

Original Research Article

ANTIFUNGAL SUSCEPTIBILITY PATTERN OF ASPERGILLUS ISOLATES FROM PATIENTS WITH LOWER RESPIRATORY TRACT INFECTIONS

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ABSTRACT

Background: Fungal infections are on rise despite of development of latest diagnostic tools and therapeutic options. Early initiation of the correct antifungal therapy may have a direct impact on the patient's quality of life and high cost of treatment and care.

Materials and Methods: *Aspergillus* species were isolated from patients with lower respiratory tract infections. Antifungal susceptibility pattern of all the *Aspergillus* isolates were assessed against Amphotericin B, Itraconazole and Caspofungin by broth micro dilution method.

Results: All *Aspergillus flavus* and *Aspergillus niger* isolates were 100% sensitive to all the three drugs. However, only 4 *Aspergillus fumigatus* isolates showed high Minimum inhibitory Concentration (MIC) value for Amphotericin B and 2 isolates for Itraconazole.

Conclusion: Broth Microdilution method is a gold standard and sensitive technique but time consuming and cumbersome to be performed routinely by clinical laboratories. Also association of increased incidences of resistant strains with treatment failure highlights the need for antifungal resistance surveillance by reference labs using such sensitive procedures.

Keywords: *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, Antifungal, Broth microdilution, Amphotericin B, Itraconazole and Caspofungin.

INTRODUCTION

Morbidity and mortality due to invasive fungal infections with *Aspergillus* and other fungi have become prominent in the United States and worldwide. These infections are also associated with high mortality even if treated with currently present anti fungal drugs.^[1-4]

Antifungal agents with potency against Invasive Aspergillosis include Amphotericin B and its lipid formulations; the triazoles Itraconazole, Voriconazole, and Posaconazole and a novel group of anti fungal echinocandins such as Caspofungin, Micafungin and Anidulafungin.^[5]

Treatment of fatal fungal infections predominantly includes Amphotericin B and two azole drugs Fluconazole and Itraconazole. Voriconazole is

approved as the initial treatment of invasive aspergillosis by guidelines as a result of its good in vitro activity, improved outcomes and limited adverse reactions as compared with Amphotericin B.^[5,6]

Although efficacious management of patients with invasive fungal diseases due to filamentous fungi as well as yeast pathogens, restricted potency and tolerability of patients for these drugs and expansion in the reports of azole resistance along with rise in MIC values to various antifungals agents for *Aspergillus*, is posing a threat to survival of the patients and have evoked need for novel drugs.^[7,8,9,10,11] *A. fumigatus* and other non-fumigatus isolates showing rise in MIC values to different anti fungal agents are being increasingly documented.^[12]

The echinocandins are a novel semisynthetic lipopeptide anti fungal derivatives, due to their proficient in- vitro activity against *Aspergillus* spp., limited toxicity and restricted drug interactions , these are available as an interesting alternative for treatment of fungal infection in response to expansion of azole resistance incidences of *Aspergillus* spp.^[13,14,15]

Azole resistance in *Aspergillus* species is attributed to cyp51A gene mutations, which may alter the targeted 14- α -sterol demethylases. Membrane permeability variations and efflux pump over-expression contribute to the same resistance profile among environmental isolates. Echinocandin resistance may be related to 1,3- β -d-glucan synthetase gene mutations, even if these molecules are occasionally used because of their fungistatic activity against *Aspergillus* species.^[16,17]

Therefore the aim of the study was to perform antifungal susceptibility of Isolated *Aspergillus* isolates against Amphotericin B, Itraconazole and Caspofungin.

MATERIALS AND METHODS

Place of the study

The study was conducted in Department of Microbiology and TB - Chest Santosh Medical College and Hospital Ghaziabad in collaboration with Department of Microbiology University College of Medical Sciences, GTB Hospital New Delhi.

Study Design

A total of 29 *Aspergillus* spp were isolated from sputum samples of 150 LRTI patients, isolates comprised of 17 *A. fumigatus*, 11 *A. flavus* and 1 *A. niger*, were subjected to Antifungal Susceptibility Testing (AST) by broth micro dilution method against Amphotericin B, Itraconazole and Caspofungin , after obtaining Ethical clearance from Institutional Ethical Committee. A reference strain *A. flavus* ATCC 204304 was included for quality control. Antifungal susceptibility was performed following reference standard M38-A2 CLSI guidelines.

Statistical Analysis

The statistical analysis was done using Statistical Package for Social Sciences Software (Statistical Package for Social Sciences – www.ibm.com)

Methodology

A) Inoculum Preparation

Inoculum was prepared following M38-A2 CLSI guidelines. Each isolate of *Aspergillus* was subcultured on potato dextrose agar slants for 3-4 days at 35 C until good sporulation is obtained. Each slant was then flood with 1 ml of sterile normal saline

along with addition of 0.01ml Tween 20. Resulting mixture was withdrawn into a sterile glass tube and heavy particles were allowed to settle down for 3-5 mins. Suspension was then passed through sterile filter tips to separate hyphal elements from non germinated conidia. At 70 % transmittance turbidity of the above solution was adjusted to an optical density (0.09- 0.013) using spectrophotometer at 530 nm by adding sterile normal saline for *Aspergillus* sp. (4×10^5 CFU/ml to 50×10^5 CFU/ml)

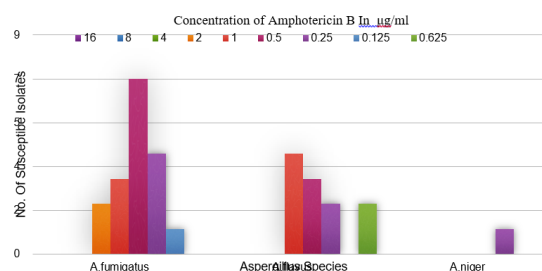
B) Broth Microdilution.

Antifungal powders of Amphotericin B (Hi media), Itraconazole (Biovision USA) and Caspofungin (Biovision USA) were obtained .Preparation of stock solutions and dilution of anti fungal drugs and inoculation of micro titre plate was done following guidelines of CLSI M38-A2 document. Inoculated micro titre plates were incubated at 35oC for 48 h. Minimum Inhibitory Concentration (MIC) for Amphotericin B and Itraconazole and Minimum Effective Concentration (MEC) for Caspofungin were interpreted as suggested by CLSI M38-A2. Results were read at 48 h for Itraconazole and Amphotericin B and at 24 h for Caspofungin.

Isolate was considered as sensitive against Itraconazole when MIC was $\leq 1 \mu\text{g/ml}$ (for *A. flavus* and *A. fumigatus*) , $\leq 2 \mu\text{g/ml}$ for Amphotericin B (for *A. fumigatus*, *A. flavus*, *A. niger*) and when MEC for Caspofungin is $\leq 0.0625 \mu\text{g/ml}$.

RESULTS

Graph 1 Shows Antifungal susceptibility of three *Aspergillus* species, *A. fumigatus*, *A. flavus* and *A. niger* against Amohotericin B by broth Microdilution method which shows, Among *A. fumigatus* isolates 88% (15/17) isolates were found to be sensitive for Amphotericin B . However, 2 isolates of *A. fumigatus* showed high MIC value of $\geq 2 \mu\text{g/ml}$ for Amphotericin B . All the *A. flavus* and *A. niger* isolates were observed to be sensitive against Amphotericin B.



Graph 1: Antifungal Susceptibility of Aspergillus spp Against Amphotericin B By Broth Microdilution Method

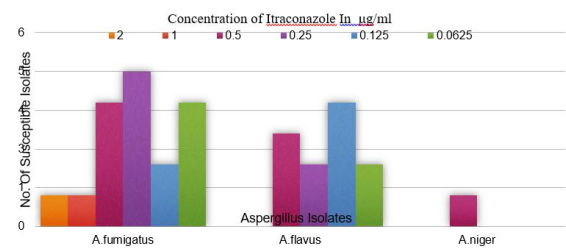
Table 1: Statistical Analysis of Aspergillus Isolates Against Amphotericin B

Total No. Of Aspergillus Isolates	Geometric Mean	Median	SD
29			
<i>A. fumigatus</i> 17	0.5	0.5	0.76
<i>A. flavus</i>	0.2	0.45	0.46

11			
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SD = Standard Deviation

Graph 2 Shows Antifungal susceptibility of three *Aspergillus* species, *A.fumigatus*, *A. flavus* and *A. niger* against Itraconazole by broth Microdilution method which shows, Among *A. fumigatus* isolates 94% (16/17 were sensitive) for Itraconazole. However among 2 isolates that showed high MIC values for Amphotericin B, one of isolate also had high MIC value of $\geq 2 \mu\text{g/ml}$ for Itraconazole also. All the *A. flavus* and *A. niger* isolates were observed to be sensitive against Itraconazole.



Graph 2: Antifungal Susceptibility of *Aspergillus* spp Against Itraconazole By Broth Micro Dilution Method

Table 2: Statistical Analysis of *Aspergillus* Isolates Against Itraconazole

Total No. Of <i>Aspergillus</i> Isolates	Geometric Mean	Median	SD
<i>A.fumigatus</i> 17	0.3	0.37	0.74
<i>A.flavus</i> 11	0.17	0.1875	0.19

SD = Standard Deviation

All *A. fumigatus*, *A. flavus* and *A. niger* isolated were found to be 100% sensitive for Caspofungin. None of the isolates was found to be resistant to any of the three anti fungal drug.

DISCUSSION

Antifungal susceptibility testing (AFS) for filamentous fungi is not easy and effortful, therefore, it is restricted to only limited laboratories. Although an ideal method of susceptibility testing must be easily replicable, correct and worthwhile. Hence, there is minimal data available on local MIC.^[18] Methods recommended for AFS testing includes broth micro-dilution (CLSI M38-A2), disk diffusion (DD), E-test (Biomeriux, USA) and other tests available commercially. However, the proposed gold standard method for AFS is CLSI broth micro-dilution. (M38- A2),^[19]

High MIC values have been documented sometimes by E – test,^[18] Reference method (M51-A) for disk diffusion antifungal susceptibility testing of non-dermatophyte filamentous fungi have been published by CLSI in 2010.^[20]

Echinocandins, such as Caspofungin, Anidulafungin, and Micafungin, inhibit β - glucan synthesis, which is a crucial component of fungal cell walls. As fungistatic agents, they compromise cell wall integrity and are used as salvage therapy for pulmonary aspergillosis.^[21]

In the present study we performed AFS of *Aspergillus* isolates by broth micro dilution due to its high accuracy and being a gold standard method against Amphotericin B, Itraconazole and Caspofungin, and determined Minimum Inhibitory Concentration for Amphotericin B and Minimum effective concentration for Caspofungin using reference standard CLSI (M38- A2) for broth micro-dilution.

Among the *A.fumigatus* isolates 88%,^[15] showed low MIC values and were found to be sensitive to Amphotericin B, 94%,^[16] against Itraconazole and 100 %, ^[17] MEC was obtained for Caspofungin. Only , 2 *A.fumigatus* isolates showed high MIC values of $\geq 2 \mu\text{g/ml}$ for Amphotericin B of which 1 isolate of *A.fumigatus* also had high MIC value for Itraconazole. Our study results were similar to a surveillance study at a tertiary care hospital by Fonseca et al which revealed that 67.2% of *Aspergillus* isolates had elevated AMB MIC, potentially leading to treatment failure.^[22]

However, in our study all the *A.flavus* and *A.niger* isolates were found to be 100% sensitive against all the three anti fungal drugs. None of our *A.flavus* and *A.niger* isolate showed high MIC and MEC values which were similar to the findings of Shiv prakash et al,^[23] but in contrast to a study from PGIMER has reported 3 strains of *A. flavus* with high MIC values of 2 mg/l for Amphotericin B and 4 mg/l for Voriconazole,^[24] Although studies have reported an increase in the number of AMB- resistant strains. A study by Fakhim et al. documented AMB resistance rates of 36.8%, 14.9%, 5.2%, and 2.01% in *A. terreus*, *A. flavus*, *A. niger*, and *A. fumigatus*, respectively.^[25]

Incidences of Caspofungin resistance in *Aspergillus* spp. are still least reported from India. This could be because Caspofungin is still the least used antifungal in India. Our showed all the *Aspergillus* isolates to be 100% sensitive for Caspofungin. Similarly, a study by Kaur et al. also reported 100% Caspofungin susceptibility in all *Aspergillus* isolates.^[26] However, Arendrup et al, had reported a clinical isolate of *A. fumigatus* to be resistant to Caspofungin, confirmed

by E-test with an MIC >32 µg/ml. This patient also failed to respond to Caspofungin.^[27]

There is a requirement for more studies, which can contribute to more data on antifungal susceptibility testing on larger number of *Aspergillus* isolates obtained from various infections, focusing on analysis and observing various trends in MIC values obtained against various Antifungal agents . Even though this is a limitation of our study but this study will help in determining antifungal MIC, epidemiological surveillance of rise in fungal infections, their therapeutic interventions. This will be help in studying the development of resistant pattern in *Aspergillus* species.

CONCLUSION

Increasing incidences resistant to Antifungal agents such as Amphotericin B and Azoles are of great concern as these are the primary antifungal agents used to treat various fungal infections caused by *Aspergillus* species. Satisfactory outcomes of patients having *Aspergillus* infection depends on early diagnosis and prompt initiation of therapeutic measures following antifungal susceptibility testing to be performed wherever possible.

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Conflicts of interests: None

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