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Importance of Speciating Aspergillusfrom Clinical Samples and Interpretation of Antifungal Susceptability Results

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ABSTRACT

Background: Aspergillosis caused by different species might affect the choice of treatment in certain species depending upon the MIC values.The aim of this study was to determine the antifungal susceptibility pattern among dif-ferent Aspergillus species to the routinely used antifungal agents in accordance with CLSI M38 A.

Methods: Aspergillus grown from various clinical sample was subcultured and identified phenotypically. Antifungal susceptibility testing (AFST) was done for 5 antifungal agents- AmphotericinB, Voriconazole, Posaconazole, Itraconazole and Caspofungin.

Results: *Aspergillus flavus* was the predominantly isolated speies. *Aspergillus flavus* and *Aspergillus niger* showed high MIC values to AMB & ITR and low MIC for VOR & POS. Some species of *Aspergillus flavus* exhibited rare phenomenon like paradoxical/trailing effect. *Aspergillus fumigatus* showed low MICs for both triazoles and AMB. *Aspergillus terreus* had high MIC for triazoles and AMB. All of the tested isolates showed low MECs against caspofungin.

Conclusion: *Aspergillus* is a fungal pathogen which is easy to treat unless it is invasive. Variation in susceptibility profile noted for the *Aspergillus* for each species to the common antifungal agents; since the azole resistance is in increasing trend hindering the effective antifungal therapy resulting in therapeutic failures.

Key Words: *Aspergillus, Antifungal susceptibility testing, Eagles effect, triazoles, Broth microdilution, CLSI M38-A2, Trailing effect, Paradoxical effect*

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INTRODUCTION

The *Aspergillus* species cause various disease manifestations such as allergic syndromes, intoxications, chronic in-fections and acute invasive diseases particularly in immunocompromised patients [1, 2]. The spectrum of aspergillosis is broadly classified into invasive life-threatening infections in immunocompromised persons; sub-acute or chronic infections in patients with structural lung abnormalities or pre-existing pulmonary or sinus disease or some subtle defect in innate immunity; allergic or eosinophilic disease manifested in many forms like allergic bronchopulmonary aspergillosis (ABPA), eosinophilic rhinosinusitis, and extrinsic allergic alveolitis; and locally invasive infections as a result of trauma or surgery such as keratitis or post-operative infections [1, 3]. *Aspergillus fumigatus* is the most common cause of invasive aspergillosis [4, 5] followed by *A. flavus, A. niger, A. terreusand A. nidulans* [6]. At least 428 species have been described in 20 groups or sections of *Aspergillus*. Among those the species belonging to sections Circumdati, Clavati, Cremei, Nigri, Restricti, Usti, and Versicolores have been predominantly isolated from the environment but the species of the Fumigati, Terrei, and Flavi sections have been predominantly isolated from clinical samples [7]. The prevalence of reduced susceptibility due to acquired resistance to antifungal drugs are common among *Aspergillus* species, also some species like *A. terreusshows* less susceptibility to common antifungal agents used in the treatment of Aspergillosis which results in high morbidity and mortality in immunocompromised patients. The purpose of this study is to determine the antifungal susceptibility pattern among different *Aspergillus* species to the routinely used antifungal agents.

MATERIALS AND METHODS

Study Setting: The study was conducted in the Department of Microbiology in Mycology division at Sri Ramachandra Institute of Higher Education and Research. The study period was from October 2018- June 2022. Ethical

clearance was obtained on OCT 2018 from SRIHER (REF:IEC-NI/18/SEP/66/64) and Apollo Hospitals (REF:IEC-CS App.No.: AMH-006/10-18).

Study Population: All clinical isolates which were positive for *Aspergillus* species from the period from October 2018 - June 2022 was taken up for the study.

Collection of Isolates: The samples were processed as per routine mycological procedures. A total of 99 isolates were collected from the Microbiolagy laboratory. The samples were subjected to 10% potassium hydroxide mount and those which showed hyphal elements were cultured in Saboraud's dextrose agar/ oat meal agar slant.

Fungal culture: All the samples which showed KOH positivity were inoculated in Saboraud's dextrose agar/ Oatmeal agar with gentamicin added at a concentration of 20 milligram/litre. Two set of tubes were incubated at both 370 C and 250C. Cultures were examined for growth, daily in the first week and then twice a week for subsequent period. The species identification was based on phenotypic characteristics like growth rate, temperature requirements and colony characteristics such as colour, texture, submerged hyphae, obverse & reverse pigmentation and microscopic identification done by LPCB mount from direct culture or using slide culture technique.

Antifungal susceptibility testing: The *in-vitro* susceptibility testing was performed according to the CLSI-M38A 2017 document titled Reference Method for Broth microdilution Antifungal susceptibility testing of Filamentous Fungi; Ap-proved standard - 3rd edition, for common filamentous fungi or moulds, including the dermatophytes. The five drugs tested were triazoles (Itraconazole, Voriconazole, Posaconazole), polyene antifungal (Amphotericin B), echinocandins (Caspo-fungin diacetate). Antifungal stock solutions were prepared at concentration of atleast 1280µg/ml or 10 times the highest concentration to be tested, whichever was greater. However, some antifungal agents with limited solubility may need lower concentration. RPMI-1640 medium after sterility check was used for plating the microtitre well. From the antifungal stock solution dilution of drugs were prepared using broth microdilution susceptibility tests for non-dermatophyte isolates. Growth control and Drug control wells were included for each drug.Inoculum was prepared for Mould (0.4-5.0 x 104) and 100 µl of conidial suspension was added to 5 ml RPMI-1640 medium (1:50 dilution) and then 100 µl of inoculum was added to each well (excluding sterility control). The plates were incubated at 350 C. For Amphotericin B, Itraconazole, Voriconazole, Posaconazole- The results were read at 46 – 50 hours. Minimum inhibitory concentration (MIC) was taken as the lowest concentration well without visible growth. For echinocandins- The results were read at 21 - 26 hours; Minimum Effective Concentration (MEC) or the point where reduction in growth was noted when compared to the growth control.

RESULTS

A total of 99 *Aspergillus* isolates from individual patients were collected from the Central Microbiology lab and the Mycology lab at Sri Ramachandra Institute of Higher Education and Research & Apollo Hospitals over a period of 18 months from October 2018 – June 2022. Among 99 patients 54 were males and 45 were females. The age of the patient ranges between 12 and 64 years. A majority of the patients were in the age group 50 – 60 years with male preponderance. Out of 99 patients most common source of isolation was from ear swabs & granulation tissue from ear followed by bronchial wash/ bronchoalveolar lavage fluid, paranasal sinus, nasal tissue, endotracheal aspirate, sputum, vocal cord tissue and pus from gluteal region. Table 1 shows the source of specimen from which *Aspergillus* was grown. Based on phenotypic identification, *Aspergillusflavus* was the most predominant species isolated from 46 patients (47%) followed by *Aspergillusniger* 23 (23%),*Aspergillusfumigatus*21 (21%), *Aspergillusterreus* 8 (8%) and *Aspergillusnidulans* 1 (1%).

Table 1: Specimen source of Aspergillus species

Specimen	**Total (n=99)**
Ear- pus & tissue	35
BAL	17
Sputum	6
Bronchial wash	12
Nasal tissue & Paranasal sinus	16
Endotracheal aspirate	10
Vocal cord tissue	1
Gluteal region- pus	1
Oral mucosa- Tissue	1

BAL- Bronchoalveolar lavage

Majority of the patients were immunocompromised primarily with diabetes mellitus (40%) followed by COPD (18%), lymphoma/leukemia (12%), Asthma (9%), Kidney disease (9%), Bronchiectasis (9%) and Liver disease (3%). Antifungal susceptibility testing was performed for representative of 63 *Aspergillus* isolates.

Minimum Inhibitory Concentration (MIC) / Minimum Effective Concentration (MEC) values for 5 Antifungal drugs – Itraconazole (ITR), Voriconazole (VOR), Posaconazole (POS), Caspofungin (CAS) and Amphotericin B (AMB) is given in Table 2 .The antifungal susceptibility results were analysed based on Epidemiological Cutoff Values (ECVs) since the clinical breakpoints have not been established for most of the *Aspergillus* species. In this study, *Aspergillus flavus* and *Aspergillus niger* showed high MIC values to AMB & ITR and low MIC for VOR & POS. Except one all other isolates of *Aspergillus fumigatus* showed low MICs for both triazoles and AMB. *Aspergillus terreus* had high MIC for triazoles (ITR, VOR & POS) and AMB. All of the tested isolates showed low MECs against caspofungin.

Table 2: showing Antifungal susceptibility pattern of *Aspergillus* species against commonly used Antifungal agents

***Aspergillus* species**	**Antifungal agent**	**Minimum inhibitory concentration(MIC) / Minimum effective concentration(MEC) in µg/ml**								
		0.0625	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**
Aspergillus flavus (27)	**ITR**		4	7	5	3	2	1	1	4
	VOR		6	10	8	1	1			1
	POS	1	10	8	5	1	1			1
	AMB			6	1	5	6	3	4	2
	CAS			18	6	3				
Aspergillus niger (20)	**ITR**		1	1	3	6	1	4	1	3
	VOR		5	4	8	1	1	1		
	POS	1	5	8	4		2			
	AMB			2	3	4	3		3	5
	CAS			11	7	2				
Aspergillus fumigatus (13)	**ITR**		1	3	5	3				1
	VOR	3	3	3	4					
	POS	4	4	3	2					
	AMB			1	8	3		1		
	CAS			9	4					
Aspergillus terreus (2)	**ITR**							2		
	VOR			1			1			
	POS			1		1				
	AMB					1			1	
	CAS			1	1					
Aspergillus nidulans (1)	**ITR**					1				
	VOR		1							
	POS			1						
	AMB								1	
	CAS			1						

ITR-Itraconazole, VOR-Voriconazole, POS-Posaconazole, AMB-Amphotericin B, CAS-Caspofungin

Table 2 showing MIC values of all the 5 tested Antifungal agents

DISCUSSION

In this study, *Aspergillus* species isolated from ear infection constituted 58% followed by bronchial wash/BAL as 21%. In contrast to our findings, a study reported bronchial secretion or sputum as most common source of *Aspergillus* species and second most common source as auditory exudates [8]. In patients with suppurative otitis media, fungal infections range from 2.1–25 % of cases among which *Aspergillus* species are the commonest cause accounting for 92.1 % [9]. The most frequent species isolated in the present study from ear swab was *Aspergillus flavus*[11] followed by *Aspergillus niger*[10]. In contrast to our study findings, Jing et al., 2021 reported *Aspergillus nig*er as the most common cause of otomycosis followed by *Aspergillus terreus*. Similarly in another study [10], *Aspergillus niger*was the most common species isolated followed by *Aspergillus fumigatus* [11]. In both the studies, *Aspergillus flavus* was isolated as third most common cause of otomycosis from patients with ear infections [11, 12].The predominant age group was between 50 to 60 years of age, which is in contrast to some studies showing 20-30 years [10, 11]. Males were higher in number (56%) as compared to the females which correlates with many other studies showing male preponderance [9, 11]. With regard to species distribution, *Aspergillus flavus*46 (47%) was the most common species isolated followed by *Aspergillus niger* 23 (23%), *Aspergillus fumigatus* 21 (21%), *Aspergillus terreus* 8 (8%) and *Aspergillus nidulans* 1 (1%). This finding correlates with the findings of Masih et al., 2016 where *Aspergillus flavus* (44.2%) was the most frequently isolated species from clinical specimen.Some variations were observed regarding incidence of *Aspergillus* infection, age wise distribution, sex wise distribution as well as species distribution. These variations in incidence and distribution may be due to the differences in climatic conditions like temperature & humidity which affects the growth of *Aspergillus* in environment. Other conditions include season of the study period, environmental conditions of the study

area like wind & dust particles in air and predisposing factors present among the patients [7, 13]. Most of the studies reported that the patients with underlying diseases like hematological malignancy, solid organ transplant, other cancer, HIV, COPD, cystic fibrosis and some other causes of immunosuppression develop invasive aspergillosis [5, 14 & 15]. In this study, majority of the *Aspergillus* species were isolated from immunocompromised patients. Immunocompetent individuals contributed only a few cases.

In a study conducted in Brazil, *Aspergillus fumigatus* strains were found to be less susceptible to Itraconazole which is contrast to this study where *Aspergillus fumigatus* showed low MIC values to all the 5 antifungal agents. In the same study, *Aspergillus flavus* strains were less susceptible to Amphotericin B which correlates with the present study [16]. All *Aspergillus* isolates tested in this study showed good activity for caspofungin by recording low MEC value [12, 16].

Eagles effect: Very few isolates among *Aspergillus flavus* exhibited Eagles effect which is also called as paradoxical effect. Eagles effect is the ability of an organism to grow above MIC value of specific antifungal agent with susceptibility to lower concentration of the same drug. This kind of Paradoxical effect (PE) was most commonly observed with CAS and often referred as Caspofungin Paradoxical Effect (CPE). CPE was described previously for *Aspergillus fumigatus* but in this study the eagle effect was observed for 2 of the *Aspergillus flavus*. The reason for development of such an effect is un-known. It may develop as a result of changes in the composition of fungal cell wall but the exact mechanism is not yet fully understood till today [17]. Similarly, Trailing effect (TE) was also observed for some strains of *Aspergillus flavus* for all the 5 antifungal drugs showing persistent growth throughout the microtitre plate without complete growth inhibition of fungus even with increasing concentration of antifungal agent [18]. TE and PE analysis may be useful when evaluated for their possible influence on outcome of the patient. These effects usually affects the interpretation of susceptibility results by altering the determination of MIC values [17–19].

Susceptibility profile aids in successful treatment. The terms susceptible and resistant is based on MIC values in accordance with the epidemiological cutoff values established by CLSI for antifungal susceptibility testing of filamentous fungi like *Aspergillus*. The epidemiological cutoff values for the upper limit of wild type MIC distributions helps in determining the possibility of defining resistance pattern in *Aspergillus* species. The ECVs proposed by CLSI guidelines have been used to interpret the results of antifungal susceptibility testing [20, 21]. Based on the clinical breakpoints mentioned in CLSI M38-A2 for antifungal susceptibility testing of filamentous fungi, the cut off values were formulated with MICs of >2 mg/L for AMB, ITR and VOR. They were termed as *in-vitro* resistant *Aspergillus* species whereas for POS, MIC >1 mg/L and for CAS with MECs of >0.5 mg/L were considered as resistant isolates [12, 22].Triazoles (ITR, VOR & POS) are the mainstay of treatment for patients with aspergillosis. Currently, a new extended spectrum triazole- Isavuconazole has been proven to be active against *Aspergillus* species. These agents are the orally available anti-Aspergillus agents for long term therapy [5]. Based on some study, the ECVs of *Aspergillus fumigatus* is 1µg/ml for ITR , VOR and Isavuconazole ; for POS it is 0.25µg/ml [23] . Voriconazole is the drug of choice for Invasive Aspergillosis. Itraconazole is the primary treatment of choice for allergic non-invasive forms of Aspergillosis like ABPA [5, 24]. The 90-60 rule of antibiotics play an important role in the treatment of infections which states that infection due to susceptible isolates may respond 90% of the time to appropriate therapy but even with inappropriate therapy i.e. infection due to resistant isolates may also respond to 60% of the time [25]. Although *Aspergillus* species were identified long back, the severity of Aspergillosis has been increasing every day. Most common causative agent of invasive Aspergillosis was *Aspergillus fumigatus* followed by *Aspergillus flavus* which is in contrast to this study in which the most common organism isolated was *Aspergillus flavus*. The other species causing infection includes *A. niger, A.terreus, A.nidulans, A. Versicolor*, etc. With the evolutionary changes of *Aspergillus* along with the development of molecular biology, the diagnosis of invasive aspergillosis has increased in the recent past. Most of the fungal infections were treated without identification of species. Speciation of *Aspergillus* should be done to choose an appropriate antifungal agent to treat invasive aspergillosis, since some of the *Aspergillus* species are intrinsically resistant to the common antifungal agent. Along with the speciation, *in-vitro* susceptibility testing if done will be beneficial incase of multi drug resistant *Aspergillus* like *A. terreus*. Current trends in treatment of *Aspergillus* is mainly triazole antifungal especially voriconazole along with therapeutic drug monitoring(TDM). However, few rare species of *Aspergillus* which were phenotypically similar to the common *Aspergillus* species were resistant to the present drug of choice like triazoles. In such cases, the treatment with newer azole like isavuconazole can be tried. Identification of such rare species was possible only with the advent of molecular diagnosis. These are called as cryptic *Aspergillus* which are phenotypically indistinguishable from the commonly isolated species. The frequency of such cryptic species have been reported as 11% in TRANSNET study and 15% in FILPOP study [14]. Molecular methods using PCR have been developed for early identification and also for identifying cryptic species; real time PCR may also be useful in identification of *Aspergillus* directly from clinical samples like Broncho alveolar lavage and blood samples [26]. Some of the cryptic species like *A.calidoustus and A.lentulus* show reduced susceptibility to multiple antifungal agents, thus accurate identification to species level is important [27].

CONCLUSIONS

Aspergillosis affects both immunocompromised as well as immunocompetent patients causing wide variety of infections, where the identification of *Aspergillus* species is possible by conventional phenotypic methods like

Microscopy as well as Culture characteristics. Nowadays molecular technique is warrented, especially when the phenotypic identification is doubtful or inconclusive, which paves the way for identification of previously unknown species of *Aspergillus*. Even though the number of isolates tested above are small, it showed a varied susceptibility profile to the common antifungal agents used in treatment. It is important to perform antifungal susceptibility testing for accurate diagnosis and early initiation of treatment to reduce the morbidity and mortality in patients especially with Invasive Aspergillosis.

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