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Brief Report

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Antifungal susceptibility of clinical *Cryptococcus gattii* isolates from Colombia varies among molecular types

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Abstract

Cryptococcosis by *Cryptococcus gattii* is endemic in Colombia, affecting mostly immunocompetent hosts. Since antifungal susceptibility differs between molecular types of cryptococcal isolates, as reported elsewhere, the aim of this study was to determine if 42 Colombian clinical isolates, VGI, VGII and VGIII, differ in the susceptibility to commonly used antifungals, using Sensititre plates. Among the molecular types, six non-wild type isolates to fluconazole, voriconazole, and 5-flucytosine, were identified. Besides, VGI and VGII were less susceptible to 5-flucytosine and azoles, respectively, than other molecular types. These findings support the applicability of practicing susceptibility testing, which could better guide treatment in cryptococcosis.

Lay Summary

Cryptococcosis gattii affects immunocompetent people. For a correct treatment, antifungal susceptibility testing is essential. This study shows differences in the susceptibility to commonly used antimycotics among genotypes of Colombian clinical *C. gattii* isolates, some of which are non-wild-type.

Key words: Antifungal susceptibility, Colombia, cryptococcosis, *Cryptococcus gattii,* molecular types.

Introduction

Cryptococcosis is a potentially fatal opportunistic fungal disease mainly caused by *Cryptococcus neoformans*. As a primary mycosis, however, cryptococcosis is mainly due to *Cryptococcus gattii*, as this species affects otherwise healthy people.[1] Although *C. gattii* infections comprise <20% of all cryptococcosis cases, they are more difficult to treat and leave more neurological sequeale.[2,3] In Colombia, *C. gattii* has long been recognized as an endemic pathogen, causing about 4% of cryptococcal meningitis cases, predominantly in people with no specific risk factors. In addition, this primary pathogen affects a considerable proportion of children and young adults (~9%) in the country, which contrasts with the epidemiology of pediatric cryptococcosis in the world.[4] Currently, there are five major molecular types in *C. gattii*: VGI to VGIII, which have been reported in several countries, including Colombia; VGIV, which is more restricted to Africa as well as some countries in America and Asia,[4,5] and VGV, which was recently reported from the environment in East Africa.[6] Besides the geographical distribution, these molecular types differ in regards of the epidemiology, virulence, population genetics, and antifungal susceptibility.[2] As there are no validated antifungal clinical breakpoints for *Cryptococcus* spp., *in vitro* susceptibility testing is therefore helpful, as it allows to identify and monitor the emergence of isolates with reduced antifungal susceptibility. Current treatment options to combat cryptococcosis include the use of polyenes, flucytosine, and azoles. However, both intrinsic and acquired resistance to these antifungals has been described in cryptococcal strains worldwide.[7] This is of great concern among

clinicians, as resistance may complicate therapy. Thus, the aim of this study was to report the antifungal susceptibility profile of *C. gattii* isolates, with different molecular types, recovered from clinical samples in Colombia. Establishing whether cryptococcal isolates, depending on the molecular type, are more or are less susceptible to commonly used antifungals, could eventually guide treatment choice, which in turn could result in a better disease outcome of the patients.

The studied *C. gattii* isolates, which were recovered from 42 patients from 15 Colombian states between 1997 and 2019, are part of the strain collection gathered as part of the National Surveillance Program for *Cryptococcus* and cryptococcosis led by the National Institute of Health, in Colombia. Most isolates (88.1%) were recovered from patients with no risk factor for cryptococcosis. Only two patients were HIV positive, and one patient each presented with diabetes, arthritis, and malnutrition. Data on serotype, and mating type of most isolates, and data on molecular type of all isolates were determined as reported elsewhere (Supplementary Table 1).[4,8] Susceptibility testing of the isolates was carried out using Sensititre® YeastOne® plates (Thermo Scientific, USA), following the manufacturer's instructions. Plates were incubated at 35°C and read after 72 h. *Candida krusei* ATCC® 6258 and *Candida parapsilosis* ATCC® 22 019, were used as quality control strains following the M27-A3 guideline of the Clinical and Laboratory Standards Institute (CLSI).[9] The range of drug concentrations tested by twofold serial dilutions was 0.125–8 μg/ml for amphotericin B, 0.0625–64 μg/ml for 5-flucytosine, 0.125–256 μg/ml for fluconazole, 0.016–16 μg/ml for itraconazole, and 0.008–8 μg/ml for voriconazole and posaconazole.

Minimal inhibitory concentrations (MICs) of each antifungal drug were determined for all isolates and per molecular type. Mode and geometric mean MICs were calculated. MICs per drug and molecular type were compared with epidemiologic cut-off values (ECV) >95%, when available, to determine if the isolates belong to the wild-type distribution, as established with isolates from around the world.[10,11] MIC differences between molecular types were compared, per drug, with the Mann–Whitney test using the program GraphPad Prism version 7.05 (La Jolla, CA, USA). Group comparisons for MIC data included VGI vs. VGII, VGI vs. VGIII, and VGII vs. VGIII, with *P*-values < 0.05 considered significant.

Most *C. gattii* isolates from Colombia distribute among the wild-type populations of each molecular type, per antifungal drug (Table 1). However, from the 42 studied isolates, 6 (14.3%) non-wild-type isolates, recovered in 2003 or later, were identified. Among the molecular type VGII, two isolates were identified to be simultaneously fluconazole and voriconazole non-wild-type. These isolates presented MICs that were two and three dilutions higher than the ECV for fluconazole (128 and 256 μg/ml) and one and two dilutions higher than the ECV for voriconazole (0.5 and 1 μg/ml). In addition, in VGI and VGIII, one 5-flucytosine and three fluconazole non-wild-type isolates were identified, respectively. In general, the susceptibility of the studied isolates to amphotericin B did not differ among molecular types. The molecular type VGI was less susceptible to 5-flucytosine than VGII. In addition, VGII was less susceptible to fluconazole, itraconazole, and voriconazole than VGI and VGIII. VGII was also less susceptible to posaconazole than VGIII (Table 2). To corroborate the identification of isolates with high MICs to fluconazole and voriconazole, non-wild type isolates were separately cultured in media containing each azole at a concentration equal to the established MIC (Table 1), which resulted in evident growth.

Identifying VGII and VGIII isolates with reduced susceptibility to fluconazole is very important, as in resource-limited settings the induction therapy for cryptococcosis consists of fluconazole, as a substitute of 5-flucytosine, in combination with amphotericin B. Additionally, fluconazole is used as a single drug for the consolidation and maintenance phases.[3,12] In Colombia, furthermore, fluconazole has been reported as monotherapy in the induction phase in ∼13% of cases.[13] In general, this agrees with the increase of fluconazole resistance occurring globally among *Cryptococcus* isolates.[7] Finding that VGII isolates have concomitant reduced susceptibility to fluconazole and voriconazole indicates, in addition, that treatment options could be further reduced in some cases. The identification of one 5-flucytosine non-wild-type VGI isolate in Colombia and the reduced susceptibility of this molecular type to this antifungal drug, which is rarely prescribed in the country, is noteworthy. Although high MICs to 5-flucytosine are rarely reported, prevalence of resistance of up to 7% has been noted in some countries in patients with prolonged exposure to the antifungal or with monotherapy.[7,14] In addition, unlike other antifungal drugs, 5-flucytosine is almost exclusively limited to the treatment of cryptococcal meningitis, hence resistance acquired for previous exposure, as it occurs with azoles, is unlikely.[15] As reported in other countries, VGII isolates from Colombia have higher MICs to fluconazole and other azoles than isolates of other molecular types.[16–18] While the association between high MICs to azoles reported in VGII, with the treatment doses and clinical prognosis of patients have not been established, there is special interest in this molecular type. In Canada and USA, VGII has been implied in outbreaks of infection and in some regions in Colombia, this molecular type spreads clonally.[19–21]

In conclusion, this study supports the importance of examining reduced drug susceptibility or resistance of cryptococcal isolates when treating cryptococcosis, and of establishing the molecular types of the isolates. MIC determination is essential, with special attention to the *in vitro* activity of fluconazole. This is a drug with long-term usage that is prescribed in the three-part strategy of induction, consolidation, and maintenance, as such, the emergence of fluconazole resistance is likely. Reduced susceptibility to itraconazole, voriconazole, and posaconazole must be

Table 1. Distribution of the minimal inhibitory concentrations (MIC) of clinical *Cryptococcus gattii* isolates from Colombia, according to the molecular type. Modes are underlined. Non-wild-type isolates, which were determined with results obtained with the Clinical and Laboratory Standards Institute method, as referenced elsewhere,[10,11] are highlighted.

				Number of isolates at MIC value (μg/ml)													
Antifungal	MT	n	GM (μg/ml)	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Amphotericin B	VGI	4	0.297				3	1									
	VGII	26	0.326			4	10	10	2								
	VGIII	12	0.236			3	7	2									
	All	42	0.295			7	20	13	2								
5-flucytosine	VGI	4	4							1	2	1					
	VGII	26	1.846				1	-	6	14	4	1					
	VGIII	12	2.520					1	1	3	7						
	All	42	2.172				1	1	7	18	13	2					
Fluconazole	VGI	4	4.757								3	1					
	VGII	26	17.332								1	5	15	3	-	1	1
	VGIII	12	8.476								2	7	3				
	All	42	12.491								6	13	18	3	-	1	1
Itraconazole	VGI	4	0.063		4												
	VGII	26	0.132	1	4	14	6	1									
	VGIII	12	0.063	3	6	3											
	All	42	0.099	4	14	17	6	1									
Voriconazole	VGI	4	0.074		3	1											
	VGII	26	0.163	1	3	10	10	1	1								
	VGIII	12	0.074	3	5	2	2										
	All	42	0.121	4	11	13	12	1	1								
Posaconazole	VGI	4	0.125			4											
	VGII	26	0.186		2	10	11	3									
	VGIII	12	0.118		4	5	3										
	All	42	0.157		6	19	14	3									

GM: Geometric mean.

Table 2. Comparison of minimal inhibitory concentrations (MIC) values for significant differences between molecular types of clinical *Cryptococcus gattii* isolates from Colombia.

	Comparison of GM (μg/ml) [*P*-value]	
Antifungal	VGI vs. VGII	VGII vs. VGIII
5-flucytosine	4 vs. 1.846 [0.0427*]	Ns
Fluconazole	4.757 vs. 17.332 [0.0011**]	17.332 vs. 8.476 [0.0022**]
Itraconazole	0.063 vs. 0.132 [0.0059**]	0.132 vs. 0.063 [0.0006***]
Voriconazole	0.074 vs. 0.163 [0.0257*]	0.163 vs. 0.074 [0.0046**]
Posaconazole	Ns	0.186 vs. 0.118 [0.0358*]

GM: Geometric mean; ns: statistically non-significant $P > 0.05$.
$^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

surveyed as well since these azoles are salvage consolidation therapies when fluconazole is not available. In general, broaden investigations of the genetic basis of reduced antifungal susceptibilities phenotypes in *C. gattii* are warranted, as it is still unknown what causes the differences in the *in vitro* antifungal susceptibilities of the molecular types and the occurrence of resistance when there has not been previous exposure to the drugs.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References

1. Kwon-Chung KJ, Fraser JA, Doering TL et al. *Cryptococcus neoformans* and *Cryptococcus gattii*, the etiologic agents of cryptococcosis. *Cold Spring Harb Perspect Med*. 2014; 4: a019760.
2. Chen SC, Meyer W, Sorrell TC. *Cryptococcus gattii* infections. *Clin Microbiol Rev*. 2014; 27: 980–1024.
3. Perfect JR, Dismukes WE, Dromer F et al. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2010; 50: 291–322.
4. Lizarazo J, Escandón P, Agudelo CI, Firacative C, Meyer W, Castañeda E. Retrospective study of the epidemiology and clinical manifestations of *Cryptococcus gattii* infections in Colombia from 1997–2011. *PLoS Negl Trop Dis*. 2014; 8: e3272.
5. Cogliati M. Global molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii*: an atlas of the molecular types. *Scientifica (Cairo)*. 2013; 2013: 675213.
6. Farrer RA, Chang M, Davis MJ et al. A new lineage of *Cryptococcus gattii* (VGV) discovered in the Central Zambezian Miombo woodlands. *mBio*. 2019; 10: e02306–19.
7. Bermas A, Geddes-McAlister J. Combatting the evolution of antifungal resistance in *Cryptococcus neoformans*. *Mol Microbiol*. 2020; 114: 721–734.
8. Firacative C, Roe CC, Malik R et al. MLST and whole-genome-based population analysis of *Cryptococcus gattii* VGIII links clinical, veterinary and environmental strains, and reveals divergent serotype specific sub-populations and distant ancestors. *PLoS Negl Trop Dis*. 2016; 10: e0004861.
9. Clinical and Laboratory Standards Institute (CLSI). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*, 3rd ed.; CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA, USA. 2008.
10. Espinel-Ingroff A, Aller AI, Canton E et al. *Cryptococcus neoformans-Cryptococcus gattii* species complex: an international study of wild-type susceptibility endpoint distributions and epidemiological cutoff values for fluconazole, itraconazole, posaconazole, and voriconazole. *Antimicrob Agents Chemother*. 2012; 56: 5898–5906.
11. Espinel-Ingroff A, Chowdhary A, Cuenca-Estrella M et al. *Cryptococcus neoformans-Cryptococcus gattii* species complex: an international study of wild-type susceptibility endpoint distributions and epidemiological cutoff values for amphotericin B and flucytosine. *Antimicrob Agents Chemother*. 2012; 56: 3107–3113.
12. Mourad A, Perfect JR. The war on cryptococcosis: a review of the antifungal arsenal. *Mem Inst Oswaldo Cruz*. 2018; 113: e170391.
13. Escandon P, Lizarazo J, Agudelo CI, Castaneda E. Cryptococcosis in Colombia: compilation and analysis of data from laboratory-based surveillance. *J Fungi (Basel)*. 2018; 4: 32.
14. Padda IS, Parmar M. Flucytosine. *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing, 2021.
15. Pfaller MA. Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. *Am J Med*. 2012; 125(1 Suppl):S3–S13.
16. Chong HS, Dagg R, Malik R, Chen S, Carter D. *In vitro* susceptibility of the yeast pathogen *Cryptococcus* to fluconazole and other azoles varies with molecular genotype. *J Clin Microbiol*. 2010; 48: 4115–4120.
17. Lockhart SR, Iqbal N, Bolden CB et al. Epidemiologic cutoff values for triazole drugs in *Cryptococcus gattii*: correlation of molecular type and *in vitro* susceptibility. *Diagn Microbiol Infect Dis*. 2012; 73: 144–148.
18. Trilles L, Meyer W, Wanke B, Guarro J, Lazera M. Correlation of antifungal susceptibility and molecular type within the *Cryptococcus neoformans*/*C. gattii* species complex. *Med Mycol*. 2012; 50: 328–332.
19. Kidd SE, Hagen F, Tscharke RL et al. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). *Proc Natl Acad Sci USA*. 2004; 101:17258–17263.
20. Byrnes EJ, Bildfell RJ, Frank SA, Mitchell TG, Marr KA, Heitman J. Molecular evidence that the range of the Vancouver Island outbreak of *Cryptococcus gattii* infection has expanded into the Pacific Northwest in the United States. *J Infect Dis*. 2009; 199: 1081–1086.
21. Firacative C, Torres G, Meyer W, Escandon P. Clonal dispersal of *Cryptococcus gattii* VGII in an endemic region of cryptococcosis in Colombia. *J Fungi (Basel)*. 2019; 5: 32.