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Azole Resistance in Moulds—Approach to Detection in a Clinical Laboratory

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Abstract The multitude of factors has contributed to the increasing number of fungal infections caused by species of difficult-to-treat opportunistic moulds, such as *Fusarium*, *Scedosporium*, and cryptic *Aspergilli*. Also, rare fungi sporadically encountered, such as *Rasamsonia argillacea*, *Penicillium oxalicum*, and melanized fungi, are now well recognized. The high mortality associated with these rare and uncommon fungi is primarily linked to the difficulty in diagnosis and limited therapeutic options, as many of them exhibit resistance to antifungals including azoles. Azole resistance in *Aspergillus fumigatus* has been increasingly reported because standardized methods for susceptibility testing and associated clinical breakpoints and epidemiological cutoff values became available. However, such advances in antifungal susceptibility testing (AFST) in non-*Aspergillus* moulds barring mucorales have been lacking. Notwithstanding the fact that the true incidence of these non-*Aspergillus* filamentous moulds in clinical settings is hitherto unknown, also data on AFST by standardized methods is largely lacking. Determination of minimum inhibitory concentration (MIC) by reference techniques is the gold standard to detect azole resistance in filamentous fungi. In recent years, some progress has been made toward the description of resistance mechanisms at molecular level especially in *Aspergillus*. This paper reviews the present state of azole resistance in *Aspergillus* and other filamentous mould species and discusses their relevance to clinical practice.

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Introduction

Filamentous fungi encompassing many genera are associated with a wide spectrum of diseases in humans ranging from superficial to life threatening invasive infections. The multitude of factors has contributed to the increasing number of fungal infections in the last two decades, especially caused by species of opportunistic moulds for which there is no reliable medical therapy. The factors mainly include increasing use of immunosuppressing agents, selection of these moulds in the setting of antifungal prophylaxis, natural disasters, and their better recognition due to advanced identification methods [1, 2, 3••]. The incidence of mould infections is much lower than candidiasis; however, infections due to filamentous fungi are a significant cause of morbidity and mortality especially among immunocompromised patients [4]. Also, the epidemiology of mycoses associated with several filamentous fungi has changed such that the most prevalent invasive mould infection is primarily due to *Aspergillus fumigatus*, but the global emergence of azole-resistant strains has been described in the last decade [5]. Also, infections caused by difficult-to-treat moulds, such as species of *Mucorales* [6], *Fusarium* [7], and *Scedosporium* [8], are increasingly reported, although true incidence of these non-*Aspergillus* filamentous moulds is hitherto unknown. Furthermore, rare fungi sporadically encountered, such as *Rasamsonia argillacea*, *Penicillium oxalicum*, and melanized fungi are now well recognized [9, 10].

The high mortality associated with the rare and uncommon fungi is primarily linked to the difficulty in diagnosis, limited

therapeutic options, and a lack of knowledge of the most effective antifungal therapy. In recent years, the challenges pertaining to identification are significantly reduced by availability of molecular tools and mass spectrometry, which is particularly helpful for the species level identification of fungi with high accuracy and also for determining their resistance profile to antifungals [11, 12••]. However, the therapeutic options for invasive fungal infections remain limited and include only three structural classes of drugs: polyenes, azoles, and echinocandins. Among antifungals, the better tolerated azoles and echinocandins have emerged as first-line agents for most common invasive fungal infections [13]. The azoles, namely fluconazole (FLU), voriconazole (VRC), and posaconazole (POS), are the most widely used antifungal to treat invasive fungal infections. Azoles inhibit ergosterol biosynthesis and, in general, are fungistatic. However, VRC is fungicidal toward *A. fumigatus*. Notably, FLU has essentially no activity against moulds. In contrast, itraconazole (ITC), VRC, POS, and recently approved isavuconazole (ISA) all have activity against moulds. Therefore, the emergence of azole antifungal resistance in moulds jeopardizes the effective treatment. The other major challenge in this respect is that antifungal susceptibility testing (AFST) is not routinely performed in many centers in the world. The true rates of global azole resistance in these pathogens are enigmatic; therefore, detection and monitoring of azole resistance are of paramount importance for the effective management of the disease. The present review aims to provide synopsis of azole resistance in clinically significant opportunistic filamentous mould species and discusses the approach of detection of azole resistance in these fungi.

Global Emergence of Azole Resistance in *Aspergillus* Species

The last decade has witnessed shift in the etiology of aspergillosis and highlighted the emergence of cryptic and rare *Aspergillus* species in various clinical settings in both immunocompromised and immunocompetent hosts [14]. The application of multilocus DNA sequence analysis in various studies has indicated the prevalence of previously unknown "cryptic" *Aspergillus* species in clinical specimens [15, 16]. In two population based prospective studies in the USA and Spain, the prevalence of cryptic *Aspergillus* species detected in clinical specimens was found to be 10 and 12 %, respectively [17, 18]. *Aspergillus* species belonging to the section *Fumigati* (*A. fumigatus* complex) are often misidentified as they cannot be distinguished from *A. fumigatus* by conventional methods. Furthermore, these species often display intrinsic resistance to azoles and other antifungal drugs. *A. lentulus*, *A. udagawae*, *A. viridinutans*, and *A. thermomutatus* (*Neosartorya pseudofischeri*) have been associated with refractory cases of invasive aspergillosis (IA) [16, 19, 20]. Azole resistance in *Aspergillus* species is not restricted to section *Fumigati* but several species in other sections also exhibit elevated minimum inhibitory concentrations (MICs) for azoles and are listed section wise in Table 1.

Primarily, ITC is used in the treatment of chronic pulmonary aspergillosis (CPA) [21], while VRC is used as first line therapy of IA [22]. Recently another azole, ISA, has been approved for the primary treatment of IA [23]. POS is indicated as prophylaxis in high-risk patients, such as acute myeloid leukemia (AML) and stem cell transplant patients with graft-versus-host-disease (GVHD) [22]. However, in recent years, emergence of azole resistance in clinically relevant filamentous moulds due to prolonged azole exposure had led to increasing reports of treatment failure and breakthrough infections. Such development of azole resistance in chronic aspergillosis patients while on prolonged azole antifungals has been well documented [24]. Regarding azole resistance in *A. fumigatus*, it was first observed in collection of the late 1980s isolates in the USA from two patients treated with ITC [25]. Later, azole resistance in *A. fumigatus* was reported from the Netherlands in the 2000s with an annual prevalence ranging from 1.7 to 6 % [26•], followed by the UK reporting 28 % of azole-resistant *A. fumigatus* (ARAF) isolates in 2008 and 2009 corresponding to 14 and 20 % of patients, respectively [27]. Surveillance studies presently suggest the global presence of azole resistance in *A. fumigatus* and include reports from Europe, the Middle East, Asia, Africa, Australia, and most recently, North and South America [28••, 29••, 30]. Azole resistance in *A. fumigatus* is frequently the result of mutations in the *cyp51A* gene. Several non-synonymous point mutations at codons G54, M220, and G138 in this gene are primarily found in patients treated long-term with azoles [31]. Many of these mutations result in resistance to multiple anti-*Aspergillus* triazole antifungals [31]. However, in contrast to the point mutations observed in the host development route of azole resistance, in the environmental driven route *A. fumigatus* strains have in addition to the mutations in the *cyp51A* gene, a tandem repeat duplication in the promoter region, which increases the expression of the gene [31]. To date, two resistance mechanisms TR_{34}/L98H and TR_{46}/Y121F/T289A are reported to be associated with the environmental *A. fumigatus* isolates from soil and air samples [30, 31]. The patients directly acquire these azole-resistant isolates from the environment by inhalation, and in high-risk patients may result in life-threatening azole-resistant IA. The environmental selection of ARAF isolates is suggested to be a consequence of the wide use of triazole fungicides in agriculture, the latter being highly similar in their structures, as proven by homology modeling, to those used in medicine [3••, 32••]. Recently, point mutations, G54 and M220, in *cyp51A* gene of *A. fumigatus* environmental isolates were reported from India, Romania, Tanzania, and Germany [28••, 29••].

Table 1 An overview of published data on antifungal susceptibility against triazoles and molecular identification methods of clinical isolates of *Aspergillus* species

S. No.	Species	Method of Identification	MIC Range (μg/ml)			Method	References
			ITC	VRC	POS		
Section *Fumigati*							
1	*Aspergillus fumigatus*[a]	ITS, *β-actin, Cmd*	2–>32	0.5–>16	0.5–>32	CLSI, E test, EUCAST	[31, 39]
2	*Aspergillus fumigatiaffinis*	*β-tubulin*	0.5–8	0.25–6.66[b]	0.064–1.16[b]	CLSI	[19, 33]
3	*Aspergillus lentulus*	*β-tubulin*, ITS	0.43–16	1–7.5[b]	0.25–1	CLSI	[17, 19, 88]
4	*Aspergillus novofumigatus*	*β-tubulin*	>8–16	8–16	1	CLSI, EUCAST	[88, 89]
5	*Aspergillus thermomutatus*	ITS, *β-tubulin, Cmd*	1–2	2–16	0.5	CLSI	[90, 91]
6	*Aspergillus udagawe*	ITS, *β-tubulin*	0.125–2	0.25–2	0.125–0.25	CLSI	[17]
7	*Aspergillus viridinutans*	*β-tubulin*	1–14.4[b]	0.38–4[b]	0.064–0.5	CLSI, EUCAST, E-test	[19, 88, 89]
8	*Neosartorya pseudofischeri*	*β-tubulin*, ITS	0.25–16	2–6.66[b]	0.25–0.5	CLSI	[17, 19]
9	*Neosartorya udagawe*	ITS, *β-tubulin, rodA*	1–4	2–116	0.25–0.5	CLSI	[92]
Section *Flavi*							
10	*Aspergillus tamarii*	ITS	0.25–2	0.125–8	0.03–2	CLSI	[93]
Section *Nidulantes*							
11	*Emericella nidulans*	ITS, *β-tubulin*	0.125–2	0.032–4	0.032–4	CLSI	[33]
12	*Emericella unguis*	ITS, *β-tubulin*	0.25–0.5	0.125–16	0.25–2	CLSI	[33, 34]
Section *Usti*							
13	*Aspergillus calidoustus*	ITS, *β-tubulin, Cmd*	0.25–>32	1–16	0.5–32	CLSI, EUCAST	[17, 33, 89, 90, 94]
14	*Aspergillus ustus*	ITS	1–8	4–8		CLSI	[95]
Section *Circumdati*							
15	*Aspergillus ochraceus*	ITS, *β-tubulin, Cmd*	0.5–4	0.5–1	0.5–2	CLSI	[33, 90]
16	*Aspergillus sclerotiorum*	ITS, *β-tubulin*	0.25	1	2	CLSI	[33]
Section *Terrei*							
17	*Aspergillus terreus*	ITS, *β-tubulin*	0.06–2	0.06–4	0.03–4	CLSI, EUCAST	[33, 96]
Section *Nigri*							
18	*Aspergillus niger*	ITS, *β-tubulin, Cmd*	0.125–0.5	0.064–0.5	0.125–32	CLSI	[33]
Section *Versicolores*							
19	*Aspergillus sydowii*	ITS, *β-tubulin*	0.25–0.5	1–2	0.25–0.5	CLSI	[17]

ITC itraconazole, *VRC* voriconazole, *POS* posaconazole

[a] Isavuconazole MIC range 0.5–>8 μg/ml [31]

[b] Geometric mean MICs

Detection of Azole Resistance in Aspergillus

The patients who have a positive *Aspergillus* culture and the clinical intention is to treat requires determination of the species level complex of the isolate. Morphological methods are not always useful in species identification of rare and uncommon *Aspergillus* isolates. Correct identification of species with in the section *Fumigati* is clinically relevant specifically in context of *A. lentulus* that appears to have higher in vitro MICs to azoles compared to *A. fumigatus*, thus altering the therapeutic decisions [19]. The major molecular taxonomic tool, ribosomal sequencing, is widely used for fungal identification. The use of internal transcribed spacer regions (ITS) for inter-section-level identification and the *β-tubulin* locus for identification of individual species within the various *Aspergillus* sections is recommended [16, 33, 34]. The AFST is very crucial in settings where antifungal therapy is to be initiated in determining not only the best clinically active antifungal agent but also to detect resistance in *Aspergillus*. The Clinical Laboratory Standard Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods based on broth microdilution are highly reproducible and recommended to detect in vitro resistance against triazoles in filamentous fungi. EUCAST has published MIC breakpoints for ITC (>2 μg/ml), VRC (>2 μg/ml), and POS (>0.25 μg/ml) for defining resistance in *A. fumigatus* [35]. Although break points are not available for CLSI method, epidemiological cutoff values (ECVs) of ITC (1 μg/ml), VRC (1 μg/ml), and POS (0.25 μg/ml) for *A. fumigatus* and other five clinically relevant *Aspergillus* species have also been established [36]. Recently, the ECVs for the ISA using CLSI M38-A2 broth microdilution method and EUCAST have been described as 1 and 2 μg/ml, respectively, for *A. fumigatus* [37, 38]. It is recommended to perform in vitro susceptibility testing on multiple colonies, as different azole susceptibility phenotypes might be present in a single culture [39].

The major lacuna in detecting azole resistance is the problem that MIC determination of *Aspergillus* is not performed routinely in many microbiological laboratories worldwide. A simple agar-based screening method containing four-well plate with a growth control and ITC, VRC, and POS added to the agar [40] (VIP Check TMBeneden-Leeuwen, the

Netherlands) has the advantage of selecting ARAF strains. The incorporation of this screening approach in laboratories may result in isolating potential resistant isolates that could be sent to referral laboratories for AFST and to identify the resistant mechanisms.

Direct Detection of Azole Resistance in Culture Negative Clinical Samples

A few studies have reported direct detection of mutations in culture-negative clinical samples using real time PCR assays [41, 42]. Recently, a multiplex real-time PCR for detection of two environment associated resistance mechanisms, i.e., TR_{34}/L98H and TR_{46}/Y121F/T289A and for detection of *Aspergillus* species has become available (AsperGenius®, PathoNostics, Maastricht, the Netherlands). In the hematologic malignancies, the authors reported sensitivity and specificity of 88.9 and 89.3 %, respectively, and in intensive care unit patients 80 and 93.3 %, respectively [43]. Considering that the AsperGenius-PCR allows identification of only two mechanisms of resistance, therefore, a negative test result does not rule out the presence of azole resistance.

Molecular Mechanism of Azole Resistance in A. fumigatus

The characterization of two different sterol demethylase genes (*Cyp51A* and *Cyp51B*) in *A. fumigatus* have led to the description of mutations in *cyp51A* leading to azole resistance. A number of mutations have been described that confers different resistance profiles in *Aspergillus*. Various point mutations cause structural changes in enzyme's active site causing decreased affinity for its ligands [44]. These mutations have been associated with resistance to one or two azoles (G54, M220) or may confer cross-resistance [29••, 45]. Resistance due to the tandem repeat coupled with mutation L98H or Y121F/T289A in *cyp51A* has been proven to upregulate the enzyme expression [46, 47•]. In addition, mutations at *cyp51* gene have been related to disturbances in protein structure causing alteration in the active site of docking of the antifungal agent thereby leading to increase MICs. Until 2008, azole resistance in *A. fumigatus* was primarily attributed to mutations in *cyp51* gene; however, in 2010, Manchester reference laboratory, UK demonstrated that 43 % of the ARAF isolates were without any mutation in the *cyp51A* gene [27]. Apart from the abovementioned mutations, the genetic disturbances, such as overexpression of ABC or MFS efflux pumps such as *atrF*, *cdr1B*, *Cyp51B* overexpression and incorporation of exogenous cholesterol into *A. fumigatus* plasma membranes, have been well studied in *A. fumigatus* [31]. Also, another mutation in CCAAT-binding transcription factor complex subunit HapE, resulting in azole resistance, have been reported in *A. fumigatus* [48•]. Furthermore, *cyp51A* expression modulated by insertion of an *Aft1* transposon 370 bp upstream of the start codon has been reported [49]. A solitary study determined the azole MICs of 50 black *Aspergilli* (section *Nigri*) using modified EUCAST and Etest methods and compared the results with *cyp51A* sequences. ITC resistance was observed in 51 % of the clinical isolates, but azole cross-resistance was unusual. The authors found G427S and K97T mutations in *cyp51A* gene of black *Aspergilli*, which warrant further investigations [50].

Azole Resistance in Non-*Aspergillus* Moulds

The epidemiology of non-*Aspergillus* mould infections is changing probably due to the wide use of molecular and proteomic diagnostic methods; some moulds previously not reported in the literature are reported to cause invasive diseases suggesting the notion of emergence. In the last decade, environmental filamentous ascomycetes other than *Aspergillus* species are increasingly reported as agents of invasive diseases specifically in profoundly immunosuppressive patients, such as during prolonged neutropenia, GVHD, and in rejection episodes among solid organ transplant (SOT) patients. They include the relatively more commonly reported species of *Fusarium* and *Scedosporium* and rarely encountered species of *Acremonium*, *Penicillium*, and *Rasamsonia* [51, 52]. Infections with these fungi are lethal because the hosts they usually infect are incapable of mounting an effective immune response and because they tend to be resistant to antifungals. Table 2 lists species of filamentous moulds reported to exhibit elevated MICs of azoles and are discussed below:

Intrinsic Azole Resistance in Species of Fusarium

Fusarium spp. are widespread filamentous fungi that are primarily soil saprophytes and plant pathogens. In humans, 74 species of *Fusarium* are incriminated to cause infections, but the most commonly reported species include *Fusarium solani* complex, *F. oxysporum* complex, and *F. (Giberella) fujikuroi* complex, which include among others *F. verticillioides* and *F. proliferatum*. Also, to a lesser extent, both *F. dimerum* and *F. incarnatum-equiseti* species complex (SC) have been reported [52, 53]. They cause wide spectrum of infections, ranging from mildly superficial to fatally disseminated disease especially in patients with profound and prolonged neutropenia and/or T cell immunodeficiency [51, 53–57]. Fusariosis is highly fatal some reports suggesting 30 % survival rates, especially among patients with persistent neutropenia [51, 58, 59]. *Fusarium* species are intrinsically resistant to azole antifungals, and some clinically relevant species are also resistant to almost all currently used antifungals, including echinocandins and polyenes [60] (Table 2).

CLSI subcommittee on AFST in the M38-A2 document includes reproducible procedure for testing the antifungal susceptibilities of *Fusarium* spp. However, species-specific

clinical breakpoints (BPs) have not been established for this pathogen due to lack of both clinical trials and knowledge about the molecular resistance mechanisms. Recently, ECVs have been established for important *Fusarium* species and azoles including the highest ECVs for the three triazoles and *F. solani* SC (32 μg/ml). Lower POS and VRC ECVs were reported for *F. verticillioides* (2 and 4 μg/ml, respectively) and *F. oxysporum* SC (8 and 16 μg/ml, respectively) [61]. These triazole ECVs are higher than the dose dependent trough levels of azole antifungals and highlight the intrinsic resistant nature of *Fusarium* spp. [62]. It is emphasized that ECVs are not BPs, therefore, cannot envisage clinical response to therapy but predict those isolates that are more likely to harbor acquired molecular mutations conferring resistance. The molecular resistance mechanism in *Fusarium* is not understood, but combination of CYP51A amino acid alteration or overexpression may be involved. Recently, Fan et al. showed that CYP51 in *Fusarium* has three paralogues (CYP51A, CYP51B, and CYP51C), with CYP51C being unique to the genus and CYP51A deletion increases the sensitivity of *F. graminearum* to azoles [63].

Scedosporium Species

Scedosporium spp. are distributed in the environment as inhabitants of soil, polluted water, and animal excreta [64]. *Scedosporium apiospermum* complex and *Lomentospora prolificans* (previously *S. prolificans*) account for most infections prevalent worldwide and are associated with poor clinical outcomes [65]. The three main species within *S. apiospermum* complex are *S. apiospermum*, *S. boydii*, and *S. aurantiacum*. The complex also encompasses uncommon species, such as *S. minutispora* and *S. dehoogii* [66]. The fungus affect diverse patient population with varied clinical manifestations and risk factors include chronic obstructive lung disease, hematologic malignancy, SOT or hematopoietic stem cell transplantation (HSCT), corticosteroid use, neutropenia, and diabetes mellitus [65]. Disseminated infections associated with high mortality are typically caused by *L. prolificans* in immunocompromised hosts [67–69]. Recently described *S. aurantiacum* colonizes or infect the respiratory tract of patients with cystic fibrosis and other chronic lung disease [68]. The species-specific differences in virulence and AFST patterns have been reported in *S. apiospermum* complex and *L. prolificans* necessitating species level identification of the causative agent [66]. Species identification requires sequencing of the *β-tubulin*, *β-actin*, and *calmodulin* gene targets [70•].

The majority of *Scedosporium* isolates exhibit multiple antifungal resistance including azoles, and data on species-specific susceptibility patterns is limited (Table 2). A comprehensive study on AFST of 332 molecularly identified *Scedosporium* isolates demonstrates that *L. prolificans* exhibits the highest GM MICs of all antifungal drugs including azoles (ITC GM MIC, 32 μg/ml; VRC GM MIC, 15.4 μg/ml; POS GM MIC, 32 μg/ml; and ISA GM MIC, 25.6 μg/ml) [71••]. All *S. apiospermum* and *S. boydii* were found to have high MIC values of AMB (MIC_{50} 4 μg/ml), ITC (MIC_{50} 16 g/ml), and ISA (MIC_{50} >4 μg/ml). POS and VRC are the most promising drugs against *Scedosporium* species other than *L. prolificans*. Limited in vitro activity of VRC was found only for *L. prolificans* and *S. dehoogii*. Furthermore, *S. aurantiacum* also exhibits elevated MICs for ITC, POS, and ISA. Overall, VRC has activity against *S. apiospermum*, *S. boydii*, and *S. aurantiacum* [71••]. The consequence of varied MICs for azoles reflects that the susceptibilities of individual isolates are difficult to predict, and thus, susceptibility testing of clinical isolates remains essential for targeted treatment. It is emphasized that clinical breakpoints, ECV, or molecular resistance mechanism for *Scedosporium* are not yet elucidated; therefore, interpretation of MIC testing remains difficult. Concordance among in vitro resistance profiles and in vivo outcome has been reported [72], and VRC treatment of *L. prolificans* infections showed a 40 % clinical response despite an MIC_{50} of 4 μg/ml [67].

Rare and Emerging Species of Rasamsonia, Paecilomyces, Penicillium, and Acremonium

Rasamsonia argillacea, previously known as *Geosmithia argillacea*, is an emerging pathogen that in the past has been misidentified as *Penicillium* or *Paecilomyces* species. *Rasamsonia argillacea* complex causes pulmonary infection in chronic granulomatous disease patients [73]. Also, fatal infection in stem cell transplant patients has been reported [74]. AFST reveals that species belonging to *Rasamsonia* complex are resistant to VRC (Table 2) and variably resistant to ITC, amphotericin B, and POS [9, 75]. Several studies highlight that *R. argillacea* infection to be ruled out in patients whose fungal infections worsen and whose cultures are reported as *Penicillium* species, especially if these patients are receiving VRC [9]. Similarly, antifungal susceptibility profiles of *Paecilomyces variotii* and other species of *Paecilomyces* including *P. formosus*, *P. dactylethromorphus*, and *P. divaricatus* are reported to be VRC resistant [76]. *Paecilomyces* infections are uncommon, but serious manifestations include pneumonia, sinusitis, osteomyelitis, disseminated infection, and fungemia [77, 78, 79•]. VRC-resistant *P. oxalicum* break through infections have recently been reported in AML and in chronic aspergillosis patients while on VRC therapy [10].

Another genus that seems to be emerging and show limited VRC activity as in the abovementioned moulds is *Acremonium*, which includes about 150 species but only a few implicated as human pathogens [80, 81]. The most common species are *A. kiliense* and *A. falciforme*, and others including *A. roseogriseum*, *A. strictum*, *A. patronii*, and *A. recifei* are reported as opportunistic pathogens mainly affecting immunocompromised hosts following HSCT, SOT,

Table 2 Literature review of triazoles antifungal susceptibility data of molecularly identified clinically significant non-*Aspergillus* moulds

S. No.	Species	Method of identification	MIC Range (μg/ml)				Method	References
			ITC	VRC	POS	ISA		
	Fusarium spp.							
	FFSC							
1.	*Fusarium fujikuroi*	*β-tubulin*, *RPB1*, *RPB2*, ITS	16->16	2–16	0.5–4	4–16	CLSI	[60, 61, 97]
2.	*Fusarium ananatum*	*RPB2*	>16	1–4	0.5–1	1–8	CLSI	[60]
3.	*Fusarium andiyazi*	*RPB2*, ITS, *β- tubulin*	>64	1–4	0.25–4	1–8	CLSI	[60, 98]
4.	*Fusarium acutatum*	*RPB2*	>16	2–8	1–2	4->16	CLSI	[60]
5.	*Fusarium anthophilum*	*RPB2*	16->16	1–4	0.25–0.5	1–8	CLSI	[60]
6.	*Fusarium napiforme*	*RPB2*, ITS	>16	1–4	2->16	2–8	CLSI	[60, 97]
7.	*Fusarium nygamai*	*RPB2*, ITS	>16	4->16	>16	8->16	CLSI	[61, 97]
8.	*Fusarium proliferatum*	*CAM*, *β-tubulin*, *RPB1*, *RPB2*, ITS	16->64	1->16	0.12->32	4->16	CLSI, EUCAST	[51, 59–61, 97–99]
9.	*Fusarium sacchari*	*RPB2*	>16	1–4	0.5->16	2–16	CLSI	[60]
10.	*Fusarium subglutinans*	*RPB2*, ITS	0.5-≥16	1–8	0.125- ≥16	2–4	CLSI	[60]
11.	*Fusarium temperatum*	*RPB2*	>16	1	0.25	1	CLSI	[60]
12.	*Fusarium thapsinum*	*RPB2*, ITS	>16	1–4	2->16	8–16	CLSI	[60, 98]
13.	*Fusarium verticillioides*	*β-tubulin*, *RPB1*, *RPB2*, ITS	1->64	0.5->16	0.12->16	1–2	CLSI, EUCAST	[51, 59–61, 97, 98]
14.	*Fusarium dimerum SC*	*β-tubulin*, *RPB1*, *RPB2*, ITS	1->16	1–16	0.5->16	ND	CLSI, EUCAST	[51, 61, 98]
15.	*Fusarium incarnatum-equiseti SC*	*β-tubulin*, *RPB1*, *RPB2*, ITS	1->16	0.5->16	0.5–16	ND	CLSI	[61]
16.	*Fusarium oxysporum SC*	*β-tubulin*, *RPB1*, *RPB2*, ITS	1->16	0.5->16	0.5–16	ND	CLSI, EUCAST, E-test	[51, 61, 98]
17.	*Fusarium solani SC*	*β-tubulin*, *RPB1*, *RPB2*, ITS	0.5->16	0.5->16	1->16	ND	CLSI, EUCAST	[51, 61, 98]
18.	*Fusarium keratoplasticum*	ITS, *RPB2*, *β-tubulin*	>64	8	>16	ND	CLSI	[98]
19.	*Fusarium petroliphilum*	ITS, *RPB2*, *β-tubulin*	>64	8–16	>16	ND	CLSI	[98]
	FTSC							
20.	*Fusarium acuminatum*	ITS, *RPB2*, *CAM*	ND	4	ND	ND	CLSI	[97]
21.	*Fusarium avenaceum*	ITS, *RPB2*, *CAM*	ND	4	ND	ND	CLSI	[97]
22.	*Fusarium graminearum SC*	ITS, *RPB2*, *CAM*	ND	4	ND	ND	CLSI	[97]
23.	*Fusarium sporotrichioides SC*	ITS, *RPB2*, *CAM*	ND	>16	ND	ND	CLSI	[97]
	Scedosporium / Pseudallescheria spp.							
24.	*Lomentospora prolificans*	rRNA sequence analysis, AFLP	8->16	4->16	8->16	4->16	CLSI	[71••, 100, 101]
25.	*Scedosporium apiospermum*	AFLP	0.25->16	0.25–8	1->16	0.25–8	CLSI	[71••, 101]
26.	*Scedosporium aurantiacum*	AFLP	1–128	0.5–32	4–16	0.5–32	CLSI	[71••, 101, 102]
27.	*Scedosporium boydii*	*β-tubulin*, *CAL*, *RPB2*	≥8–32	1–64	ND	1–64	CLSI	[71••, 102, 103]
28.	*Scedosoprium dehoogi*	AFLP	0.5->16	0.5–>16	0.5–>16	2–>16	CLSI	[71••]
29.	*Pseudallescheria ellipsoidea*	AFLP	0.25–32	0.125–16	1->16	0.125–16	CLSI	[71••, 101, 102]
30.	*Pseudallescheria angusta*	AFLP	0.25–128	0.25–32	1->16	0.25–32	CLSI	[71••, 102]
31.	*Pseudallescheria minutispora*	AFLP	0.5->16	0.25–2	0.5–>16	2–16	CLSI	[71••]
	Acremonium spp.							
32.	*Acremonium kiliense*	ITS	>16	0.125–4	2	ND	CLSI	[82••, 104]
33.	*Acremonium sclerotigenum-A. egyptiacum*	ITS	>16[b]	2[b]	2[b]	ND	CLSI	[82••]
34.	*Acremonium implicatum*	ITS	>16[b]	8[b]	>16[b]	ND	CLSI	[82••]
35.	*Acremonium persicinum*	ITS	>16[b]	8[b]	8[b]	ND	CLSI	[82••]
36.	*Acremonium atrogriseum*	ITS	8[b]	2[b]	2[b]	ND	CLSI	[82••]
37.	*Acremonium fusidioides*	ITS	>16[b]	2[b]	2[b]	ND	CLSI	[82••]
38.	*Acremonium strictum*	ITS	>8	ND	8	ND	CLSI	[105]
	Rasamsonia spp.							
39.	*Rasamsonia aegroticola*	ITS, *β-tubulin*, *Cmd*	1–2	>16	1–4	ND	CLSI	[106]
40.	*Rasamsonia argillacea*	ITS, *β-tubulin*, *Cmd*	1–32	16->16	0.25–8	ND	CLSI, EUCAST	[9, 106–108]
41.	*Rasamsonia cylindrospora*	ITS, *β-tubulin*, *Cmd*	1–2	>16	1–8	ND	CLSI	[106]
42.	*Rasamsonia eburnean*	ITS, *β-tubulin*, *Cmd*	1–4	>16	1–4	ND	CLSI	[106]
43.	*Rasamsonia piperina*	ITS, *β-tubulin*, *Cmd*	0.5–1	8->16	0.06–2	ND	CLSI	[106]
	Paecilomyces spp.							
44.	*Paecilomyces variotii*	ITS	0.008–4	1–32	0.008–0.125	ND	CLSI	[76]
45.	*Paecilomyces dactyletromorphus*	ITS	0.031–0.125	32	0.03	ND	CLSI	[76]
46.	*Paecilomyces divaricatus*	ITS	0.25–1	32	0.125–0.25	ND	CLSI	[76]
47.	*Paecilomyces formosus*	ITS	0.063–1	16–32	0.031–0.25	ND	CLSI	[76]
48.	*Penicillium oxalicum*	*β-tubulin*	0.5–2	2->16	0.125–0.5	8	CLSI	[10]
	Melanized fungi							
49.	*Curvularia aeria*	ITS	>16[a]	8[a]	1[a]	ND	CLSI	[109]
50.	*Curvularia lunata*	ITS	0.03–32	0.25–1	<0.03–0.5	ND	CLSI	[109]
51.	*Curvularia protuberata*	ITS	>16	8–16	0.5–1	ND	CLSI	[109]
52.	*Ochroconis mirabilis*	ITS, D1D2, *β-tubulin*	7[a]	11.09[a]	18.23[a]	ND	CLSI	[110]

Table 2 (continued)

S. No.	Species	Method of identification	MIC Range (μg/ml)				Method	References
			ITC	VRC	POS	ISA		
53.	*Pyrenochaeta romeroi*	ITS	0.5	4->8	0.25–0.5	0.125	CLSI, EUCAST	[111, 112]
54.	*Veronaea botryosa*	ITS	0.25–1	1–8	0.031–0.25	4–16	CLSI	[113, 114]

Abbreviations: ITC Itraconazole, *VRC* Voriconazole, *POS* Posaconazole, *ISA* Isavuconazole, *FFSC F. fujikuroi* species complex, *FSSC F. solani* species complex, *FTSC F. tricinctum* species complex, *ND* Not Done, *AFLP* amplified fragment length polymorphism

[a] Geometric mean MIC data of these isolates is given here

[b] MIC_{90} data of these isolates is given

and hematologic malignancies. The fungus causes varied manifestations including pneumonia, arthritis, osteomyelitis, endocarditis, meningitis, peritonitis, and fungemia [82••]. The limited in vitro AFST data show high MICs for POS, VRC, and ITC (Table 2) when compared to those found for other hyaline molds, such as *A. fumigatus* [83].

Melanized Fungi and Azoles

Melanized fungi are ubiquitous saprophytic fungi in the environment. Their prevalence estimated in clinical samples showed only 10 % of them being clinically significant [84]. However, their clinical significance in the last few years is increasing not only in immunocompetent individuals but also in immunocompromised patients. Their clinical manifestations span from respiratory tract inflictions like allergies, superficial infections to fatal disseminated cases [85••]. In SOT subjects, dark pigmented fungi are recognized as most recent emerging opportunistic pathogens. In SOT recipients, species of *Verrucosa* and *Ochroconis* are mainly reported from lung and liver transplants patients. Emergence of melanized fungi in pathologies requires the use of accurate diagnostic tools, such as molecular methods. The limited data on the in vitro AFST of melanized fungi are available in the literature, mainly inferred from clinical cases, with potential variations due to the use of different methodologies [86]. It is emphasized that there are no defined BPs and no established correlation between MIC and clinical outcome, and also, the marked differences in the in vitro susceptibility results both at the genus and at the species levels reflect the phylogenetic diversity of these fungi. Therefore, presently exploring azole resistance in melanized moulds is challenging. Table 2 lists melanized fungi reported to exhibit elevated azole antifungal MICs.

Perspectives and Conclusions

Intrinsic or acquired antifungal resistance in pathogenic fungi may be encountered in both antifungal drug exposed and antifungal drug-naive patient. Furthermore, prior antifungal treatment confers a selection pressure and increases the possibility of resistance in patients failing therapy. Thus, in both scenarios, detection of resistant isolates requires appropriate and carefully performed susceptibility testing and endpoint interpretation. Intrinsically resistant species can be diagnosed through correct species identification, but their identification is challenging using phenotypic methods, as non-*Aspergillus* moulds may poorly sporulate, e.g., *Fusarium* and *Scedosporium* species complexes on routine media. As discussed above, molecular identification tests could reliably identify the isolates but are cumbersome and not performed in routine microbiology laboratories. Nevertheless, with the availability of isolate, less cumbersome mass spectrometric species identification is possible. Identification of fungi using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS is rapid and potentially economical compared to sequence-based technologies, after equipment is purchased. However, current commercial MALDI-TOF MS reference databases contain a limited number of filamentous fungal spectra. Thus, substantial augmentation of the spectral library is required for routine laboratory and several studies have highlighted the importance of in-house database creation for species of *Aspergillus*, *Fusarium*, and *Scedosporium* filamentous fungi for reaching a consensus between proteomic and sequence-based identifications [12••, 34, 87••]. It is emphasized that without the creation of a highly stringent supplemental database, MALDI-TOF MS analysis is often unable to achieve species, and sometimes genus level identification compared to that of sequencing.

Regarding the progress made toward the description of azole resistance mechanisms at molecular level, barring *A. fumigatus*, the underlying mechanism remains unknown in a number of resistant filamentous moulds. MIC determination is still the most reliable procedure for surveillance of azole resistance in clinical isolates; however, molecular methods allowing detection of resistance warrants further standardization of techniques for effective integration in the routine laboratories. Also, standardized techniques detecting azole resistance in culture-negative specimens for rapid

diagnosis and effective therapy need to be developed. Finally, regarding filamentous fungi, continued efforts to improve the reliability of AFST followed by analysis of the prevalence of resistance at molecular level are warranted.

Compliance with Ethical Standards

Conflict of Interest Anuradha Chowdhary, Aradhana Masih, Cheshta Sharma declare that they have no conflict of interest. Cheshta Sharma was supported by a research grant from University Grants Commission Research Fellowship, India (F.2-15/2003 SA-I).

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- • Of importance
- •• Of major importance

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