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Original Article

ANTIFUNGAL SUSCEPTIBILITY OF *CRYPTOCOCCUS NEOFORMANS* ISOLATES FROM FUNGAL MENINGITIS IN AIDS PATIENTS IN INDIA

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ABSTRACT

Objectives: This study was undertaken to determine the antifungal susceptibility of fifteen cryptococcal isolates from cerebrospinal fluid (CSF) of HIV seropositive patients with cryptococcal meningitis.

Methods: Antifungal susceptibility testing of the isolates was done by CLSI M27 A-3 methodology for amphotericin B, fluconazole, voriconazole, itraconazole and 5-flucytosine.

Results: Our study demonstrates no evidence of resistance among clinical isolates of *Cryptococcus neoformans* for any of the above mentioned antifungal drugs. Only two isolates showed slightly higher minimum inhibitory concentration (MIC) to 5-flucytosine (8 µg/ml).

Conclusion: Routine *In vitro* antifungal susceptibility for cryptococcal isolates is important, in order to obtain a baseline data and to predict any shift in the susceptibility patterns in the Indian population.

Keywords: Antifungal susceptibility, Cryptococcal meningitis, HIV, India, MICs.

INTRODUCTION

Cryptococcus neoformans is an environmental saprophyte that commonly infects immunocompromised patients causing cryptococcal meningitis (1). The incidence of cryptococcal infections has increased dramatically over the years in many countries due to the HIV epidemic (2, 3). With wider use of antifungal drugs for treatment of cryptococcal meningitis with increase in HIV positive population, antifungal resistance is likely to be on the rise. This would increase treatment costs in resource poor countries like ours where healthcare infrastructure is already strained (4). We conducted this study because of the gravity of the potential problems posed by antifungal drug resistance and the need for more antifungal susceptibility data in India. For the conduct of this study we measured the *In vitro* antifungal drug susceptibilities of *Cryptococcus neoformans* isolates from AIDS patients at a tertiary care hospital in New Delhi.

MATERIALS AND METHODS

A total of 15 *Cryptococcus* isolates from the cerebrospinal fluid (CSF) of HIV positive patients from P.G.I.M.E.R. & DR. R.M.L.Hospital, New Delhi were taken for this study. CSF was cultured on Sabouraud's dextrose agar (High Media) to obtain the isolates. The presence of C*ryptococcus neoformans* was confirmed by various microbiological tests like sugar assimilation and fermentation, growth on Niger seed agar and on Canavanine glycine bromothymol blue agar. These isolates were then tested for antifungal susceptibility. Demographic data including gender, age, CD4 counts, socioeconomic status and in-hospital mortality were obtained using a structured proforma and patient medical records. Informed consent was taken from all the patients to have their CSF collected and their clinical records reviewed.

Antifungal susceptibility testing by CLSI M27 A-3 methodology (5):

We tested five antifungal drugs from different manufacturers as follows: Amphotericin B (Sigma), Fluconazole, voriconazole (Pfizer), Itraconazole (LeePharma) and 5-flucytosine (Sigma). Stock solutions were prepared in distilled 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^{0}C until needed. The final concentration of the drugs ranged from 0.125 to64 ug/ml for fluconazole and 5-flucytosine, 0.03 to 16 ug/ml for amphotericin B and 0.015 to 8 ug/ml for itraconazole and voriconazole.

The yeast inoculums were adjusted to a concentration of 0.5x10^3-2.5x10^3 cells/ml in RPMI1640 as measured by spectrophotometry. 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 were incubated at 35^{0}C for 72 hours. 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 minimum inhibitory concentration (MIC) for all strains twice on two different days. The MIC end points were read visually after 72 hours 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.

RESULTS

Fifteen isolates of *Cryptococcus neoformans* were obtained over a period of one year from clinically suspected chronic meningitis cases in HIV seropositive population. The age of the patients with cryptococcal meningitis in this study was ranged from 19 to 55 years. The male: female ratio was 13:2. Cryptococcal meningitis was found to be more common in 30-39 yrs. age group. Amongst the fifteen cases of cryptococcal meningitis, 10 had CD4 count <50 cells/µl followed by 3 patients with CD4 counts between 50-100 cells/µl and remaining 2 had CD4 counts between 100-200 cells/µl. Outcome was fatal in 6 out of 15 cryptococcal meningitis cases, thus mortality rate being 40%. The patients with CD4 count < 50 cells/µl had highest mortaliy rate i.e. 50%

Most of the cryptococcal isolates were found sensitive to the above mentioned antifungal drugs. Only 2 isolates showed slightly higher MIC to 5-flucytosine (8 µg/ml). Individual MIC values to various antifungal drugs are shown in Table 1.

For all cryptococcal isolates, we recorded the MIC at which 50% of the isolates were inhibited (MIC_{50}) and the MIC at which 90% of the isolates were inhibited (MIC_{90}) and determined the MIC geometric mean for each therapeutic agent [Table 2].

Table 1: Antifungal susceptibilities of 15 *Cryptococcus* isolates

Drugs Case No.	Amphotericin B (µg/ml)	Fluconazole (µg/ml)	Voriconazole (µg/ml)	Itraconazole (µg/ml)	5-Flucytosine (µg/ml)
1	0.125	2	<0.03	<0.03	8
2	0.125	4	0.06	<0.03	4
3	0.06	4	<0.03	<0.03	4
4	0.125	4	0.03	0.06	4
5	0.25	4	0.06	0.125	8
6	0.125	4	0.06	0.125	4
7	0.5	2	0.06	0.06	4
8	0.25	4	0.125	0.125	4
9	0.5	4	0.06	0.06	4
10	1	4	0.06	0.125	4
11	0.5	2	0.125	0.06	4
12	0.5	4	0.03	0.03	2
13	1	4	0.03	0.03	2
14	0.5	1	0.03	0.03	2
15	1	4	0.06	0.125	4

Table 2: MIC value and Geometric mean of MIC

S. No.	Drug.	*GM	MIC range (µg/ml)	*MIC_{50} (µg/ml)	*MIC_{90} (µg/ml)
1	Amphotericin B	0.429	0.06 – 1	0.25	0.5
2	Fluconazole	3.4	1 – 4	4	4
3	Itraconazole	0.069	0.03 – 0.125	0.06	0.125
4	Voriconazole	0.056	0.03 – 0.125	0.06	0.06
5	5-Flucytosine	4.13	2 – 8	4	4

*GM – Geometric mean,
All the isolates of *Cryptococcus* showed low MICs for all the antifungal drugs tested.

DISCUSSION

Cryptococcus is an opportunistic fungal infection and cryptococcal meningitis has become the most common lethal opportunistic fungal infection in people with HIV and AIDS (6). In these patients, cryptococcal meningitis is usually incurable, and individuals who survive the initial infection are given lifelong antifungal therapy to reduce the likelihood of relapse (7). In these circumstances there are more chances of development of resistance to antifungal drugs especially to fluconazole which is used for long term maintenance therapy. Resistance to fluconazole or amphotericin B have already been reported in some studies from developed countries. This situation could be more problematic in developing countries where large numbers of HIV-infected patients exist, and resources to treat the disease are inadequate. In addition to this, the surveillance for antifungal resistance is still poor or ignored in developing countries. Therefore, in this study, an attempt was made to find out the trend of *In vitro* antifungal susceptibility with clinical cryptococcal isolates from AIDS patients.

Our study demonstrates no evidence of resistance among clinical isolates of *Cryptococcus neoformans.* Isolates remained highly susceptible to 5-flucytosine (100% susceptible at MIC of 4 µg/ml), fluconazole (100% susceptible at MIC of 4ug/ml) as well as to other azoles (voriconazole & itraconazole). Voriconazole exhibited the highest inhibitory activity (GM 0.056 mg per ml) followed by itraconazole with GM of 0.069 mg per ml. Voriconazole and other newer azoles like posaconazole have consistently been shown to have good activity against *Cryptococcus neoformans* (8, 9, 10). However, the use of voriconazole and posaconazole is still restricted as no clinical trials have been undertaken to compare these agents to first-line drugs and these agents remain prohibitively expensive for use in resource-limited settings (11).

Our observation of the absence of resistance among *Cryptococcus neoformans* isolates is consistent with the data published by the Centre for Disease Control and Prevention, which showed *In vitro* resistance to antifungal agents to be uncommon and unchanged among *Cryptococcus neoformans* isolates from 1992 to 1998 (2). An extensive survey of the susceptibility profiles of clinical isolates of *Cryptococcus neoformans* at a University hospital during 1987 to 1994 observed no emergence of resistance (12). Similar results were obtained by Archibald *et al* (13) who found no evidence of resistance among clinical isolates of *Cryptococcus neoformans* from Thailand, Malawi, and the United States.

There is paucity of reports on *In vitro* antifungal susceptibilities of clinical isolates of *Cryptococcus neoformans* in India. Most of these reports involve resistance to fluconazole emerging in the setting of meningitis in AIDS patients after long term treatment or prophylaxis with fluconazole. Study in AIIMS by Datta *et al*. (14), demonstrated intermediate susceptibility to fluconazole in 16% of clinical isolates which is the first report from India on higher fluconazole MIC values in clinical cryptococcal isolates. Another study by Anuradha *et al* (15) from Delhi involving both clinical and environmental isolates, reported fourfold increase in fluconazole MICs over a period of 1.5–2.5 months in 4 serial isolates obtained from four patients receiving antifungal therapy of amphotericin B for 3 weeks followed by fluconazole prophylaxis (400 mg daily). They found similar fourfold increase for itraconazole and amphotericin B also.

Casadevall *et al*. (16), found an increase in the fluconazole MICs for serial *Cryptococcus neoformans* isolates that were recovered from five patients with recurrent cryptococcal meningitis. Paugam *et al*. (17), reported clinical and *In vitro* fluconazole resistance in three AIDS patients with recurrent cryptococcal meningitis (increases in MICs from 4 to 64, 16 to 128, and 0.25 to 16 mg/ml, respectively).

Antifungal susceptibility testing is required to monitor the susceptibility pattern of clinical isolates to existing antifungals for the prevention of any sudden emergence of drug-resistant pathogens. For these reasons, it is necessary to develop routine surveillance of susceptibility to antifungal treatment *In vitro*. Only a few antifungal drugs may be available in developing countries because of limited resources or cost restrictions, this further necessitates the surveillance for resistance to available drug

treatment for appropriate patient care and improved patient outcome. Our study has few limitations. Firstly, the sample size is small and second, we did not tested serial isolates from recurrent or relapse cases as the patient follow-up was limited to the duration of hospital admission. This might be a reason that we could not find resistance to any of the antifungal drug because resistance is more likely to arise in circumstances where patients have been treated with suboptimal induction-phase regimen and where long-term, low-dose fluconazole is prescribed for suppression of disease.

CONCLUSION

To conclude, we found no evidence of resistance to any of the antifungal drug tested for clinical isolates of *Cryptococcus neoformans* using a standardized testing method but antifungal testing may be more helpful to document the emergence of resistance over time among patients with serially collected isolates.

CONFLICT OF INTERESTS

Declared None

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