In vitro antifungal susceptibility profiles and genotypes of 308 clinical and environmental isolates of *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii* serotype B from north-western India

Anuradha Chowdhary,¹ Harbans Singh Randhawa,¹ Gandhi Sundar,¹ Shallu Kathuria,¹ Anupam Prakash,¹ Ziauddin Khan,² Sheng Sun³ and Jianping Xu³

Correspondence

Anuradha Chowdhary
dranuradha@hotmail.com

¹Department of Medical Mycology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi 11007, India

²Mycology Reference Laboratory, Department of Microbiology, Faculty of Medicine, Kuwait University, Safat, Kuwait

³Institute of Infectious Disease Research, Michael G. DeGroote School of Medicine, and Department of Biology, McMaster University, Hamilton, ON L8S 4K1, Canada

Cryptococcus neoformans and *Cryptococcus gattii* are aetiological agents of cryptococcosis, a major opportunistic systemic mycosis of increasing global importance. This study reports the antifungal susceptibility profiles of clinical and environmental isolates of *C. neoformans* var. *grubii*, genotype VNI/AFLP1 ($n=246$), and *C. gattii* serotype B, genotype VGI/AFLP4 ($n=62$), originating from patients and environmental sources in north-western India. All of the *C. neoformans* var. *grubii* and *C. gattii* isolates were mating type α . Using the broth microdilution method, both species were found to be susceptible to the antifungals tested except for two clinical *C. neoformans* var. *grubii* isolates that were resistant to 5-flucytosine (MIC $>64 \mu\text{g ml}^{-1}$). Data on the geometric mean of MICs revealed that *C. gattii* was significantly less susceptible than *C. neoformans* var. *grubii* to fluconazole, itraconazole and voriconazole ($P<0.0001$). In addition, the MIC₉₀ of *C. gattii* was twofold higher than that of *C. neoformans* var. *grubii* for fluconazole, itraconazole and voriconazole. However, no statistically significant difference in susceptibility of the two *Cryptococcus* species was observed against amphotericin B and 5-flucytosine. Furthermore, the environmental *C. neoformans* var. *grubii* isolates were significantly less susceptible to fluconazole, itraconazole and 5-flucytosine ($P<0.0001$) than the clinical isolates. A continued surveillance of antifungal susceptibility of clinical and environmental strains of *C. neoformans* and *C. gattii* is desirable to monitor the emergence of any resistant strains in order to ensure more successful therapy of cryptococcosis.

Received 13 December 2010

Accepted 7 March 2011

INTRODUCTION

Cryptococcosis is a life-threatening, opportunistic fungal infection of worldwide distribution, including India, especially in the human immunodeficiency virus (HIV) positive population (Casadevall & Perfect, 1998; Chakrabarti *et al.*, 2000; Khanna *et al.*, 2000; Lakshmi

et al., 2007; Thakur *et al.*, 2008). It has two major aetiological agents, namely *Cryptococcus neoformans* and *Cryptococcus gattii*. *C. neoformans* has serotypes A, D and AD, whereas *C. gattii* has serotypes B and C. The strains belonging to serotype A represent *C. neoformans* var. *grubii*, whereas those of serotype D represent *C. neoformans* var. *neoformans*. *C. neoformans* and *C. gattii* differ significantly with regard to their geographical distribution and ecological niches (Casadevall & Perfect, 1998; Kwon-Chung *et al.*, 2002). Based on molecular studies, using PCR fingerprinting, amplified fragment length polymorphism (AFLP) analysis, analysis of the orotidine monophosphate pyrophosphorylase (*URA5*) and phospholipase (*PLBI*)

Abbreviations: AFLP, amplified fragment length polymorphism; CLSI, Clinical and Laboratory Standards Institute; GM, geometric mean.

The GenBank/EMBL/DDBJ accession numbers for the *URA5* sequences of the *C. gattii* isolates determined in this study are FJ604536–FJ604593 and HM059634–HM059637.

genes by RFLP and multilocus sequence typing, *C. neoformans* and *C. gattii* have been further classified into several distinct genotypes: VNI/AFLP1 and VNII/AFLP1A/AFLP1B (*C. neoformans* var. *grubii*, serotype A), VNIV/AFLP2 (*C. neoformans* var. *neoformans*, serotype D), VNIII/AFLP3 (hybrid serotype AD), VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5, VGIV/AFLP7 and AFLP10 (*C. gattii*, serotype B/C). In addition, hybrids of *C. neoformans* var. *neoformans* and *C. gattii* and of *C. neoformans* var. *grubii* and *C. gattii* belong to genotypes AFLP8 and AFLP9, respectively.

The vast majority of cryptococcal infections, particularly in immunocompromised patients, are caused by *C. neoformans*, whereas *C. gattii* accounts for a smaller proportion of cases, often occurring in immunocompetent patients in tropical and subtropical regions. However, in the past decade, a more virulent genotype of *C. gattii*, VGIIa/VGIIc, has emerged as a primary pathogen on Vancouver Island and its adjoining areas in Canada and the USA, indicating extension of its geographical domain to the temperate climate (Kidd *et al.*, 2004; Byrnes *et al.*, 2010). The outbreak of human and animal cryptococcosis on Vancouver Island due to *C. gattii* indicated that exposure to environmental sources such as colonized trees and soil led to pulmonary and disseminated cryptococcosis. In India, we have reported a widespread colonization of decayed wood inside trunk hollows of diverse tree species by *C. neoformans* var. *grubii* and *C. gattii* serotype B (Randhawa *et al.*, 2006, 2008; Hiremath *et al.*, 2008). The objective of this study was to compare antifungal susceptibility profiles of clinical isolates with those of environmental isolates of *C. neoformans* var. *grubii* and *C. gattii* serotype B originating from decayed wood of diverse tree species and from their surrounding soil in north-western India.

METHODS

Fungal isolates. Three hundred and eight isolates, comprising 246 *C. neoformans* var. *grubii* and 62 *C. gattii* serotype B, originating from clinical and environmental sources were included in the study. All of the *C. neoformans* isolates ($n=246$) were serotype A, genotype VNI/AFLP1 and mating type α , whereas all of the *C. gattii* isolates belonged to serotype B, genotype VGI/AFLP4 and mating type α . Of the 246 *C. neoformans* var. *grubii* isolates, 160 were clinical, originating from 130 patients, and the remaining 86 were from decayed wood of trees and soil. Among the 62 *C. gattii* isolates, 60 were from environmental and two were from clinical sources. The clinical isolates had been collected from 2002 to 2009 from various hospitals in the Union Territories of Delhi and Chandigarh, and the states of Uttar Pradesh and Himachal Pradesh. Of the 162 clinical isolates, 140 (86%) originated from cerebrospinal fluid, 11 (7%) from blood, seven (4%) from sputum, three (2%) from urine and one (1%) from an endotracheal secretion. Of these, 109 (67%) isolates were obtained from initial clinical specimens from patients with cryptococcosis, whereas 53 (33%) were repeat isolations (two or more isolates from an individual patient collected at least 1 month apart) after the patients were put on amphotericin B or fluconazole therapy. The number of isolates obtained from 92 HIV-positive patients was 116 (72%), whereas 21 isolates (13%) were from 15 HIV-negative patients. The HIV status

of the 25 patients yielding the remaining isolates of *C. neoformans* var. *grubii* was unknown.

Among the 146 environmental isolates, 86 (59%) were *C. neoformans* var. *grubii* and 60 (41%) were *C. gattii* serotype B. They had been collected and stocked during our investigation of decayed wood inside trunk hollows of a wide spectrum of tree species and soil samples in proximity to some of the positive trees (Randhawa *et al.*, 2006, 2008; Hiremath *et al.*, 2008). Also included for comparison were eight reference strains procured from global culture collections. These were *C. neoformans* (serotype A) ATCC 90112, *C. gattii* (serotype B) CBS1930 and CDC 3175 (Centers for Disease Control and Prevention, Atlanta), *C. gattii* (serotype B) B4495 and B4499 and *C. gattii* (serotype C) B4546, JF109 and JF101 (McMaster University, Hamilton, Ontario, Canada).

All isolates of *C. neoformans* var. *grubii* and *C. gattii* tested here were identified by standard mycological procedures (Randhawa *et al.*, 2006, 2008; Hiremath *et al.*, 2008). Their genotypes were determined based on two methods: (i) PCR fingerprinting using (GACA)₄ and M13 phage core sequences as single primers (Meyer *et al.*, 1999); and (ii) DNA sequences at the *URA5* locus (Meyer *et al.*, 2009). The mating type of isolates was determined by PCR amplification, using primer pairs designed from the sequences of the mating type-specific *STE12* and *STE20* genes (Hiremath *et al.*, 2008).

Antifungal susceptibility testing. *In vitro* antifungal susceptibility testing was carried out using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (CLSI, 2008). The antifungal drugs tested were amphotericin B (Sigma), fluconazole, voriconazole (Pfizer), itraconazole (LeePharma) and 5-flucytosine (Sigma). Stock solutions were prepared in water (fluconazole and 5-flucytosine) or DMSO (itraconazole, voriconazole and amphotericin B). Further dilutions of each antifungal agent were prepared with RPMI 1640 with glutamine without bicarbonate (Sigma), buffered to pH 7 with 0.165 M MOPS (Sigma). The drug dilutions were dispensed in 96-well microdilution plates, sealed and frozen at -70°C until needed. The final concentrations of the drugs ranged from 0.125 to 64 $\mu\text{g ml}^{-1}$ for fluconazole and 5-flucytosine, 0.03 to 16 $\mu\text{g ml}^{-1}$ for amphotericin B and 0.015 to 8 $\mu\text{g ml}^{-1}$ for itraconazole and voriconazole. The yeast inoculum was adjusted to a concentration of 0.5×10^3 – 2.5×10^3 cells ml^{-1} in RPMI 1640 as measured by spectrophotometry, and an aliquot of 0.1 ml was added to each well containing various concentrations of the antifungal drugs. Drug-free and yeast-free controls were included, and microplates were incubated at 35°C for 72 h.

Following the CLSI recommendations, two quality-control strains, *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019), were used with each test. The reproducibility of the *in vitro* results was assessed by determining MICs for all strains twice on two different days. The MIC end points were read visually after 72 h and defined for fluconazole, voriconazole, itraconazole and 5-flucytosine as the lowest drug concentration that caused a prominent decrease in growth (50%) compared with the controls. For amphotericin B, the MIC was defined as the lowest concentration at which there was 100% inhibition of growth compared with the drug-free control wells.

Statistical analysis. Statistical differences between MIC values of various groups of strains were assessed using the Mann–Whitney test. Statistical significance was defined as $P<0.05$. Statistical analyses were performed with GraphPad Prism version 5.00 (GraphPad Software).

RESULTS

The results of antifungal susceptibility testing of *C. neoformans* var. *grubii*, genotype VNI/AFLP1, and *C. gattii*

Table 1. *In vitro* antifungal susceptibilities of clinical and environmental isolates of *C. neoformans* var. *grubii* (*n*=246) and *C. gattii* (*n*=62) to amphotericin B, 5-flucytosine and some azolesMIC values are given in µg ml⁻¹.

Test species and source	Amphotericin B				Fluconazole				Itraconazole				Voriconazole				5-Flucytosine			
	GM	MIC range	MIC ₅₀	MIC ₉₀	GM	MIC range	MIC ₅₀	MIC ₉₀	GM	MIC range	MIC ₅₀	MIC ₉₀	GM	MIC range	MIC ₅₀	MIC ₉₀	GM	MIC range	MIC ₅₀	MIC ₉₀
<i>C. neoformans</i> var. <i>grubii</i>																				
Clinical (<i>n</i> =160)	0.235	0.031–1	0.250	0.5	2.190	0.5–8	2	4	0.099	0.031–0.250	0.125	0.250	0.053	0.015–0.125	0.062	0.125	1.450	0.031–64	2	4
Environmental (<i>n</i> =86)	0.232	0.062–0.5	0.250	0.5	3.639	2–8	4	4	0.142	0.031–0.5	0.125	0.250	0.062	0.031–0.250	0.062	0.125	3.785	1–16	4	8
Total (<i>n</i> =246)	0.235	0.031–1	0.250	0.5	2.614	0.5–8	2	4	0.112	0.031–0.5	0.125	0.250	0.056	0.015–0.125	0.062	0.125	2.033	0.031–64	2	8
<i>C. gattii</i> serotype B																				
Clinical (<i>n</i> =2)	0.125	0.125	0.125	0.125	1.545	1–2	1.5	1.9	0.062	0.062	0.062	0.062	0.062	0.015–0.125	0.07	0.114	0.773	0.5–1	0.75	0.95
Environmental (<i>n</i> =60)	0.256	0.125–1	0.250	0.250	7.379	2–16	8	8	0.256	0.125–0.5	0.250	0.5	0.143	0.062–0.250	0.125	0.250	2	1–4	2	2
Total (<i>n</i> =62)	0.250	0.125–1	0.250	0.250	6.996	1–16	8	8	0.244	0.062–0.5	0.250	0.5	0.138	0.015–0.250	0.125	0.250	1.934	0.5–4	2	2

serotype B, genotype VGI/AFLP4, are summarized in Table 1. All of the isolates of *C. neoformans* var. *grubii* and *C. gattii* showed low MICs for all the antifungals tested except for two clinical isolates of *C. neoformans* var. *grubii*, which had high MICs against 5-flucytosine (MIC $>64 \mu\text{g ml}^{-1}$). However, there were some notable differences in antifungal susceptibility of the two species and within each species depending on the origin of strains from environmental or clinical sources. Specifically, the geometric means (GMs) of MICs for the *C. gattii* serotype B sample were significantly higher than those of *C. neoformans* var. *grubii* for fluconazole (GM 6.996 vs $2.614 \mu\text{g ml}^{-1}$, $P<0.0001$), itraconazole (GM 0.244 vs $0.112 \mu\text{g ml}^{-1}$, $P<0.0001$) and voriconazole (GM 0.138 vs $0.056 \mu\text{g ml}^{-1}$, $P<0.0001$). Similarly, the MIC₉₀ values for *C. gattii* were twofold higher than those for *C. neoformans* var. *grubii* for fluconazole (8 vs $4 \mu\text{g ml}^{-1}$), itraconazole (0.5 vs $0.25 \mu\text{g ml}^{-1}$) and voriconazole (0.25 vs $0.125 \mu\text{g ml}^{-1}$). However, no statistically significant difference in susceptibility of the two *Cryptococcus* species was observed against amphotericin B and 5-flucytosine. Interestingly, in comparison with clinical isolates, the environmental *C. neoformans* var. *grubii* isolates exhibited significantly reduced susceptibility to fluconazole (GM 3.639 vs $2.190 \mu\text{g ml}^{-1}$, $P<0.0001$), itraconazole (GM 0.142 vs $0.099 \mu\text{g ml}^{-1}$, $P<0.0001$) and 5-flucytosine (GM 3.785 vs $1.450 \mu\text{g ml}^{-1}$, $P<0.0001$).

Of the 53 serial isolates of *C. neoformans* var. *grubii* in this study, collected at least 1 month apart from 23 patients, four serial isolates obtained from four patients receiving antifungal therapy of amphotericin B for 3 weeks and followed by fluconazole prophylaxis (400 mg daily) showed a fourfold increase in fluconazole MICs over a period of 1.5–2.5 months. However, the MIC values did not exceed $4 \mu\text{g ml}^{-1}$. Likewise, one serial *C. neoformans* var. *grubii* isolate from a patient showed a fourfold increase in itraconazole MIC but not in fluconazole MIC. A similar increase in amphotericin B MICs was found, which ranged from fourfold in two serial isolates to eightfold in four serial isolates over 1–3 months for strains originating from six patients who had received this antifungal drug for 3 weeks. Here again, the increase in the amphotericin B MICs did not exceed $1 \mu\text{g ml}^{-1}$. Interestingly, three serial isolates from three patients showed a fourfold increase in 5-flucytosine MICs, although none of the patients had received 5-flucytosine previously.

DISCUSSION

This study is noteworthy for documenting the antifungal susceptibility profiles of the highest number of *C. gattii* genotype VGI/AFLP4 isolates investigated in any study reported so far from India, employing the microbroth dilution method. An earlier study from India reported only the fluconazole susceptibility profiles and genotypes of 57 clinical isolates, comprising 51 *C. neoformans* var. *grubii*, genotype VNI/AFLP1, one *C. neoformans* var. *neoformans*, genotype VNIV/AFLP2, and five *C. gattii*, genotype VGII/

AFLP6 (Jain *et al.*, 2005). The reported MICs ranged from 8 to $16 \mu\text{g ml}^{-1}$ for *C. neoformans* var. *grubii* and 2 to $64 \mu\text{g ml}^{-1}$ for *C. gattii*, whereas in the present study none of the 160 clinical and 86 environmental isolates of *C. neoformans* var. *grubii* revealed MICs $>8 \mu\text{g ml}^{-1}$ for *C. neoformans* var. *grubii* (range 0.5– $8 \mu\text{g ml}^{-1}$) and 1– $16 \mu\text{g ml}^{-1}$ for *C. gattii* serotype B. The low MICs observed in our clinical isolates of both species may be attributed to the fact that the majority originated from patients without history of exposure to fluconazole.

The results demonstrated that *C. gattii* isolates were significantly less susceptible to azoles than *C. neoformans* var. *grubii*, which agrees with several previous reports (Fernandes *et al.*, 2003; Trilles *et al.*, 2004; Khan *et al.*, 2007, 2009; Gomez-Lopez *et al.*, 2008). However, there is divergence of results concerning the antifungal susceptibility patterns of the two species in some other studies, which reported no such difference (Chen *et al.*, 2000; Calvo *et al.*, 2001; Morgan *et al.*, 2006; Tay *et al.*, 2006; Thompson *et al.*, 2009). This divergence in results may be due to a possible lack of uniformity in the methodologies of testing adopted by different investigators. Although the Etest has been recommended as a good alternative to the CLSI microbroth dilution method for antifungal susceptibility of yeasts, the results obtained with *Cryptococcus* species have been inconsistent (Khan *et al.*, 2007). It should be pointed out that the reported discrepancies in results pertain to antifungal drugs that inhibit or bind ergosterol in the cell membrane. Therapy of cryptococcosis due to *C. gattii* with these antifungals has also been reported to be more difficult than therapy of the disease caused by *C. neoformans* (Mitchell *et al.*, 1995; Speed & Dunt, 1995). Attention should be given to the report by Varma & Kwon-Chung (2010), showing that 86 % of the *C. gattii* strains expressed a heteroresistance level of $>16 \mu\text{g ml}^{-1}$ to fluconazole compared with 46 % of *C. neoformans* strains. They found that all of the clinical isolates not exposed to azoles as well as the environmental strains manifested heteroresistance to fluconazole. Furthermore, this heteroresistance of the test strains was an intrinsic character that was associated with their virulence. Thus, the inherently higher level of heteroresistance to fluconazole of *C. gattii* strains may be another factor that influences the MICs of the strains resulting in the variability of results of *in vitro* antifungal susceptibility reported in different studies.

As reported previously (Yildiran *et al.*, 2000; van Duin *et al.*, 2004; Souza *et al.*, 2005; Khan *et al.*, 2007), voriconazole exhibited the highest inhibitory activity against the isolates of *C. neoformans* var. *grubii* (GM $0.056 \mu\text{g ml}^{-1}$) and *C. gattii* (GM $0.138 \mu\text{g ml}^{-1}$). Itraconazole MICs were in the range of 0.25– $0.5 \mu\text{g ml}^{-1}$ in 84 % of *C. gattii* serotype B and in 14 % of *C. neoformans* var. *grubii* isolates. This is in agreement with the results of Iqbal *et al.* (2010), who tested 43 clinical isolates of *C. gattii* from patients in Oregon, USA. Twenty-three per cent of their isolates had itraconazole MICs $>1 \mu\text{g ml}^{-1}$, whereas 55.8 % revealed MICs in the range of 0.25– $0.5 \mu\text{g ml}^{-1}$.

Interestingly, their VGI and VGIII isolates had comparatively low fluconazole MICs, whilst the majority with MICs of 16–32 $\mu\text{g ml}^{-1}$ were of subtype VGIIc. Similarly, all of our VGI/AFLP4 *C. gattii* isolates revealed MICs of $\leq 16 \mu\text{g ml}^{-1}$, consistent with the report by Hagen *et al.* (2010) on *C. gattii*, which showed lower MICs for AFLP4/VGI isolates (1.401 and 2.467 $\mu\text{g ml}^{-1}$) versus the higher MICs for AFLP6/VGII isolates (4.961 and 5.638 $\mu\text{g ml}^{-1}$) against 5-flucytosine and fluconazole, respectively. Concerning the susceptibility to 5-flucytosine, <2% of *C. neoformans* isolates have been reported as resistant to this drug prior to treatment (Scholer & Polak, 1984), which is comparable to our results of 1.2% (2/162) for clinical *C. neoformans* var. *grubii* isolates. However, concern about the emergence of resistance during treatment with this drug alone has led to its use in combination with amphotericin B in patients with cryptococcosis (Perfect *et al.*, 2010). All of the patients whose serial isolates showed an increase in azole and amphotericin B MICs were HIV-positive. It may be added in this context that relapses in patients with AIDS-associated cryptococcosis are often due to deterioration of the host immune function rather than to an increase in MICs (Witt *et al.*, 1996). However, a rising MIC of fluconazole has been implicated in clinical relapse in patients with AIDS-associated cryptococcal meningitis (Paugam *et al.*, 1994; Birley *et al.*, 1995; Currie *et al.*, 1995; Armengou *et al.*, 1996; Berg *et al.*, 1998; Davey *et al.*, 1998; Aller *et al.*, 2000).

Our significantly lower susceptibility of environmental *C. neoformans* var. *grubii* isolates to fluconazole, itraconazole and 5-flucytosine compared with that of the clinical isolates is in contrast to the findings of some investigators who found that antifungal susceptibility was not related to the clinical or environmental origin of strains (Franzot & Hamdan, 1996; Moraes *et al.*, 2003; Trilles *et al.*, 2004). Of relevance here is the report by Soares *et al.* (2005) stating that a solitary isolate of *C. neoformans* var. *grubii* from pigeon excreta was resistant to fluconazole (MIC 64 $\mu\text{g ml}^{-1}$). Likewise, in another report from Brazil, one of their environmental isolates of *C. neoformans* var. *neoformans* was found to be resistant to itraconazole and three additional isolates exhibited high MICs of 16–32 $\mu\text{g ml}^{-1}$ against fluconazole (Costa *et al.*, 2010). Furthermore, a fluconazole-resistant strain isolated from an immunocompetent patient without exposure to this azole has also been reported, indicating the existence of primary resistance in environmental strains to fluconazole (Orni-Wasserlauf *et al.*, 1999). Keeping in mind these emerging reports of resistance in environmental strains, continued surveillance for the emergence of antifungal resistance in clinical and environmental strains of *C. neoformans* and *C. gattii* is desirable for more successful therapy of cryptococcosis.

ACKNOWLEDGEMENTS

Acknowledgement is made to the Indian National Science Academy, New Delhi, for the award of a Hony Scientist position to H. S. R., and

to Dr M. Rahman for assistance with statistical analysis of the data. This work was financially supported by the Department of Science and Technology, Government of India (F. no. SR/SO/HS-62/2008) and the Indian Council of Medical Research, New Delhi (HIV/50/107/2008).

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