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In Vitro Susceptibility Pattern Of Aspergillus Flavus To Newer Azoles (Isavuconazole & Ravuconazole) And Comparison With Conventional Azole Antifungal Agents (Itraconazole, Voriconazole, And Posaconazole)

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ABSTRACT

Invasive and allergic infections by Aspergillus flavus are more common in tropical and subtropical countries. Triazoles are first-line antifungal agents for the treatment of infections caused by Aspergillus spp. The emergence of voriconazole resistance in A. flavus impacts the management of aspergillosis. Aim of this study is to determine the antifungal susceptibility pattern of conventional (itraconazole, voriconazole, posaconazole) and newer azoles (isavuconazole, ravuconazole) among the A. flavus isolates by broth microdilution method. A total of 90 A. flavus isolates obtained from various clinical samples were identified and antifungal susceptibility testing was performed against itraconazole, voriconazole, posaconazole, isavuconazole and ravuconazole by broth microdilution method. The percentage of resistance was highest in itraconazole i.e. 45.39% with highest mean Minimum Inhibitory Concentration (MIC) of 3.09µg/ml. This was followed by voriconazole and isavuconazole with a similar resistance percentage of 13.3% and also their mean MIC values were close to each other (1.42 µg/ml and 1.34 µg/ml respectively). Posaconaole and ravuconazole had very low MICs against A. flavus isolates with mean MICs 0.20µg/ml and 0.51 µg/ml respectively. Among the tested isolates pan azole resistance was noted in 5/90 (5.6%) isolates. Since resistance to commonly used azole antifungal agents are on the rise, performing an antifungal susceptibility test would be a better option in choosing the appropriate antifungal agent for treatment of invasive aspergillosis and helps in preventing the development of resistance in future. Also, knowledge about the resistance pattern of the Aspergillus species will help in developing better treatment protocol.

Keywords: *Susceptibility, Aspergillus, resistance, isavuconazole, ravuconazole*

INTRODUCTION

The genus *Aspergillus* encompasses more than 250 species and is one of the largest genera of filamentous fungi causing human diseases [1,2]. Invasive aspergillosis infections are an important cause of morbidity and mortality globally, mainly due to increasing number of immunocompromised individuals at risk for fungal infections [3]. Worldwide, *Aspergillus fumigatus* is the most common agent of invasive aspergillosis and has been widely studied and reviewed. Apart from *A. fumigatus*, the most prevalent species involved are *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus versicolor*. Infection due *to A. flavus* is predominant in Asia, the Middle East and Africa possibly due to its better ability to survive in hot and arid climatic conditions compared to other *Aspergillus* spp [4]. Triazoles are the mainstay of treatment for invasive aspergillosis. At the same time resistance to this class of antifungal agents is also on rise [5,6]. Mainly, the emergence of voriconazole resistance in *A. flavus* impacts the management of invasive aspergillosis [7].

Patients may develop resistance due to acquired resistance mechanism or due to intrinsic resistance [7,8]. Acquired resistance can be detected through suitable and precisely done susceptibility tests and endpoint interpretation [8]. Acquired resistance patterns are observed, with multi-azole and pan-azole resistance being

more prevalent than resistance to a single triazole [9]. A global study involving 19 countries reported overall prevalence of azole resistance of 3.2%, ranging from 0 to 25.5% among the participating centres [10]. Evidence also shows that inappropriate and suboptimal azole exposure in patients on long-term azole therapy is a powerful predictor for the emergence of resistance [11,12]. Since the availability of newer azole antifungal agents provides the opportunity for developing better treatment strategies for patients with invasive aspergillosis caused by azole resistant species, their *in vitro* effectiveness against clinical isolates is of great interest.

Conventional antifungal agents such as itraconazole, voriconazole, posaconazole, and isavuconazole, a recently licenced drug for aspergillosis, were examined in this study. Furthermore, the investigational triazole, ravuconazole has also been evaluated. Hence this study emphasizes the need of susceptibility testing to evaluate the burden of azole resistance in this most commonly isolated mould, *A. flavus*.

MATERIALS AND METHODS

The present cross sectional study was undertaken in the Department of Microbiology in a tertiary care centre after obtaining Institutional ethical clearance. A total number of 90 isolates from various specimens were collected for the study with precision of 4.5%, level of significance of 5% and with an assumed prevalence of 5% using nMaster software. For this study, *Aspergillus* isolates grown from all clinical samples of patients from March 2020 to June 2021, were considered for the study. Patients without any prior intake of antifungals were included and repetitive isolates from same patients were excluded from the study. The *Aspergillus* isolates were inoculated into Sabouraud Dextrose Agar (SDA) plate and incubated at 25°C for five days with regular examination once in two days. The isolates were systematically identified as *A. flavus*, based on their macroscopic colony morphology on SDA and microscopic morphology on LPCB tease mount and/or slide culture technique.

DNA EXTRACTION

Isolates identified as *A. flavus* by conventional techniques were further confirmed by molecular method. DNA was extracted from all the *A. flavus* isolates by Phenol-Chloroform method with minor modifications. A small bit of growth was taken from the SDA culture and finely grounded in a sterile mortar and pestle and mixed with 500µl of TESS buffer (Tris HCl, Ethylenediamine tetra acetic acid, Sodium chloride and sodium dodecyl sulphte lysis buffer) in microcentrifuge tube and was heated at 100°C in waterbath for 10 min. Equal volumes of 500µl of phenol: chloroform mixture (1:1) was added and vortexed. The tubes were centrifuged at 10,000 rpm for 10 min. The upper aqueous layer was transferred into a new microcentrifuge tube. Equal volume of Chloroform was added to the supernatant and vortexed. The tubes were centrifuged at 10,000 rpm for 10 min. Equal volume of absolute Isopropyl alcohol was added to the tubes and mixed gently. The tubes were centrifuged at 10,000 rpm for 10 min. The aqueous layer was discarded and 200µl of 70% ethanol was added to the microcentrifuge tube. The tubes were centrifuged at 10,000 rpm for 10 min. The alcohol was discarded and the tubes were air dried. The DNA pellet was resuspended in 50µl of nuclease free water and stored at -20°C until use.

PCR AMPLIFICATION

PCR amplification of Internal Transcribed Spacer - ITS1 and ITS2 regions was carried out using universal fungal primers ITS 1 (5' – TCC GTA GGT GAA CCT GCG G – 3') and ITS4 (5' – TCC TCC GCT TAT TGA TAT GC – 3'). PCR master mix was prepared containing 25µl of PCR mix (Takara, Japan), 1µl of forward (ITS1) and reverse primer (ITS4), 5µl of template DNA, and the volume made up to 50µl with sterile nuclease free water. The reaction mixtures were amplified in a thermal cycler (Veriti 96 well, Applied Biosystems, USA). The amplification conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec, with a final extension period at 72°C for 10 min. The PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized with ultraviolet light. PCR amplification of the ITS region yielded a 595 base pair band [13]. Representative isolates were further confirmed by sequencing.

The strains were maintained in glycerol stock media at 4°C with regular sub-culture for once in three months for further testing [14]. Basic demographic details were obtained for all the patients. Further testing was done for all isolates to determine their antifungal susceptibility pattern.

ANTIFUNGAL SUSCEPTIBILITY TESTING

Aspergillus flavus ATCC 9643 and *Aspergillus fumigatus* ATCC 204305 was the control strains used for antifungal susceptibility testing. The antifungal susceptibility testing was done using broth micro dilution method, adhering to Clinical Laboratory Standards Institute (CLSI) M38-A2 guidelines [15] The antifungal agents and the range of concentration tested were

Itraconazole (16657-100MG, Sigma-Aldrich, USA) - 0.125 to 16µg/ml
Voriconazole (PZ0005-5MG, Sigma-Aldrich, USA) - 0.125 to 16µg/ml
Posaconazole (32103-25MG, Sigma-Aldrich, USA) - 0.125 to 16µg/ml
Isavuconazole (SML2357-5MG, Sigma-Aldrich, USA) - 0.125 to 16µg/ml
Ravuconazole (SML1216-5MG, Sigma-Aldrich, USA) - 0.125 to 16µg/ml

The MIC is read as the lowest drug concentration that prevents any discernible growth. The results were analysed and interpreted according to CLSI guidelines, after 48 hours of incubation. As a quality check, the complete absence of turbidity in the media control well was checked each time the test was performed. Potential bias was eliminated through the interpretation of the results by two independent observers for all the strains that were tested. The MIC data obtained were reported as the ranges, mean, standard deviation, MIC_{50} and MIC_{90} values.

Statistical analysis

Statistical testing was conducted with the statistical package for the social science system version SPSS 17.0. The distribution of mean MIC values of *Aspergillus* isolates for all the azoles was analyzed by repeated measures of ANOVA and P values < 0.05 were considered statistically significant.

RESULTS

Among the *Aspergillus* isolates that were collected, 90 isolates were confirmed as *A. flavus*. Based on the EORTC guidelines, 90 confirmed *A. flavus* isolates obtained from various sample sources were grouped as proven and probable invasive aspergillosis [16]. (Table 1).

Table 1: Source of isolates

Sample Source	No. of Isolates(N)	Invasive Aspergillosis (EORTC Guidelines)
Tissue	48	Proven
Sinus	5	
Brain abscess	2	
Biopsy	2	
Bronchial wash	12	Probable
Pus	10	
Broncho alveolar lavage fluid	4	
Endotracheal aspirate	3	
Tracheal aspirate	2	
Sputum	2	

In this study, the percentage of males 55.56% (n=50) were slightly higher than the females 44.44% (n=40). The age of the patients ranged between 5 to 69 years. Most of the patients were in the age group 41 to 60 years (41.11%). The mean age was found to be 41 years. The distribution of age and gender among the study population is shown in the Table 2.

Table 2: Distribution of age and gender among the study population

Age group (years)	No. of females	No. of males	Total
<20	6 (15%)	5 (10%)	11 (12.22%)
21-40	15 (37.5%)	18 (36%)	33 (36.67%)
41-60	18 (45%)	19 (38%)	37 (41.11%)
>60	1(2.5%)	8 (16%)	9 (10%)

Total	40(44.44%)	50(55.56%)	90

The most common underlying comorbid condition among the patients was found to be post-surgery (20%) followed by diabetes (11%). About 32% of the patients had bacterial infections and 9% had Covid 19 infection.

ANTIFUNGAL SUSCEPTIBILITY TESTING

The control strains used in this study showed satisfactory results. The mean MIC values of *A.flavus* and *A. fumigatus* control strains for itraconazole, voriconazole, posaconazole, isavuconazole and ravuconazole were 0.25, 0.5µg/ml; 0.25, 1µg/ml; 0.125, 0.25 µg/ml; 0.25, 0.5 µg/ml and 0.06, 0.125 µg/ml respectively. In the present study, *A. flavus* isolates had high mean MIC value to itraconazole (3.09µg/ml), than the other conventional triazoles like voriconazole (1.42µg/ml) and posaconazole (0.20µg/ml). Among the conventional and newer azole antifungal agents, posaconaole and ravuconazole had very low MICs against *A. flavus* isolates with mean MICs 0.20µg/ml and 0.51 µg/ml. respectively. The MIC_{50} and MIC_{90} values of itraconazole were found to be higher (1µg/ml and 8µg/ml), whereas posaconazole and ravuconazole had low MIC_{50} and MIC_{90} values. It was also noted that voriconazole and isavuconazole had almost similar MIC_{50} and MIC_{90} values. The *in vitro* activities of each antifungal drugs against the *A. flavus* strains along with their MIC range, mean MIC, SD, MIC_{50}, MIC_{90} values and percentages of resistant against azoles considering the breakpoints as described above are represented in the Table 3

Table 3: Range, Mean MIC, SD, MIC_{50}, MIC_{90} values & resistant percentages for the azole antifungal agents

Antifungal Agents	Minimum Inhibitory Concentration (µg/ml)					Resistance (%)*
	Range	Mean MIC	Standard Deviation (SD) (+/-)	MIC_{50}	MIC_{90}	
ITRACONAZOLE	16 - 0.25	3.09	4.40	1	8	45.39%
VORICONAZOLE	16 -0.125	1.42	2.90	1	2	13.3%
POSACONAZOLE	2 - 0.125	0.20	0.26	0.125	0.25	6.7%
ISAVUCONAZOLE	16 -0.125	1.34	2.91	0.5	2	13.3%
RAVUCONAZOLE	2 - 0.125	0.51	0.36	0.5	1	Not Determined

* % of MICs ≥ Epidemiological Cut off Values (ECV). (ECV = 1 µg/ml for itraconazole, voriconazole and isavuconazole, 0.25 µg/ml for posaconazole). No ECVs were available for ravuconazole.

The percentage of resistance was highest for itraconazole (n=59) i.e. 45.39% whereas posaconazole (n=6) had the lowest resistance percentage of 6.7%. Also, voriconazole and isavuconazole had the same resistance percentage of 13.3%. Ravuconazole being the investigational triazole, breakpoints for mould testing have not been established for the same. MIC_{50} and MIC_{90} values of ravuconazole were found to be slightly similar to isavuconazole.

Despite the fact that five (5.6%) out of the 90 isolates demonstrated pan azole resistance, which is defined as MICs that were within the resistance range for all currently active azoles, [17] they were all ravuconazole-sensitive and had very low MICs. Moreover, it is found that there is a statistically significant difference of $P < 0.005$.

DISCUSSION

The development of *A. fumigatus* azole resistance is becoming a major concern around the world [18]. Due to the fact that *A. flavus* is the second most common species to cause invasive infections in immunocompromised patients and the most common species to be isolated in some parts of the world, azole resistance in this species has begun to rise causing similar concern [19].

In our study, male patients were more commonly affected (50/90 i.e.55.56%) when compared to females (40/90 i.e. 44.44%). This was in agreement with a previous study which also showed male predominance [20]. The age-specific incidence rate in our study was lowest among children less than 10 years old, and the rate increased with age. Also, most of the patients in our study were in the age group of 41 to 60 years (41.11%). According to Marr et al., older age was associated with an increased risk of invasive aspergillosis among 1,682 patients [21]. Older

age people are more likely to have weakened immune system, making them more vulnerable to fungal infections when they inhale fungal spores that are naturally present in the environment.

The most common comorbid condition in our study was post-surgery (17.78%) which was in accordance with few other studies where the authors found post-surgery as the major underlying comorbid condition among the study participants [22,23]. The high-risk factors, such as weakened immunity and prolonged antibiotic use, could have also caused break-through infections in patients during the postoperative period. Additionally it is essential to screen for the presence of *Aspergillus* spores in the operating room air, which could also be a possible source of infection. In our study, diabetes was found to be the second most common underlying comorbid condition observed in 11% of the patients. This was similar to previous study, in which diabetes was the most prevalent comorbid condition observed [24]. Patients with diabetes are at increased risk to contract fungal infections and also have increased drug resistance [25]. Because high blood sugar level is associated with impaired leucocyte function, which renders them more vulnerable to invasive aspergillosis. Among our patients almost 32% had bacterial infections, which was in accordance with another study, where bacterial and/or viral infections were the most common associated infections [26].

In our study, the *A. flavus* isolates showed high mean MIC value for itraconazole. This was in agreement with a recent study conducted in the year 2020 by Mostafa Chadeganipour *et al*, among *A. flavus/oryzae* isolates, they identified a rate of itraconazole resistance of about 78%, indicating to the widespread use of triazoles for the treatment of aspergillus infections and azole fungicides [27]. Isolates with decreased susceptibility to itraconazole are regularly cross-resistant to other triazoles, such as voriconazole [28]. Accordingly, in the current study, 13.3% of *A. flavus* isolates exhibited voriconazole resistance. These findings were consistent with a recent study in which the authors reported 28% of *A. flavus/oryzae* cross resistance to voriconazole. In contrast to *A. fumigatus*, the emergence of drug resistance in *A. flavus* has not previously been reported. A study conducted by Tra My N. Duong *et al*, on environmental sampling has reported an unexpectedly high prevalence of resistance, which over 85% of *A. flavus* isolates were resistant to at least one azole, and half of them were resistant to itraconazole [29]. The prevalence of aspergillosis, particularly chronic pulmonary aspergillosis, is becoming more well known, and azole-resistant *A. fumigatus* has been emphasised as a health issue. Even though, a significant proportion of aspergillosis is caused by *A. flavus*, which is thought to be azole sensitive but is rarely included in surveillance. Triazoles for agricultural usage have gained popularity recently due to their affordability, broad spectrum systemic mode of action, and ability to withstand changes in their molecular structure, which allows them to last in the environment for a long time [30]. Azole fungicides are key elements in the emergence of azole resistant *Aspergillus* isolates originating from the environment that eventually develop cross resistance to medical azoles [31]. In a recent study, conducted by Verweiji *et al*., pan azole resistant strains, which are resistant to multiple azole drugs, were detected in azole naïve patients with aspergillosis in 2007 [32]. Cross resistance to medical and agricultural azoles emerged as a result of their similar structures and activities, as well as similar selective pressure pressures for azole resistance in human hosts and the environment when the fungus reproduces abundantly and is exposed to azoles [33,34]. In our study, 47 patients (52%) resided in close proximity to agricultural areas where pesticides for plant pathogens were widely used. As a result of their long term exposure to agricultural azoles, it is possible that this facilitated the development of cross resistance to clinical azoles like itraconazole. To identify whether these high level azole resistance, especially itraconazole resistance is associated to agricultural azole use, as has been described for *A. fumigatus*, more investigation is required in *A. flavus*. Due to the limitations of covid 19 and the fact that our study was conducted during this time period, environmental monitoring was not done in accordance with the constraints set forth in Covid 19, which could have established value for such data. Since azoles are the commonly used drugs for the treatment of invasive aspergillosis, to prevent treatment failure, it is better to perform antifungal susceptibility testing routinely before starting the patient with treatment.

Among the azoles tested, isavuconazole and voriconazole mean MIC values were found to be similar. This was in accordance with another study by Buil et al which showed strong similarity between isavuconazole and voriconazole MIC values [35]. This antifungal susceptibility pattern might be due to the similarity in the chemical structures of isavuconazole and voriconazole.

Posaconazole had the lowest mean MIC value of 0.20µg/ml in this study. These findings were consistent with few other studies, which showed posaconazole with a lower mean MIC as well as MIC_{50} and MIC_{90} values when compared to all the azoles tested against *A. flavus* isolates [36,19]. A recent study by Walsh TJ et al, reported that

posaconazole was utilised as salvage therapy in patients with invasive aspergillosis who were refractory or intolerant to conventional antifungals [37]. Likewise posaconazole was found to be active against *A. fumigatus* resistant to itraconazole, voriconazole and Amphotericin B [38]. Even for voriconazole-resistant *Aspergillus* isolates, posaconazole was found to have low MIC values in our investigation, indicating that it would be a preferable alternative in cases of infections caused by voriconazole-resistant *A. flavus*.

Ravuconazole had an excellent *in vitro* activity against *Aspergillus* spp. In our study, ravuconazole's MIC_{50} and MIC_{90} values were the lowest among the tested azoles (0.5 µg/ml and 1 µg/ml), which was comparable to few previous studies which also stated the same MIC_{50} and MIC_{90} values for ravuconazole [39,40]. Pan azole resistance was found to be 5.6% (n=5/90) in our study, which was in accordance with a previous study where the authors had reported azole resistance [7]. However, pan azole resistant isolates showed significantly lower MIC values for ravuconazole. These results suggests that ravuconazole being an investigational azole, with lowest MIC values, once licensed will be the preferred drug of choice for resistant *Aspergillus* infections. The lack of molecular resistance investigations of the *A.flavus* isolates in this study is a major limitation, which could have aided in understanding the resistance mechanism.

CONCLUSION

Currently, azoles, particularly voriconazole, remain the preferred agent for treatment of invasive aspergillosis. Since, resistance to the commonly used azole antifungal agents are on the rise, performing antifungal susceptibility test would be a better option in choosing the appropriate antifungal agent for treatment of invasive aspergillosis, as well as preventing the development of resistance in future. Additionally, the development of more efficient treatment protocols for pan azole resistant isolates is facilitated by the approval and commercialization of newer azole antifungal drugs like ravuconazole.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ETHICS STATEMENT - This study was approved by the Institutional Ethics Committee (Conscience Independent Ethics committee (IEC-NI/20/FEB/74/09).

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