Eur J Clin Microbiol Infect Dis (2012) 31:1647–1652
DOI 10.1007/s10096-011-1488-3

ARTICLE

Microbiological characteristics of clinical isolates of *Cryptococcus* spp. in Bahia, Brazil: molecular types and antifungal susceptibilities

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Received: 19 April 2011 / Accepted: 5 November 2011 / Published online: 26 January 2012

Abstract To determine the profiles of susceptibility to antifungal and the genotypes of clinical isolates of *Cryptococcus* in Bahia, Brazil, 62 isolates were collected from cases of meningitis in the period from 2006 to 2010. Their susceptibilities to fluconazole, itraconazole, amphotericin B and 5-flucytosine were determined by the broth microdilution technique described by the Clinical and Laboratory Standards Institute and genotyping of the *URA5* gene was accomplished by restriction fragment length polymorphism. *C. neoformans* accounted for 79% of the identified yeast and *C. gattii* represented the remaining 21%. Evaluation of the genotypes determined that 100% of the *C. gattii* isolates belong to the VGII genotype, and 98% of the *C. neoformans* isolates belong to the VNI genotype. Determination of susceptibility revealed isolates resistant to fluconazole (4.8%), 5-flucytosine (1.6%) and amphotericin B (3.2%); the stratification of sensitivity results for each species showed significant differences in susceptibility to azoles. This study is the first to describe the susceptibility profiles of molecular and clinical isolates of *Cryptococcus* in Bahia, Brazil. The high percentage of *C. gattii* isolates belonging to the VGII genotype and its lower susceptibility to antifungal agents highlight the importance of knowing which species are involved in cryptococcal infections in northeastern Brazil.

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Introduction

Cryptococcal meningitis, an important opportunistic infection in HIV-positive patients in developing countries, is caused by *Cryptococcus* spp., which are encapsulated yeasts that are spread worldwide. The genus has two species most commonly associated with infection in humans, *Cryptococcus neoformans* (serotypes A, D and the AD hybrid; VNI – IV genotypes) and *Cryptococcus gattii* (serotypes B and C; VGI – IV genotypes), which differ genotypically, phenotypically and epidemiologically [1–3].

Immunocompromised patients are more frequently infected by *C. neoformans*, while *C. gattii* has emerged as an important cause of infection in immunocompetent individuals, as illustrated by the recent outbreak on Vancouver Island (Canada) by the VGII genotype, with molecular evidence of spread to the northwestern United States [4–6]. In Brazil, there are few studies that illustrate the distribution of this genotype [7, 8]. The yeast is

acquired from the environment, and its tropism for the central nervous system leads to meningitis [9, 10]. The American Society of Infectious Diseases suggests that the treatment of cryptococcal meningitis be initiated with an induction therapy of amphotericin B alone or combined with 5-flucytosine, followed by consolidation and maintenance therapy with an azole [11].

There are few reports of antifungal resistance in isolates of *Cryptococcus* [12–14]. Although some authors suggest that it remains stable [15–17], the ARTEMIS global antifungal surveillance study showed increased fungal resistance to fluconazole. In the period from 1997–2000, the rate of resistance to fluconazole was 7.3%; in 2005–2007, this rate increased to 11.7%, with the highest rates found in Latin America (13.6%) and Africa (12.4%) [18]. The authors propose that the increased resistance may be limited to areas where antiretroviral therapy is still not effective, such as Spain [13], Cambodia [12] and Africa [19, 20].

Little is known about the frequency and susceptibility profile of *Cryptococcus* species in northeastern Brazil. Thus, this study aims to determine the antifungal susceptibility profiles and the frequencies of species and genotypes of clinical isolates of *Cryptococcus* spp. in the state of Bahia in northeastern Brazil.

Materials and methods

Source and identification of clinical isolates

We collected 62 clinical isolates from 62 cases of cryptococcal meningitis diagnosed at the Hospital Couto Maia, Bahia, from 2006 to 2010. After 48 hours of growth on Sabouraud dextrose agar (Acumed, New York, NY), the genus was identified based on the demonstration of typical encapsulated cells in India ink preparations and the development of brown pigmentation on the surface of Niger seed agar; classical biochemical tests were also conducted for the identification of yeasts [21]. The identification of *C. neoformans* and *C. gattii* was accomplished using canavanine-glycine-bromothymol blue agar [22]. This study was approved by the ethics committee of the Hospital Couto Maia.

Reference strains

Cryptococcus complex reference strains were kindly provided by the Mycology Laboratory of the Institute of Clinical Research Evandro Chagas - Oswaldo Cruz Foundation and included WM 148 (VNI), WM 626 (VNII), WM 628 (VNII), WM 629 (VNIV), WM 179 (VGI), WM 178 (VGII), WM 161 (VGIII) and WM 779 (VGIV) [23].

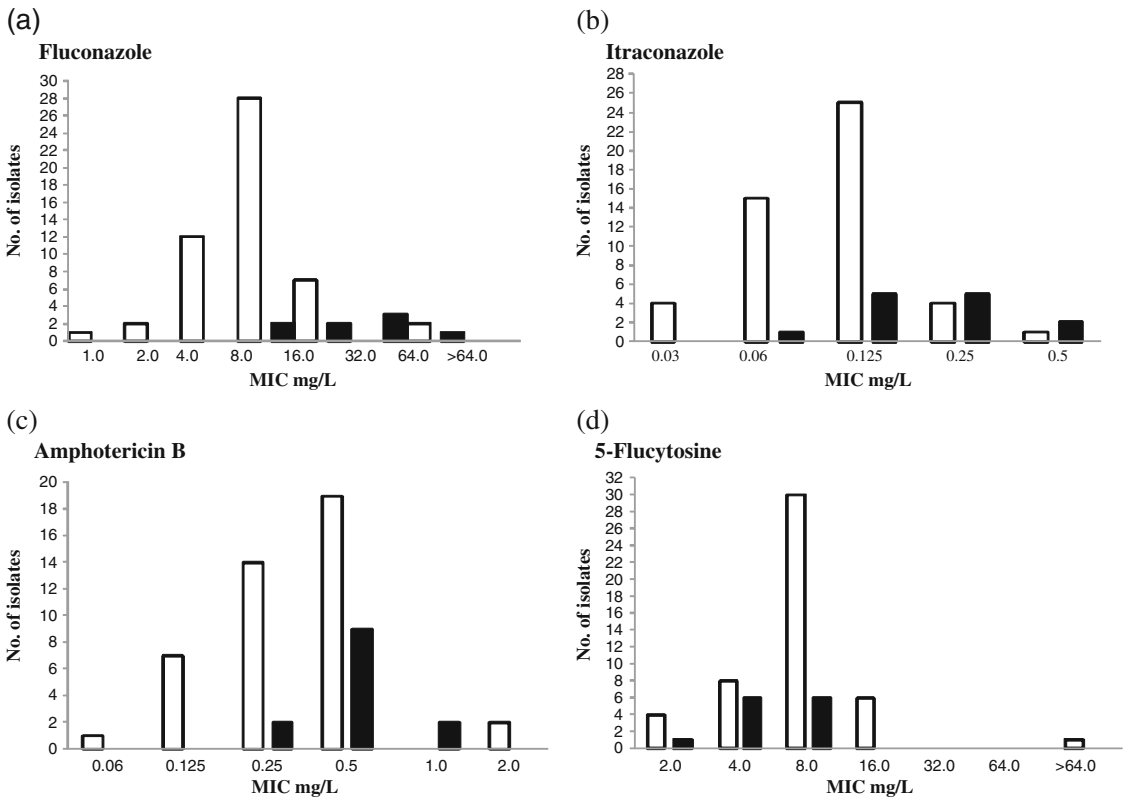

Fig. 1 The distributions of MIC (mg/L) values for fluconazole (**a**), itraconazole (**b**), amphotericin B (**c**) and 5-flucytosine (**d**) for 62 isolates of *Cryptococcus* spp. *C. neoformans* (*empty bars*) and *C. gattii* (*black bars*)

Table 1 Susceptibility profiles and in vitro activities of antifungal agents against clinical isolates of *Cryptococcus* spp

Antifungal agent	Species	Susceptibility profile %			*p* value*
		S	S-DD	R	
Fluconazole	*C. neoformans*	83.7 (41/49)	14.3 (7/49)	2 (1/49)	0.013
	C. gattii	38.5 (5/13)	46 (6/13)	15.5 (2/13)	
Itraconazole	*C. neoformans*	89.8 (44/49)	10.2 (5/49)	0	0.001
	C. gattii	46.1 (6/13)	53.9 (7/13)	0	
Amphotericin B	*C. neoformans*	96 (48/49)	0	4 (2/49)	0.622
	C. gattii	100 (13/13)	0	0	
5-Flucytosine	*C. neoformans*	24.5 (12/49)	7.5 (36/49)	2 (1/49)	0.163
	C. gattii	53.9 (7/13)	46.1 (6/13)	0	

S susceptible, *S-DD* susceptible, dose-dependent, *R* resistant

Reference strains from the American Type Culture Collection (ATCC) included *Candida parapsilosis* 22019 and *C. krusei* 6258, which were also used for quality and reproducibility controls during susceptibility testing.

Antifungal susceptibility tests

The susceptibility tests against fluconazole, itraconazole, amphotericin B and 5-flucytosine (Sigma Aldrich Quimica SA, St. Louis, MO) were performed as described in document M27-A3 of the Clinical and Laboratory Standards Institute (CLSI) [24]. The minimum inhibitory concentrations (MICs) were determined by the lowest antifungal agent concentrations that inhibited 50% fungal growth compared to the control growth (without antifungal) for fluconazole, itraconazole and 5-flucytosine; the MICs for amphotericin B were determined by the lowest concentrations that inhibited 100% growth.

DNA extraction

For the extraction of genomic DNA, the mechanical lysis method was used after digestion of the capsule with urea buffer [25].

Restriction fragment length polymorphism (RFLP)—*URA5* gene

RFLP analysis using the *URA5* gene was performed as described by Meyer and colleagues (2003) using the primers URA5 (5′ATGTCCTCCCAAGCCCTC GACTCCG3′) and SJ01 (5′ TTAAGACCTCTGAA CACCGTACTC3′) [23]. RFLP patterns were assigned visually by comparing them to the patterns obtained from the standard-type strains (VNI-VNIV and VGI-VGIV).

Statistical analysis

Statistical analysis was performed by the least squares method using SPSS 17.0 software (SPSS Inc, Chicago, Illinois). Statistical significance was defined at $p<0.05$.

Results

All yeasts were characterized as belonging to the genus *Cryptococcus* spp., and there were no biochemical differences among isolates; 79% (49/62) of the isolates were *C. neoformans* and 21% (13/62) were *C. gattii*. The distributions of the MIC values of the 62 *Cryptococcus* spp., separated by species, to the antifungals tested are shown in Fig. 1. The antifungal susceptibility profiles are summarized in Table 1, showing isolates of *C. neoformans* that were resistant to fluconazole, amphotericin B and 5-flucytosine and *C. gattii* isolates that were significantly resistant to fluconazole.

The *URA5* gene RFLP analysis revealed that 98% (48/49) of isolates of *C. neoformans* presented the profile of the molecular genotype VNI and 2% (1/49) of genotype VNII,

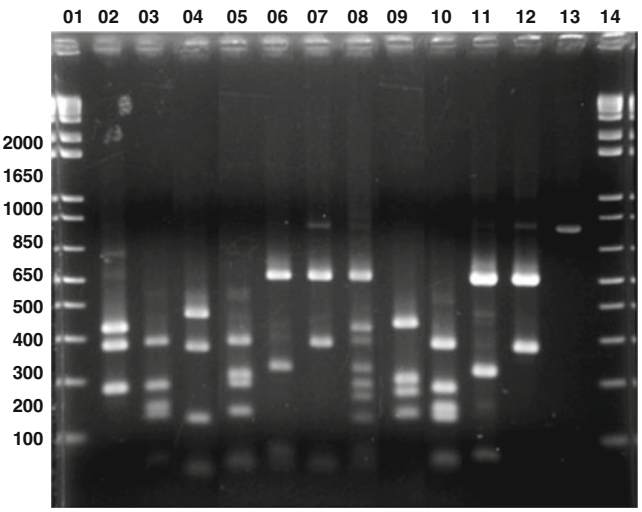

Fig. 2 Representative RFLP profiles of the *URA5* genes from *Cryptococcus* spp. obtained by double-digestion with *Hha*I and *Sau*96I. *Lanes 1 and 14* are the molecular markers; *lanes 2, 3, 4, and 5* are genotypes VGI, VGII, VGIII and VGIV, respectively; *lanes 6, 7, 8, and 9* are genotypes VNI, VNII, VNII and VNIV, respectively; *lane 10* is the clinical isolate of *C. gattii*; *lanes 11 and 12* are clinical isolates of *C. neoformans*; *lane 13* is the *URA5* gene amplified product

while 100% (13/13) of isolates of *C. gattii* showed VGII genotype profile (Fig. 2).

Discussion

The epidemiology of *Cryptococcus* has been widely studied around the world, but in Brazil, information regarding the distribution of species is still fragmentary and incomplete, reflecting only differences in frequency. Cryptococcosis caused by *C. neoformans* is an important cause of morbidity and mortality in immunocompromised individuals worldwide, but the number of published reports of infection by *C. gattii* in patients without immunosuppression is increasing [3–5, 26].

C. gattii was initially considered to be restricted to tropical and subtropical Australia, Southeast Asia and some African regions. Currently, however, it is expanding to the northwest Pacific and North America, which suggests it is adapting to a new climatic niche or that global warming may provide favorable conditions for its growth [5, 27, 28]. In Brazil, the analysis of *Cryptococcus* spp. isolates from various regions has shown that *C. gattii* is significantly more prevalent in the north-northeast of the country and that *C. neoformans* is more prevalent in the south-southeast of the country; however, there are few studies in the north-northeast that report the distributions of these pathogens [29].

Santos and colleagues (2008) analyzed the distribution of species in 56 isolates from 43 patients with meningitis in the state of Pará and identified a frequency of 35.7% (20/56) for *C. gattii* infection [30]. In the southeast, all isolates from different areas of São Paulo belonged to *C. neoformans* [31]. However, Almeida and colleagues (2007) analyzed 83 samples from 38 patients and found only four isolates of *C. gattii* from only one patient [32]. In Minas Gerais, 11.4% of 35 isolates belonged to the species *C. gattii* [33]. In the state of Mato Grosso, in the Midwest, 16.6% of 26 samples of 26 cases of cryptococcosis were *C. gattii* isolates [34]. These results reveal a high number (13) of isolates of *C. gattii* (21%) compared with other studies [30–34] and suggest that Bahia is an important source of infection by *C. gattii*.

Genotypic determination revealed a predominance of the VNI genotype among *C. neoformans* isolates, which have been frequently reported in various regions of the world [35–40]. All *C. gattii* isolates belonged to the VGII genotype. This genotype was reported as the causal agent of the cryptococcosis outbreak that occurred on Vancouver Island (BC, Canada) [4, 41]. This genotype has also been reported in Brazil [7, 8] demonstrating its potential to cause severe disease in immunocompetent hosts, as it was recognized as the main agent of a primary cryptococcosis endemic in the north [29, 30].

The determination of susceptibility profiles revealed the presence of antifungal-resistant *C. neoformans* isolates. Antifungal resistance is among the factors that can contribute to treatment failure because previous exposure to a risk factor often leads to resistance [42, 43]. Our findings are consistent with authors who suggest that there are differences between *C. neoformans* and *C. gattii* sensitivities [44–46], although others disagree [14, 47, 48]. It is known that an infection caused by *C. gattii* has a less favorable response to antifungal therapy and a relatively worse prognosis compared to infection by *C. neoformans* [49, 50]; however, the mechanisms underlying this difference are not clear.

Conclusion

This study is the first to describe the susceptibility profiles of molecular and clinical isolates of *Cryptococcus* in Bahia, Brazil. The high percentage of isolates from the *C. gattii* genotype VGII, in addition to its lower susceptibility to antifungal agents, highlights the importance of knowing the species involved in cryptococcal infection in this state. Surveillance studies are needed to elucidate the environmental niche of this yeast and to monitor trends in antifungal susceptibility and the distribution of species in Bahia, Brazil.

Acknowledgements We thank the Mycology Laboratory of the Institute of Clinical Research Evandro Chagas - Oswaldo Cruz Foundation (FIOCRUZ) - Rio de Janeiro, Brazil for the standard isolates of *Cryptococcus* used in this study.

We also thank the Foundation for Research of the State of Bahia and the Coordination for the Improvement of Higher Education Personnel for the financial support for this project.

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