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

Ruchi Sethi Gutch1,2, Shesh Rao Nawange1,2, Shankar Mohan Singh1,2, Ruchika Yadu1,2, Aditi Tiwari1,2, Richa Gumasta1,2, Arvind Kavishwar3

1Department of Biological Sciences, Rani Durgavati University, Madhya Pradesh, India.
2Centre for Medical Mycology, Fungal Disease Diagnostic and Research Center, Madhya Pradesh, India.
3National Institute For Research In Tribal Health, Madhya Pradesh, 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 immunocompromised patients with AIDS, and also in immunocompromised 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 withazole 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; Pfäffer et al., 1998; Pfäffer 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 x 10⁴-2.5 x 10⁵ 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. (Pfäffer et al., 1995; Rex et al., 1996; Rodríguez-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 intermediate susceptible, 60.3% were susceptible, the MIC50 was 8 \( \mu \text{g/mL} \), the MIC90 was 32 \( \mu \text{g/mL} \), geometric mean was 6.93 \( \mu \text{g/mL} \), and MIC range was 0.063-64 \( \mu \text{g/mL} \). The MIC results for *C. gattii* (5) strains were as follows: 40% were resistant, 20% were intermediate susceptible, 40% were susceptible, the MIC50 was 16 \( \mu \text{g/mL} \) and the MIC90 64 \( \mu \text{g/mL} \), geometric mean was 13.93 \( \mu \text{g/mL} \) and the MIC range was 2-64 \( \mu \text{g/mL} \).

The MIC results for itraconazole for 58 *C. neoformans* strains exhibited that 5.2% were resistant, 24.1% were intermediate susceptible, 70.7% susceptible, 0.125 was the MIC50 \( \mu \text{g/mL} \) and the MIC90 0.5 \( \mu \text{g/mL} \). The geometric mean was 0.124 \( \mu \text{g/mL} \) and MIC range 0.03-1 \( \mu \text{g/mL} \). The MIC values for *C. gattii* (5) strains against itraconazole showed no resistance and 40% were intermediate susceptible. However, 60% were susceptible, 0.125 \( \mu \text{g/mL} \) was the MIC50, the MIC90 was 0.5 \( \mu \text{g/mL} \), geometric mean was 0.125 \( \mu \text{g/mL} \), the MIC range was 0.03-0.5 \( \mu \text{g/mL} \).

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 against itraconazole showed no resistance and 40% were intermediate susceptible. However, 60% were susceptible, 0.125 \( \mu \text{g/mL} \) was the MIC50, the MIC90 was 0.5 \( \mu \text{g/mL} \), geometric mean was 0.125 \( \mu \text{g/mL} \), and MIC range was 0.03-0.125 \( \mu \text{g/mL} \).

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

| Antifungal agent & organism | No. Tested | % 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      | 0.03-0.125|
| *C. gattii*                 | 5          | 0 NS| 40    | 60  | 0.125  | 0.5   | 0.125      | 0.03-0.125|
| 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 |
| *Cn* (58)| Clin. (8) | Geometric Mean | 8.72  | 0.210 | 0.06 |
|         | Env. (50) | Geometric Mean | 6.68  | 0.114 | 0.049 |
| Total (63)| Clin. (9) | Geometric Mean | 9.33  | 0.198 | 0.06 |
|          | Env. (54) | Geometric Mean | 7.04  | 0.115 | 0.049 |

*Cg* - *Cryptococcus gattii* (B); *Cn* - *Cryptococcus neoformans* (A/D); Flu - Fluconazole; Itra - Itraconazole; Ket - Ketoconazole; Clin. - Clinical; Env. - Environmental.
was $16 \mu g/mL$, itraconazole $0.125 \mu g/mL$ and ketoconazole $0.03 \mu g/mL$. Likewise, the geometric means of environmental isolates for fluconazole was $13.45 \mu g/mL$, itraconazole $0.124 \mu g/mL$ and for ketoconazole, it was $0.05 \mu g/mL$. 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 $8.72 \mu g/mL$, for itraconazole was $0.21 \mu g/mL$ and for ketoconazole, it was $0.06 \mu g/mL$. For *C. neoformans* environmental isolates (50), the geometric mean for fluconazole was $6.68 \mu g/mL$, for itraconazole, it was $0.114 \mu g/mL$, and for ketoconazole, $0.049 \mu g/mL$. 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 $\mu g/mL$ was the mean MIC value for the drug fluconazole and 4-16 $\mu g/mL$ was the range. The mean MIC for the drug fluconazole was 0.22 $\mu g/mL$ and 0.03-1 $\mu g/mL$ was the MIC range. The mean MIC for the drug itraconazole was 0.08 $\mu g/mL$ and the MIC range was 0.03-0.25 $\mu g/mL$. There were 4 *C. neoformans* strains isolated from soil contaminated by hospital waste; 18.5 $\mu g/mL$ was the mean MIC and 2-32 $\mu g/mL$ was the range for the drug fluconazole. The mean MIC value for the drug itraconazole was 0.06 $\mu g/mL$ and 0.03-0.125 $\mu g/mL$ was the range. The mean MIC for the drug ketoconazole was 0.16 $\mu g/mL$ and 0.03-0.125 $\mu g/mL$ was the range. The mean MIC value for the drug ketoconazole was 0.085 $\mu g/mL$ and 0.03-0.06 $\mu g/mL$ was the range for this drug. There were 6 strains of *C. neoformans* isolated from soil contaminated by other bird excreta; 14.01 $\mu g/mL$ was the mean MIC value for fluconazole and 0.063-32 $\mu g/mL$ was the MIC range for this drug. The mean MIC for the drug itraconazole was 0.29 $\mu g/mL$ and 0.032-0.125 $\mu g/mL$ was the range for this drug. The mean MIC value for the drug ketoconazole was 0.06 $\mu g/mL$. There were 11 *C. neoformans* strains isolated from pigeon excreta; 20.47 $\mu g/mL$ was the mean MIC value for the drug fluconazole with the MIC range of 0.125-64 $\mu g/mL$ for this drug. The mean MIC value for the drug itraconazole was 0.15 $\mu g/mL$ and the range was 0.06-0.25 $\mu g/mL$. The mean MIC value for the drug ketoconazole was 0.052 $\mu g/mL$ and 0.03-0.06 $\mu g/mL$ was the range. There were 3 *C. gattii* strains isolated from tree trunk hollows from *Tamarindus indica*; 23.3 $\mu g/mL$ was the mean MIC for the drug fluconazole and 2-64 $\mu g/mL$ was the range. The mean MIC value for itraconazole was 0.34 $\mu g/mL$ and 0.03-0.5 $\mu g/mL$ the range for this drug. The mean MIC value for ketoconazole was 0.072 $\mu g/mL$ and 0.03-0.125 $\mu g/mL$ was the range for this drug. There were 2 *C. neoformans* strains isolated from tree trunk hollows of *Mangifera indica*; 8.25 $\mu g/mL$ was the mean MIC for fluconazole and 0.5-16 $\mu g/mL$ was the range for this drug. The mean MIC for itraconazole was 0.155 $\mu g/mL$ and 0.06-0.25 $\mu g/mL$ was the range for this drug. The mean MIC range for ketoconazole was 0.06 $\mu g/mL$. 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 $\mu g/mL$ respectively. The mean MIC value for *C. neoformans* strains, for fluconazole was 3.67 $\mu g/mL$ and 1-8 $\mu g/mL$ was the range for this drug. The mean MIC for drug itraconazole was 0.072 $\mu g/mL$ and 0.03-0.125 $\mu g/mL$ was the range. The mean MIC for the drug ketoconazole was 0.05 $\mu g/mL$ and the range was 0.03-0.06 $\mu g/mL$. There were 4 strains isolated from *Eucalyptus* spp.; 10.25 $\mu g/mL$ was the mean MIC for the drug fluconazole and 1-32 $\mu g/mL$ was the range. The mean MIC for the drug itraconazole was 0.59 $\mu g/mL$ and 1-0.25 $\mu g/mL$ was the range for this drug. The mean MIC value for the drug ketoconazole was 0.06 $\mu g/mL$. There was only a single *C. neoformans* strain isolated from the soil contaminated by bird droppings; 8 $\mu g/mL$ was the mean MIC for fluconazole, 0.125 $\mu g/mL$ was the mean MIC for itraconazole and 0.03 $\mu g/mL$ was the mean MIC for the drug ketoconazole. There were 3 *C. neoformans* strains procured from the soil contaminated in poultry farm; 4.67 $\mu g/mL$ 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<sup>2</sup> (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<sup>3</sup> (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<sup>2</sup> (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<sup>8</sup> (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<sup>6</sup> (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<sup>1</sup> (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<sup>9</sup> (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<sup>8</sup> (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<sup>1</sup> (4) | C.g (1) Serotype B | 64 μg/mL | 0.032 μg/mL | 0.032 μg/mL |
|                      | Isolates from Eucalyptus Spp.<sup>9</sup> (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<sup>1</sup> (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) | Sputum (3) | C.n (1); Serotype D | 0.5 μg/mL | 0.5 μg/mL | 0.06 μg/mL |
|                      | Blood (4) | 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 |
|                      | Blood (4) | C.g (1); | 16 μg/mL | 0.25 μg/mL | 0.03 μg/mL |
|                      | Urine (1) | 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.

<sup>1</sup>BCCM/IHEM 20327, BCCM/IHEM 20328, MTCC 4414, MTCC 4413, MTCC 4415, MTCC 4417.
<sup>2</sup>BCCM/IHEM 20334, BCCM/IHEM 21672.
<sup>3</sup>BCCM/IHEM 20335, BCCM/IHEM 21670, MTCC 4411, MTCC 4410, MTCC 4406, MTCC 4409, MTCC 4412, MTCC 6358.
<sup>4</sup>BCCM/IHEM 20336, MTCC 4418, MTCC 4419, MTCC 4420, MTCC 4421, MTCC 4422.
<sup>5</sup>BCCM/IHEM 20337, BCCM/IHEM 20338, BCCM/IHEM 20339, BCCM/IHEM 20340, BCCM/IHEM 20341, MTCC 4403, MTCC 4404, MTCC 4405, MTCC 4407.
<sup>6</sup>BCCM/IHEM 22846.
<sup>7</sup>BCCM/IHEM 22838, BCCM/IHEM 22845.
<sup>8</sup>BCCM/IHEM 22836, BCCM/IHEM 22837, BCCM/IHEM 22849.
<sup>9</sup>MTCC 6359, MTCC 6357, MTCC 6356.
<sup>10</sup>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 0.03-0.25 µg/mL. The mean MIC for itraconazole was 0.0375 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 strains isolated from blood were 33.33 g/mL; the MIC range was 4-32 g/mL and the MIC for the drug itraconazole was 0.125 µg/mL and 0.06-0.25 µg/mL and 0.06-0.125 µg/mL respectively. The 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/NCCCLS-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-fluorocytosine 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.

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than their clinical isolates. This is contrary to our results which exhibited that differences in the clinical and environmental isolates of \textit{C. neoformans} var. \textit{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 \textit{C. neoformans} var. \textit{grubii} from soil contaminated by pigeon excreta, pigeon droppings and from the trunk hollows of \textit{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 \textit{C. gattii} (serotype B) from the trunk hollow of \textit{S. cumini} tree to be resistant to fluconazole (MIC 64 \(\mu\)g/mL). Similarly, Khan et al. (2009) and Chowdhary et al. (2011) have also reported that \textit{C. gattii} isolates were significantly less susceptible than those of \textit{C. neoformans} var. \textit{grubii} to fluconazole.

Dutta et al. (2003) assessed fluconazole and itraconazole susceptibilities of clinical isolates of \textit{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 \(\mu\)g/mL respectively. In the present study, we found 60.3% and 70.7% susceptibilities of environmental isolates of \textit{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 \textit{C. neoformans} for fluconazole. Similarly, MIC50 value for itraconazole in the present study was 0.125 \(\mu\)g/mL, much higher than 0.032 \(\mu\)g/mL reported by Dutta et al. (2003). Khan et al. (2007) reported 4 \(\mu\)g/mL as MIC90 for \textit{C. neoformans} isolates from decayed wood of trunk hollows against fluconazole. On the contrary, our environmental isolates exhibited much higher values 32 \(\mu\)g/mL MIC90 value for fluconazole; likewise for itraconazole our MIC90 value was 0.05 \(\mu\)g/mL much lower (0.094 \(\mu\)g/mL) reported by Khan et al. (2007) respectively. Interestingly, our value of MIC90 for ketoconazole was similar, i.e. 0.064 \(\mu\)g/mL to that of Khan et al. (2007).

We conclude, that the susceptibilities of \textit{C. neoformans} and \textit{C. gattii} isolates differed, with \textit{C. gattii} strains showing higher resistance percentages (for fluconazole and ketoconazole drugs) than those of \textit{C. neoformans}. Trpkovic et al. (2012) reported lowest activity of fluconazole \textit{in vitro} (48.4% susceptibility). Our results also indicate lowest activity of fluconazole for \textit{C. gattii} strains (40%), however lowest susceptibility of 37.9% was observed in case of \textit{C. neoformans} strains for the drug ketoconazole.

Tangwattanachuleepon et al. (2013) analyzed the prevalence and antifungal susceptibilities of \textit{C. neoformans} isolated from pigeon excreta from Eastern Thailand. This group studied 50 pigeon excreta samples; 100% of \textit{C. neoformans} isolated from pigeon excreta were of serotype A. Tangwattanachuleepon 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 \textit{C. neoformans} and 40% for \textit{C. gattii} strains, while 5.2% resistance was observed for the drug itraconazole for \textit{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 \textit{C. neoformans} and 40% in case of \textit{C. gattii} for the drug fluconazole. This study also revealed the high percentage of \textit{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 \textit{C. neoformans} vs. \textit{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 \textit{in-vitro} antifungal susceptibilities of \textit{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 \textit{C. neoformans} was 0.063-64 \(\mu\)g/mL and was 2-64 \(\mu\)g/mL for \textit{C. gattii} for the drug fluconazole. The MIC range for the drug itraconazole was 0.03-1 \(\mu\)g/mL for \textit{C. neoformans} and 0.03-0.5 \(\mu\)g/mL for \textit{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 \textit{C. neoformans} and \textit{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.

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