Research Paper

Antifungal susceptibility of clinical and environmental *Cryptococcus neoformans* and *Cryptococcus gattii* isolates in Jabalpur, a city of Madhya Pradesh in Central India

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Abstract

In this study, we present antifungal susceptibility data of clinical and environmental isolates of Central Indian *Cryptococcus neoformans* (Serotype A, n = 8 and n = 50 respectively) and *Cryptococcus gattii* (Serotype B, n = 01 and n = 04 respectively). Susceptibilities to fluconazole, itraconazole and ketoconazole were determined by using NCCLS broth micro-dilution methodology. The total number of resistant strains for fluconazole in case of *C. neoformans* and *C. gattii* showed a significant difference by using chi-square test (p < 0.05*), while considering fisher's exact p value was non-significant (p > 0.05). However, the total number of resistant strains for itraconazole and ketoconazole was not found statistically significant. A comparison of geometric means of clinical and environmental strains of *C. gattii* and *C. neoformans* was not found statistically significant using student 't' test (p value > 0.05 NS). Though less, the antifungal data obtained in this study suggests that primary resistance among environmental and clinical isolates of *C. neoformans* and *C. gattii* against tested antifungal was present and *C. gattii* comparatively was less susceptible than *C. neoformans* var. *grubii* isolates to fluconazole than to itraconazole and ketoconazole. A continuous surveillance of antifungal susceptibility of clinical and environmental isolates of *C. neoformans* and *C. gattii* is desirable to monitor the emergence of any resistant strains for better management of cryptococcosis patients.

Key words: *Cryptococcus neoformans*, *Cryptococcus gattii*, minimum inhibitory concentration, azoles, Central India.

Introduction

Cryptococcus neoformans (Serotype A, D, AD) and *C. gattii* (Serotype B and C) are opportunistic fungal pathogens that cause cryptococcosis predominantly in immuno-compromised patients with AIDS, and also in immuno-compromised patients with the underlying predisposing factors, such as haematological malignancies and organ transplantation (Mitchell and Perfect, 1995). Epidemiologically these species differ from each other mainly in geographic distribution, natural habitat, host infectivity and genetics (Casadevall and Perfect, 1998; Kwon-Chung and Varma, 2006). On the basis of molecular studies *C. neoformans* and *C. gattii* have been classified into several distinct genotypes.

Major antifungals available for therapy of cryptococcosis are limited to amphotericin B, 5 - flurocytosine and fluconazole. However, side effects associated with amphotericin-B and flurocytosine restrict their use. Azoles, on the other hand, offer a safer and efficacious option. Yet, one major concern with azole use has been the possible emergence of clinical resistance (Friese *et al.*, 2001). Clinical resistance may be the result of infection with a resistant strain, or a relapse of infection due to natural selective pressure exerted on the pathogen by routine, inappropriate or excessive use of antimicrobial drugs are major factors in

the development of antimicrobial resistance (Archibald *et al.*, 2004). In tropical developing countries, availability of drugs without prescription, sub-optimal therapeutic regimes, blind empirical prescribing practices that are not epidemiologically directed and lack of laboratory capacity or skilled personnel for susceptibility testing contribute to the antimicrobial resistance (Shears, 2001).

Although numerous studies have examined bacterial and mycobacterial resistance in tropics, less is known about the susceptibility profiles of medically important fungi to antifungal drugs (Davey *et al.*, 1998; Pfaller *et al.*, 1998; Pfaller *et al.*, 1999;). In developing countries like India, because of limited resources or cost restrictions, the continuous surveillance for resistance to available antifungal drugs treatment is essential for appropriate patient care and improved patient's outcome.

The purpose of the present investigation was to analyse the in vitro susceptibilities of clinical and environmental isolates of *C. neoformans* and *C. gattii* to three azole antifungal drugs. The reference micro-dilution method (NCCLS-M27A) proposed by the National Committee for Clinical Laboratory Standards (presently CLSI-Clinical and Laboratory Standards Institute) was used for susceptibility testing.

Materials and Methods

Fungal strains

A total of 63 *Cryptococcus* isolates was used, in the study of which 9 were recovered from blood, urine, sputum and cerebrospinal fluid of HIV +ve patients. Out of these 9 clinical isolates, 8 were *C. neoformans* (Serotype A) and 1 was *C. gattii*. There were 54 environmental isolates, 50 were *C. neoformans* (Serotype A) and 4 were isolates of *C. gattii.* These isolates were isolated from the trunk hollows of (*Tamarindus indica*, *Mangifera indica* and *Syzygium cumini*) living trees in Jabalpur, Madhya Pradesh in Central India. These isolates were isolated and cultured over a period of seven years at Jabalpur city (Nawange *et al.*, 2006; Grover *et al.*, 2007; Nawange *et al.*, 2011). For identification, the isolates were grown on niger seed agar medium for developing characteristic brown pigment. Physiological characteristics were determined as per Kwon-Chung and Bennett (Kwon-Chung and Bennett, 1992). Confirmation of *C. gattii* isolates was done by their ability to grow on canavanine glycine bromothymol blue medium, which was marked by a change in colour of the medium from greenish yellow to blue. Serotyping was done by crypto check kit. (Iatron laboratory Inc. Tokyo, Japan).

Minimum inhibitory concentration (MIC) determination

Antifungal susceptibilities were assessed using broth micro-dilution method of NCCLS/CLSI (National Committee for Clinical Laboratory Standards) M27-A guide-

lines. (NCCLS/CLSI-M27A). There were 58 *C. neoformans* and 5 *C. gattii* (environmental and clinical) strains. The MIC was determined for the three commonly used azoles (fluconazole, ketoconazole, itraconazole). A serial dilution was made from the stock solution of the antifungal agents to have final concentration ranges of 0.03-64 μg/mL for ketoconazole, fluconazole and itraconazole. The following antifungal drugs were used as assay powders: fluconazole (Flustan™; Dr. Reddy's Lab. Ltd.)™ - trademark under registration, itraconazole (Candistat; E Merck India Ltd.; Licensed user of T.M.), ketoconazole (Nizral-Johnson and Johnson Ethnor).

The yeast inocula were adjusted to a concentration of 0.5×10^3-2.5×10^3 cfu/mL in RPMI medium as measured by spectrophotometer, and an aliquot of 0.1 mL was added to each well containing various concentrations of antifungal drugs. These plates were incubated at 35 °C in air ambient incubator with positive (drug free well) and negative (broth well) control. End-points were read visually after 72 hours; the MIC's of ketoconazole, itraconazole and fluconazole were defined as the lowest concentration at which there was 50% inhibition of growth (*i.e.* slightly hazy) compared with that of drug free controls (NCCLS/CLSI M27-A). Interpretative break-points are as follows: Fluconazole - ≤ 8 μg/mL - susceptible, 16-32 μg/mL - intermediate, ≥ 64 μg/mL - resistant. Itraconazole- ≤ 0.125 μg/mL - susceptible, 0.25-0.5 μg/mL - intermediate, ≥ 1 μg/mL - resistant. Ketoconazole-0.0625 μg/mL - susceptible, ≥ 0.125 μg/mL - resistant. The results were tabulated and analyzed with the version 5.5 of the software WHONET.

Quality control strains

Quality control and reference strains incorporated during every testing batch of broth micro-dilution were *Candida albicans*-ATCC 90028, *C. tropicalis*-ATCC-750 and *C. glabrata*-90030. The MICs of these strains were compared with the published control limits and used to guide antifungal susceptibility testing and validation according to National Committee for Clinical Laboratory Standards (presently CLSI) guidelines.

Clinically relevant break points for azoles were not available for *C. neoformans*, hence tentative break points available for *C. albicans* have been used for interpretation. (Pfaller *et al.*, 1995; Rex *et al.*, 1996; Rodriquez-Tudela *et al.*, 1995).

Statistical analysis

The Chi-square test was applied to find significance between number of resistant *C. neoformans* and *C. gattii* strains for all the three azoles (fluconazole, itraconazole and ketoconazole). Fisher's exact p value was calculated when number of isolates was less. Student 't' test was applied to find the level of significance between clinical *vs.* environmental *C. neoformans* and *C. gattii* strains.

Results

Table 1 shows the MIC results of fluconazole drug for 58 *C. neoformans.* The results revealed that 8.6% were resistant, 31.1% were intermediately susceptible, 60.3% were susceptible, the MIC50 was 8 µg/mL, the MIC90 was 32 µg/mL, geometric mean was 6.93 µg/mL, and MIC range was 0.063-64 µg/mL. The MIC results for *C. gattii* (5) strains were as follows: 40% were resistant, 20% were intermediately susceptible, 40% were susceptible, the MIC50 was 16 µg/mL and the MIC90 64 µg/mL, geometric mean was 13.93 µg/mL and the MIC range was 2-64 µg/mL.

The MIC results for itraconazole for 58 *C. neoformans* strains exhibited that 5.2% were resistant, 24.1% were intermediately susceptible, 70.7% susceptible, 0.125 was the MIC50 µg/mL and the MIC90 0.5 µg/mL. The geometric mean was 0.124 µg/mL and MIC range 0.03-1 µg/mL. The MIC values for *C. gattii* (5) strains

against itraconazole showed no resistance and 40% were intermediately susceptible. However, 60% were susceptible, 0.125 µg/mL was the MIC50, the MIC90 was 0.5 µg/mL, geometric mean was 0.125 µg/mL and the MIC range was 0.03-0.5 µg/mL. The MIC results for drug ketoconazole for 58 *C. neoformans* strains exhibited 6.9% resistance, 55.2% were intermediately susceptible, 37.9% were susceptible, the MIC50 was 0.064 µg/mL, the MIC90 was 0.064 µg/mL, geometric mean was 0.051 µg/mL, the MIC range was 0.03-0.25 µg/mL. The MIC results of *C. gattii* (5) strains for ketoconazole exhibited 20% resistance, 20% were intermediately susceptible, 60% were susceptible, the MIC50 was 0.032 µg/mL, the MIC90 was 0.125 µg/mL, geometric mean was 0.047 µg/mL and the MIC range was 0.03-0.125 µg/mL.

Table 2 shows a comparative data of geometric means of *C. gattii* clinical (1) and environmental strains (4). The geometric mean of clinical *C. gattii* strains for fluconazole

Table 1 - Antifungal susceptibility results of three azoles against 58 *C. neoformans* and 5 *C. gattii* isolates in Jabalpur Madhya Pradesh Centaral India.

Antifungal agent & organism	No. Tested	MIC (µg/mL)						
		% R	% I-S	% S	MIC 50	MIC90	Geom. Mean	MIC Range
Fluconazole								
C. neoformans	58	8.6	31.1	60.3	8	32	6.93	0.063-64
C. gattii	5	40*	20	40	16	64	13.93	2-64
Itraconazole								
C. neoformans	58	5.2	24.1	70.7	0.125	0.5	0.124	0.03-1
C. gattii	5	0 NS	40	60	0.125	0.5	0.125	0.03-0.5
Ketoconazole								
C. neoformans	58	6.9	55.2	37.9	0.064	0.064	0.051	0.03-0.25
C. gattii	5	20 NS	20	60	0.032	0.125	0.047	0.03-0.125

* $\chi^2 = 4.589$, (p = 0.01609), p < 0.05* (Significant) and fisher's exact p value = 0.09077 (NS).
NS - Not significant; % R - Percent resistant; % I-S - Percent intermediate susceptible; % S - Percent susceptible.

Table 2 - A Comparisons of geometric means of clinical and environmental isolates of *C. gattii* and *C. neoformans* isolates in Jabalpur Madhya Pradesh Central India.

Organism	TYPE		FLU	ITR	KET
Cg (5)	Clin. (1)	Geometric Mean	16.00	0.125	0.03
	Env. (4)	Geometric Mean	13.45	0.124	0.05
		't' test p value	p > 0.05	p > 0.05	p > 0.05
Cn (58)	Clin. (8)	Geometric Mean	8.72	0.210	0.06
	Env. (50)	Geometric Mean	6.68	0.114	0.049
		't' test p value	p > 0.05	p > 0.05	p > 0.05
Total (63)	Clin. (9)	Geometric Mean	9.33	0.198	0.06
	Env. (54)	Geometric Mean	7.04	0.115	0.049
		't' test p value	p > 0.05	p > 0.05	p > 0.05

Cg - *Cryptococcus gattii* (B) ; *Cn* - *Cryptococcus neoformans* (A/D); Flu - Fluconazole; Itra - Itraconazole; Ket o -Ketoconazole; Clin. - Clinical; Env. - Environmental.

was 16 µg/mL, itraconazole 0.125 µg/mL and ketoconazole 0.03 µg/mL. Likewise, the geometric means of environmental isolates for fluconazole was 13.45 µg/mL, itraconazole 0.124 µg/mL and for ketoconazole, it was 0.05 µg/mL. On comparison, the geometric means for all the three drugs was not found statistically significant for both clinical and environmental strains.

Likewise, for *C. neoformans* clinical (8) strains, the geometric mean for fluconazole was 8.72 µg/mL, for itraconazole was 0.21 µg/mL and for ketoconazole, it was 0.06 µg/mL. For *C. neoformans* environmental isolates (50), the geometric mean for fluconazole was 6.68 µg/mL, for itraconazole, it was 0.114 µg/mL, and for ketoconazole, 0.049 µg/mL. In comparison, the geometric means for all the three drugs was not found statistically significant for both clinical and environmental strains.

The MIC geometric means of clinical strains (9) for fluconazole was 9.33, for itraconazole was 0.198, and for drug ketoconazole was 0.06. Similarly, the environmental strains (54) had a geometric mean of 7.04 for fluconazole, 0.115 for itraconazole and for drug ketoconazole 0.049 respectively. The comparison of geometric means for all the three drugs was not found statistically significant for total clinical (9) and environmental (54) strains.

Table 3, categorizes the sources of strains, the abundance of *C. neoformans* and *C. gattii,* with their mean MIC values and the MIC range of fluconazole, itraconazole and ketoconazole drugs. There were 54 environmental strains, out of which 50 were *C. neoformans* and 4 were *C. gattii.*

There were 6 *C. neoformans* strains isolated from soil contaminated by house hold garbage; 12.66 µg/mL was the mean MIC value for the drug fluconazole and 4-16 µg/mL was the range. The mean MIC for itraconazole was 0.22 µg/mL and 0.03-1 µg/mL was the MIC range. The mean MIC for the drug ketoconazole was 0.08 µg/mL and the MIC range was 0.03-0.25 µg/mL. There were 4 *C. neoformans* strains isolated from soil contaminated by hospital waste; 18.5 µg/mL was the mean MIC and 2-32 µg/mL was the range for the drug fluconazole. The mean MIC value for the drug itraconazole was 0.06 µg/mL and 0.03-0.125 µg/mL was the range. The mean MIC for the drug ketoconazole was 0.03 µg/mL. There were 2 *C. neoformans* strains isolated from soil soaked with human urine; 4.25 µg/mL was the mean MIC for the drug fluconazole and 0.5-8 µg/mL was the MIC range; 0.125 µg/mL was the mean MIC value for the drug itraconazole; 0.045 µg/mL was the mean MIC for the drug ketoconazole and the range was 0.03-0.06 µg/mL. There were 8 *C. neoformans* strains collected from soil contaminated by pigeon droppings; 25.75 µg/mL was the mean MIC value for the drug fluconazole and 2-64 µg/mL was the range for this drug. The mean MIC for the drug itraconazole was

0.16 µg/mL and 0.03-0.125 µg/mL was the range. The mean MIC value for the drug ketoconazole was 0.085 µg/mL and 0.03-0.06 µg/mL was the range for this drug. There were 6 strains of *C. neoformans* isolated from soil contaminated by other bird excreta; 14.01 µg/mL was the mean MIC value for fluconazole and 0.063-32 µg/mL was the MIC range for this drug. The mean MIC for the drug itraconazole was 0.29 µg/mL and 0.032-0.125 µg/mL was the range for this drug. The mean MIC value for the drug ketoconazole was 0.06 µg/mL. There were 11 *C. neoformans* strains isolated from pigeon excreta; 20.47 µg/mL was the mean MIC value for the drug fluconazole with the MIC range of 0.125-64 µg/mL for this drug. The mean MIC value for the drug itraconazole was 0.15 µg/mL and the range was 0.06-0.25 µg/mL. The mean MIC value for the drug ketoconazole was 0.052 µg/mL and 0.03-0.06 µg/mL was the range. There were 3 *C. gattii* strains isolated from tree trunk hollows from *Tamarindus indica*; 23.3 µg/mL was the mean MIC for the drug fluconazole and 2-64 µg/mL was the range. The mean MIC value for itraconazole was 0.34 µg/mL and 0.03-0.5 µg/mL the range for this drug. The mean MIC value for ketoconazole was 0.072 µg/mL and 0.03-0.125 µg/mL was the range for this drug. There were 2 *C. neoformans* strains isolated from tree trunk hollows of *Mangifera indica*; 8.25 µg/mL was the mean MIC for fluconazole and 0.5-16 µg/mL was the range for this drug. The mean MIC for itraconazole was 0.155 µg/mL and 0.06-0.25 µg/mL was the range for this drug. The mean MIC range for ketoconazole was 0.06 µg/mL. There were 4 strains isolated from tree trunk hollow of *Syzygium cumini*; 1 was *C. gattii* and 3 were *C. neoformans*; the mean MIC values for *C. gattii* strains for the three drugs fluconazole, itraconazole and ketoconazole were 64, 0.032, and 0.032 µg/mL respectively. The mean MIC value for *C. neoformans* strains, for fluconazole was 3.67 µg/mL and 1-8 µg/mL was the range for this drug. The mean MIC for drug itraconazole was 0.072 µg/mL and 0.03-0.125 µg/mL was the range. The mean MIC for the drug ketoconazole was 0.05 µg/mL and the range was 0.03-0.06 µg/mL. There were 4 strains isolated from *Eucalyptus* spp.; 10.25 µg/mL was the mean MIC for the drug fluconazole and 1-32 µg/mL was the range. The mean MIC for the drug itraconazole was 0.59 µg/mL and 1-0.25 µg/mL was the range for this drug. The mean MIC value for the drug ketoconazole was 0.06 µg/mL. There was only a single *C. neoformans* strain isolated from the soil contaminated by bird droppings; 8 µg/mL was the mean MIC for fluconazole, 0.125 µg/mL was the mean MIC for itraconazole and 0.03 µg/mL was the mean MIC for the drug ketoconazole. There were 3 *C. neoformans* strains procured from the soil contaminated in poultry farm; 4.67 µg/mL was the mean MIC for fluco-

Table 3 - Clinical and environmental (*C. neoformans* and *C. gattii*) strains with respect to their source, mean MICs to azole drugs and the MIC range.

Type (n); Strain (n)	Individual Source (n)	Strain (n); Serotype	Mean (Range) Flu	Mean (Range) Itra	Mean (Range) Keto
Env.(54); *C.n* (50), *C.g* (4)	House hold garbage contaminated Soil[α] (6)	*C.n* (6)	12.66 (4-16 µg/mL)	0.22 (0.03-1 µg/mL)	0.08 (0.03-0.25 µg/mL)
	Hospital waste contaminated soil[β] (4)	*C.n* (4)	18.5 (2-32 µg/mL)	0.06 (0.03-0.125 µg/mL)	0.03 (-)µg/mL
	Soil soaked with human urine[γ] (2)	*C.n* (2)	4.25 (0.5-8 µg/mL)	0.125 (-)µg/mL	0.045 (0.03-0.06 µg/mL)
	Soil contaminated by pigeon droppings[δ](8)	*C.n* (8)	25.75 (2-64) µg/mL	0.16 (0.03-0.125) µg/mL	0.085 (0.03-0.06) µg/mL
	Soil contaminated by other bird excreta[ε] (6)	*C.n* (6)	14.01 (0.063-32 µg/mL)	0.29 (0.032-0.125 µg/mL)	0.06 µg/mL (-)
	Pigeon excreta[ζ] (11)	*C.n* (11)	20.47 (0.125-64 µg/mL)	0.15 (0.06-0.25 µg/mL)	0.052 (0.03-0.06 µg/mL)
	Tree trunk hollows from *T. indica*[η] (3)	*C.g* (3)	23.3 (2-64 µg/mL)	0.34 (0.03-0.5 µg/mL)	0.072 (0.03-0.125 µg/mL)
	Tree trunk hollows from *M. indica*[θ] (2)	*C.n* (2)	8.25 (0.5-16) µg/mL	0.155 (0.06-0.25) µg/mL	0.06 µg/mL (-)
	Tree trunk hollows from *S. cumini*[ι] (4)	*C.g* (1) Serotype B	64 µg/mL	0.032 µg/mL	0.032 µg/mL
		C.n (3)	3.67 (1-8) µg/mL	0.072 (0.03-0.125) µg/mL	0.05 (0.03-0.06) µg/mL
	Isolates from *Eucalyptus* Spp.[κ] (4)	*C.n* (4)	10.25 (1-32) µg/mL	0.59 (1-0.25) µg/mL	0.06 (-) µg/mL
	Bird droppings contaminated soil[λ] (1)	*C.n* (1)	8 µg/mL	0.125 µg/mL	0.03 µg/mL
	Poultry farm contaminated Soil (3)	*C.n* (3)	4.67 (2-8) µg/mL	0.09 (0.03-0.125) µg/mL	0.06 (-) µg/mL
Clin.(9); *C.n* (8), *C.g* (1)	CSF (3)	*C.n* (1); Serotype D	0.5 µg/mL µg/mL	0.5 µg/mL µg/mL	0.06 µg/mL
		C.n (2) Serotype A	4.25; (0.5-8) µg/mL	0.375; (0.5-0.25 µg/mL	0.045; (0.03-0.06) µg/mL
	Sputum (1)	*C.n* (1)	16 µg/mL	0.25 µg/mL	0.03 µg/mL
	Blood (4)	*C.g* (1);	16 µg/mL	0.125 µg/mL	0.03 µg/mL
		C.n (3)	33.33 (4-32) µg/mL	0.145 (0.06-0.25) µg/mL	0.176 (0.03-0.25) µg/mL
	Urine (1)	*C.n* (1)	8 µg/mL	0.125 µg/mL	0.03 µg/mL

C.g - *Cryptococcus gattii* (B) ; *C.n* - *Cryptococcus neoformans* (A/D).

Flu - Fluconazole; Itra - Itraconazole; Keto - Ketoconazole; Clin. - Clinical; Env. - Environmental.

T. indica - *Tamarindus indica*., *M. indica* - *Mangifera indica*., *S. cumini* - *Syzygium cumini*.

[α]BCCM/IHEM 20327, BCCM/IHEM 20328, MTCC 4414, MTCC 4413, MTCC 4415, MTCC 4417.

[β]BCCM/IHEM 20332, MTCC 4423, MTCC 4424 MTCC 4425.

[γ]BCCM/IHEM 20334, BCCM/IHEM 21672.

[δ]BCCM/IHEM 20335, BCCM/IHEM 21670, MTCC 4411, MTCC 4410, MTCC 4409, MTCC 4412, MTCC 6358.

[ε]BCCM/IHEM 20336, MTCC 4418, MTCC 4419, MTCC 4420, MTCC 4421, MTCC 4422.

[ζ]BCCM/IHEM 20337, BCCM/IHEM 20338, BCCM/IHEM 20339, BCCM/IHEM 20340, BCCM/IHEM 20341, MTCC 4403, MTCC 4404, MTCC 4405, MTCC 4407.

[η]BCCM/IHEM 22846.

[θ]BCCM/IHEM 22838, BCCM/IHEM 22845.

[ι]BCCM/IHEM 22836, BCCM/IHEM 22837, BCCM/IHEM 22849.

[κ]MTCC 6359, MTCC 6357, MTCC 6356.

[λ]BCCM/IHEM 20336.

[BCCM/IHEM-Belgian Co-ordinated Collections of Micro-organisms, Belgium;

MTCC-Microbial Type Culture Collection, Chandigarh India.]

nazole and 2-8 µg/mL was the range. The mean MIC for the drug itraconazole was 0.09 µg/mL and 0.03-0.125 µg/mL was the range. The mean MIC for the drug ketoconazole was 0.06 µg/mL.

There were 9 clinical strains out of which 8 were *C. neoformans* and 1 was *C. gattii*. There were 3 *C. neoformans* strains isolated from cerebrospinal fluid, in which 1 strain was serotype D; the MIC for the three drugs fluconazole, itraconazole and ketoconazole were 0.5 µg/mL, 0.5 µg/mL and 0.06 µg/mL respectively. The other 2 strains from cerebrospinal fluid were serotype A; the mean MIC for fluconazole was 4.25 µg/mL and the range was 0.5-8 µg/mL. The mean MIC for itraconazole was 0.375 µg/mL and the MIC range for this drug was 0.5-0.25 µg/mL. The mean MIC for the drug ketoconazole was 0.045 µg/mL and the MIC range was 0.03-0.06 µg/mL. There was a single *C. neoformans* strain isolated from sputum the MIC for the three drugs were 16, 0.25 and 0.03 µg/mL respectively. There were 4 strains isolated from blood (1 *C. gattii* and 3 *C. neoformans*). The single *C. gattii* strain isolated from blood had the MIC values for the three drugs as follows: 16 µg/mL, 0.125 µg/mL and 0.03 µg/mL respectively. The mean MIC values for 3 *C. neoformans* strains isolated from blood were 33.33 µg/mL; the MIC range was 4-32 µg/mL for drug fluconazole, 0.145 µg/mL was the mean MIC for the drug itraconazole with a range of 0.06-0.25 µg/mL and 0.176 µg/mL was the mean MIC for the drug ketoconazole with range of 0.03-0.25 µg/mL.

There was only one *C. neoformans* strain isolated from urine. The MIC values for fluconazole, itraconazole and ketoconazole drugs for this isolate were 8, 0.125 and 0.03 µg/mL respectively.

Statistical results

Antifungal susceptibility results of three azoles against 58 *C. neoformans* and 5 *C. gattii* isolates have been summarized in Table 1. *C. gattii* showed higher percentage of resistance (40%) compared with that of *C. neoformans* for fluconazole and statistically this difference was significant ($\chi^2 = 4.589$; p = 0.01609*), while considering fisher's exact p value = 0.09077, showed non-significance.

The total number of resistant cases for itraconazole and ketoconazole did not show any significant difference compared to *C. neoformans* and *C. gattii.*

A comparison of geometric means of clinical and environmental isolates of *C. gattii* and *C. neoformans* shown in Table 2 was not statistically significant using student 't' test (p value > 0.05 NS). Comparison of clinical (9) *vs.* environmental (54) isolates was also not statistically significant using student 't' test (p value > 0.05 NS).

Discussion

Review of literature on the subject revealed that *in-vitro* susceptibilities of *C. neoformans* and *C. gattii* strains to antifungal drugs have been studied by a number of investigators in India and abroad (Archibald *et al.*, 2004; Chowdhary *et al.*, 2011; Govender *et al.*, 2011; Khan *et al.*, 2009; Sar *et al.*, 2004; Souza *et al.*, 2005). However, few studies on the antifungal susceptibilities of clinical and environmental strains of *C. neoformans* and *C. gattii* have been reported (Chowdhary *et al.*, 2011; Franzot and Hamdan, 1996; Souza *et al.*, 2005). This study is noteworthy for documenting the antifungal susceptibility profiles of environmental and clinical isolates of *C. neoformans* and *C. gattii* against fluconazole, itraconazole and ketoconazole in Central India employing micro-dilution method of CLSI/NCCLS-M27A guidelines (NCCLS-M27A). Earlier studies from India like that of Khan *et al.* (2007) reported antifungal susceptibility profiles of clinical and environmental isolates of *C. neoformans* (serotype A) and *C. gattii* (serotype B) against amphotericin B, fluconazole, itraconazole, voriconazole and 5-flurocytosine from northeastern India. Their data on geometric mean of MICs revealed that *C. gattii* was significantly less susceptible than *C. neoformans* to fluconazole, itraconazole and voriconazole (p < 0.0001). In the same study MIC90 of *C. gattii* was two fold higher than that of *C. neoformans* for fluconazole, itraconazole and voriconazole. In this study, a comparison of the geometric mean of MIC revealed that environmental isolates of *C. neoformans* were less susceptible than environmental isolates of *C. gattii* to fluconazole. There was also no significant difference found between the antifungal susceptibilities profiles of clinical and environmental isolates of *C. neoformans* and *C. gattii* (p < 0.05). On the contrary, Chowdhary *et al.* (2011) reported that environmental *C. neoformans* variety *grubii* were significantly less susceptible to fluconazole, itraconazole than the clinical isolates. However, Souza *et al.* (2005) reported that the MIC results obtained from their clinical and environmental isolates showed similar pattern of susceptibility and no resistance was found. Earlier similar results were obtained from Brazil (Franzot and Hamdan, 1996).

The present study demonstrated that susceptibilities of *C. neoformans* var. *grubii* and *C. gattii* differed, with *C. gattii* isolates showing high resistance for fluconazole and ketoconazole than those of *C. neoformans* var. *grubii*. This is in conformity with several earlier reports (Chowdhary *et al.*, 2011; Fernandes *et al.*, 2003; Gomez-Lopez *et al.*, 2008; Trilles *et al.*, 2004). However, contrary results showing no such differences in antifungal susceptibilities of the two species have been reported by some workers (Morgan *et al.*, 2006; Tay *et al.*, 2006; Thompson *et al.*, 2009). This could be due to a possible lack of uniformity in the methodologies used by different workers. Chowdhary *et al.* (2011) reported lower susceptibility of environmental isolates of *C. neoformans* var. *grubii* to fluconazole and itraconazole

than their clinical isolates. This is contrary to our results which exhibited that differences in the clinical and environmental isolates of *C. neoformans* var. *grubii* was not statistically significant. Thus, we corroborate the findings of some investigators who found that antifungal susceptibility was not related to the clinical or environmental origin of strains. (Franzot and Hamdan, 1996; Moraes *et al.*, 2003; Trilles *et al.*, 2004).

Interestingly, our results revealed that environmental isolates of *C. neoformans* var. *grubii* from soil contaminated by pigeon excreta, pigeon droppings and from the trunk hollows of *Tamarindus indica* tree exhibited lower susceptibility to fluconazole compared to other environmental sources and clinical isolates. This is quite similar to the finding of Chowdhary *et al.* (2011). However, on the contrary, the findings of some other investigators showed that antifungal susceptibility was not related to the clinical or environmental origin of strains (Franzot and Hamdan, 1996; Moraes *et al.*, 2003; Trilles *et al.*, 2004). In the present study, we have also found a solitary isolate of *C. gattii* (serotype B) from the trunk hollow of *S. cumini* tree to be resistant to fluconazole (MIC 64 μg/mL). Similarly, Khan *et al.* (2009) and Chowdhary *et al.* (2011) have also reported that *C. gattii* isolates were significantly less susceptible than those of *C. neoformans* var. *grubii* to fluconazole.

Dutta *et al.* (2003) assessed fluconazole and itraconazole susceptibilities of clinical isolates of *C. neoformans* in India. They reported that susceptibilities to fluconazole and itraconazole were 84.1% and 93.2% respectively. MIC50 and MIC90 values for fluconazole were 4 and 16 μg/mL respectively. In the present study, we found 60.3% and 70.7% susceptibilities of environmental isolates of *C. neoformans* to fluconazole and itraconazole respectively. In our case, MIC 50 and MIC 90 values for fluconazole were 8 and 32 mg/L. It is just double the values reported by Dutta *et al.* (2003) for clinical isolates of *C. neoformans* for fluconazole. Similarly, MIC50 value for itraconazole in the present study was 0.125 μg/mL, much higher than 0.032 μg/mL reported by Dutta *et al.* (2003). Khan *et al.* (2007) reported 4 μg/mL as MIC90 for *C. neoformans* isolates from decayed wood of trunk hollows against fluconazole. On the contrary, our environmental isolates exhibited much higher values 32 μg/mL MIC90 value for fluconazole; likewise for itraconazole our MIC90 value was 0.05 μg/mL much lower (0.094 μg/mL) reported by Khan *et al.* (2007) respectively. Interestingly, our value of MIC90 for ketoconazole was similar, *i.e.* 0.064 μg/mL to that of Khan *et al.* (2007).

We conclude, that the susceptibilities of *C. neoformans* and *C. gattii* isolates differed, with *C. gattii* strains showing higher resistance percentages (for fluconazole and ketoconazole drugs) than those of *C. neoformans*.

Trpkovic *et al.* (2012) reported lowest activity of fluconazole *in vitro* (48.4% susceptibility). Our results also indicate lowest activity of fluconazole for *C. gattii* strains (40%), however lowest susceptibility of 37.9% was observed in case of *C. neoformans* strains for the drug ketoconazole.

Tangwattanachuleeporn *et al.* (2013) analyzed the prevalence and antifungal susceptibilities of *C. neoformans* isolated from pigeon excreta from Eastern Thailand. This group studied 50 pigeon excreta samples; 100% of *C. neoformans* isolated from pigeon excreta were of serotype A. Tangwattanachuleeporn *et al.* (2013) also observed decreased susceptibility towards fluconazole; still all strains tested were sensitive towards fluconazole and itraconazole. However, in the present study we observed 8.6% resistance for *C. neoformans* and 40% for *C. gattii* strains, while 5.2% resistance was observed for the drug itraconazole for *C. neoformans* strains.

Antifungal susceptibilities and genotypes of clinical isolates were analyzed in Brazil by Matos *et al.* (2012). Their study revealed resistance to fluconazole (4.8%), which is lower than our results of 8.6% resistance in *C. neoformans* and 40% in case of *C. gattii* for the drug fluconazole. This study also revealed the high percentage of *C. gattii* strains belonged to VGII genotype and its low susceptibility to antifungal agents is worth considering.

The low susceptibility of VGII genotype was also shown by Trilles *et al.* (2012). In their study geometric means for the drugs fluconazole, itraconazole and ketoconazole for VGII genotype were 6.08, 0.15 and 0.06 respectively. Our results revealed geometric means for these three drugs for *C. neoformans* vs. *C. gattii* strains were as follows: 6.93 *vs.* 13.93, 0.124 *vs.* 0.125 and 0.051 *vs.* 0.047 for fluconazole, itraconazole and ketoconazole respectively.

Favalessa *et al.* (2014) analyzed the molecular types and *in-vitro* antifungal susceptibilities of *Cryptococcus* spp. from patients in Mid west Brazil. Their MICs ranges for antifungal drugs were as follows fluconazole was 1-16 mg/L, itraconazole was 0.25-0.12 mg/L, while in the present study the MIC range for *C. neoformans* was 0.063-64 μg/mL and was 2-64 μg/mL for *C. gattii* for the drug fluconazole. The MIC range for the drug itraconazole was 0.03-1 μg/mL for *C. neoformans* and 0.03-0.5 μg/mL for *C. gattii* strains respectively. Favalessa *et al.* (2014) also concluded that the predominant genotype affecting HIV-negative individuals in Cuiaba is AFLP6/VGII.

The MIC results of our Central Indian strains are higher in comparison with other related national and international studies. Recent studies have highlighted that the genotypes and origin of *C. neoformans* and *C. gattii* had profound influence on their antifungal susceptibilities (Chong *et al.*, 2010; Iqbal *et al.*, 2010). In this present work, we were not able to work out the molecular types of the present strains. Hence, our next goal would be to determine

these molecular types and to draw a valid conclusion about the same.

Acknowledgments

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