of cryptococcosis has been poorly evaluated. The presence of several genotypes can only be detected when more than one sample is analyzed from the same patient. In this study, 21 episodes (20 patients) of cryptococcosis yielded at least two isolates from different samples. Although we only stored one colony from each sample, we were able to show that two of the episodes were caused by two different AFLP genotypes, suggesting that cryptococcosis can be a co-infection of multiple *C. neoformans* genotypes. There are few reports of infections caused by multiple-genotype *C. neoformans* isolates. Haynes *et al*. [12] described the involvement of different *C. neoformans* genotypes in recurrent cryptococcosis, which was explained by the fact that either the patient was re-infected with a distinguishable *C. neoformans* strain during and/or after antifungal treatment, or that one of the strains causing the primary infection was more persistent than the other. Another explanation is that the karyotype became unstable during infection. This phenomenon was reported by Fries *et al*. [13], who observed that chromosomal arrangements and/or deletions took place in a murine model. However, it seems unlikely that the karyotype became unstable in the mixed infections described as it implies that the strain with the hybrid genotype AFLP3 (serotype AD) has lost approximately half of its genetic content during the infection to become a strain with genotype AFLP1 (serotype A). Therefore, a mixed infection with two different genotypes of *C. neoformans* seems to be the most plausible explanation.

Although several studies have evaluated the antifungal susceptibility of *C. neoformans* and *C. gattii* [2,14–18], the introduction of new drugs means that data on antifungal susceptibility should be updated. We found a low rate of resistance to fluconazole in our 58 *C. neoformans* isolates. There is some evidence that the MICs of fluconazole (≥16 μg/ml) and amphotericin B (≥2 μg/ml) against *C. neoformans* can be a predictor of poor patient outcomes in cases of cryptococcosis [19–21]. Therefore, all isolates in the present study were classified as susceptible to amphotericin B, and 3.6% were fluconazole-resistant (≥ 16 μg/ml). The higher MICs of amphotericin B of some serotypes or genotypes may not have any clinical consequences. No history of treatment with fluconazole could be demonstrated in the two patients with isolates showing an MIC of 16 μg/ml for fluconazole. Although some authors found a low level of fluconazole resistance in *C. neoformans* when analyzing a large number of clinical strains [22–24], others reported an increasing proportion of resistant strains [25]. Interestingly, Perkins *et al*. [26] reported in 2005 that 46.6% (≥16 μg/ml) of the strains in a collection comprising 317 clinical isolates of *C. neoformans* var. *neoformans* from a Spanish mycology reference laboratory were fluconazole-resistant. This proportion of fluconazole-resistant *C. neoformans* strains is considerably higher than that observed in the present study (3.6%) and by other Spanish investigators [27]. The discrepancies may be explained by variations in the antifungal susceptibility testing procedure (EUCAST vs. CLSI) and a bias in the selection of strains submitted to the reference laboratory.

The new triazoles studied (voriconazole, posaconazole, and isavuconazole) showed potent antifungal activity against all the evaluated strains, with MICs ≤ 0.125 μg/ml. These findings are consistent with previous reports [27–31].

In conclusion, we found that most cases of cryptococcosis in our hospital were caused by strains of *C. neoformans*, with a predominance of *C. neoformans* var. *grubii*. The *C. neoformans* isolates showed a low level of resistance to fluconazole, and were highly susceptible to the new triazoles, including isavuconazole.

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