

Review Article

Acquired antifungal drug resistance in *Aspergillus fumigatus*: epidemiology and detection

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Voriconazole is the recommended agent for invasive aspergillosis, with lipid amphotericin B or caspofungin as second line treatment choices. Being the only agents available in oral formulation, azoles are used in chronic infections and often over longer time periods. In addition to being used in clinical medicine, azoles are employed extensively in agriculture. Azole-resistant *Aspergillus* has been isolated in azole naïve patients, in azole exposed patients and in the environment. The primary underlying mechanism of resistance is as a result of alterations in the *cyp51A* target gene, with a variety of mutations found in clinical isolates but just one identified in an environmental strain (a point mutation at codon 98 accompanied by a tandem repeat in the promoter region). Much less is currently known about echinocandin resistance in *Aspergillus*, in part because susceptibility testing is not routinely performed and because the methods suffer from technical difficulties and suboptimal reproducibility. Clinical breakthrough cases have been reported however, and resistance has been confirmed *in vivo*. In this paper we review the current knowledge on epidemiology, susceptibility testing and underlying mechanisms involved in azole and echinocandin resistance in *Aspergillus*.

Keywords *Aspergillus fumigatus*, echinocandin, azole, resistance

Epidemiology

Therapeutic options for aspergillosis are limited, particularly so for oral formulations, with azole drugs forming the backbone of therapy [1]. Many patients that develop resistant infections fail treatment, so resistance is an important factor in the outcome of these cases [2]. Azole resistance has predominantly been reported for *Aspergillus fumigatus*, the species which accounts for approximately 80% of invasive infections [3]. Yet itraconazole resistance has also been detected in other species in the *Fumigati* section such as *Aspergillus lentulus* and *Neosartorya pseudofischeri* [4], as well as species in other taxonomic

sections including *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus flavus* [5,6]. Most of the susceptibility data relates to itraconazole, although increasing reports describe resistance to the newer azole agents including posaconazole and voriconazole.

The first cases of itraconazole resistance were from the late 1980s [7], yet the vast majority have been detected since the turn of the millennium. The frequency is largely undefined, as many centres do not routinely test the susceptibility of their aspergilla isolates. Resistance has currently been reported in Belgium, Canada, China, Denmark, France, Norway, Spain, Sweden, The Netherlands, UK and the USA. Where reported, the frequency of resistance differs significantly. A meta-analysis (S. J. Howard, unpublished results) was conducted of *A. fumigatus* susceptibility surveillance studies, including those with more than 50 clinical isolates and covering the last two decades. The frequency in these 13 reports (amounting to almost 5,000 isolates) ranged from 0–6%, with an arithmetic mean of 2%.

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However, centres in the UK (Manchester) and The Netherlands (Nijmegen) have described particularly high frequencies (5 and 6% respectively), and a significant increase in azole resistance in recent years [2,8,9]. The high incidence may be partly explained by the patient population (primarily chronic aspergillosis cases on long term therapy in Manchester), and high use of agricultural azoles (Nijmegen), but the reason for the change in frequency remains unclear.

Much less is known regarding acquired resistance to echinocandins. Caspofungin is licensed for salvage treatment of invasive aspergillosis and is recommended as second line treatment by the IDSA (Infectious Diseases Society of America) [1]. Breakthrough infections with *A. fumigatus* isolates with elevated minimum effective concentrations (MECs) have been reported sporadically [10–12], but may be under-diagnosed as echinocandin susceptibility testing of *Aspergillus* is much less straight forward than for azoles (as described below) and less frequently performed.

Primary resistance to amphotericin B is well recognized in *A. terreus*, and in some *A. flavus* and *A. ustus* isolates [13,14]. More recently it has also been demonstrated in several species in the *Fumigati* section including *A. lentulus* and *A. fumigatiaffinis* [4]. However, thus far, evidence of development of acquired resistance is lacking, therefore this will not be discussed further in this review.

Susceptibility and breakpoints

Standardization of *in vitro* susceptibility testing methodology for filamentous fungi has aided research in this area, including publication of the CLSI (Clinical and Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) standards [15,16]. Both reference methods are broth dilution based but differ with respect to glucose concentration in the medium (0.2 vs. 2% for CLSI and EUCAST respectively), fungal inoculum size ($0.4\text{--}5 \times 10^4$ vs. $2\text{--}5 \times 10^5$ CFU/ml) and shape of the microtitre wells (U-shaped vs. flat bottom). Minimum inhibitory concentration (MIC) determinations are relatively straightforward for azoles and amphotericin B due to the growth-versus-no-growth pattern of inhibition, and MICs (defined as the lowest concentration leading to a visually clear well) obtained by the reference methods are in close agreement. Clinical breakpoints (taking pharmacokinetics/pharmacodynamics parameters and MIC-outcome relationships into account) have not yet been established. However, epidemiological cut off values have been proposed but not yet ratified by the CLSI and EUCAST committees for *A. fumigatus* and the licensed azoles, i.e., itraconazole: $\leq 1 \mu\text{g/ml}$, voriconazole $\leq 1 \mu\text{g/ml}$ and posaconazole $\leq 0.25 \mu\text{g/ml}$ [8,17,18]. These may serve as breakpoints for susceptibility until clinical breakpoints are available.

With increased prevalence of azole resistance, routine testing of aspergilli has become more important. The broth dilution format of the reference methods is not suitable for use in most clinical laboratories (too laborious and requires extensive training), but rather can be performed by specialist mycology centres. However, the commercially available Etests which are commonly used for antibacterial susceptibility testing in routine laboratories, may be useful. Furthermore Etests have been evaluated against CLSI methodology and show excellent correlation for voriconazole [19]. For itraconazole and posaconazole the results are somewhat more variable and at best 80–100% agreement is observed for *A. fumigatus* when the Etest is read at 48 hours [20–23]. These agreement figures however represent best performance as the comparative studies have been undertaken in specialist mycology laboratories. Another approach is the use of azole containing agars (for example with itraconazole 4 mg/l, voriconazole 1 mg/l and posaconazole 0.5 mg/l) as screening test for the identification of potentially resistant isolates. This technique has been evaluated for *A. fumigatus* and may prove a useful future screening tool in routine laboratories, identifying isolates that need further investigation (Van der Linden JWM, Arendrup MC, Van der Lee HAL, *et al.* Azole containing agar plates as a screening tool for azole resistance of *Aspergillus fumigatus*. Trends in Medical Mycology, Athens Greece, 2009, Abstract O1.1.).

The susceptibility end points for the echinocandins are more difficult to determine due to significant trailing growth. Thus, an MEC is defined as the lowest concentration leading to aberrant growth. MEC reading is labour intensive as it requires microscopic evaluation of micro-morphological changes. Furthermore, significant differences were observed comparing published caspofungin MEC₅₀ values for *A. fumigatus* determined at different centres to the CLSI method suggesting further work is needed to increase reproducibility and interlaboratory agreement [24,25]. Finally, the correlation between MEC and *in vivo* susceptibility has been questioned in a recent paper reporting clinical failure and *in vivo* resistance in an animal model for an isolate with an MEC in the wild type range [12].

Mechanisms of azole resistance

The majority of azole resistant *A. fumigatus* isolates studied to date have been found to contain an alteration in the target protein sterol 14 α -demethylase (Cyp51), potentially inhibiting drug binding. These structural changes are as a result of single nucleotide polymorphisms in the gene (*cyp51A*) encoding the protein leading to amino acid substitutions.

Cyp51A mutations described in *A. fumigatus* isolates currently are shown in Table 1. A limited number have thus

Table 1 Mutations in the *cyp51A* gene identified in azole resistant *Aspergillus fumigatus* isolates.

Genotype	Amino acid substitutions	Typical phenotype	References
<i>Hot spot mutations commonly found in azole resistant isolates^a</i>			
G54	E, K, R, V, W	Itraconazole and posaconazole resistant, voriconazole susceptible	[2,17]
L98	H	Pan-azole resistant	[2,9,30]
M220	K, I, T, V	Itraconazole and posaconazole resistant, voriconazole variable	[2,17]
<i>Mutations found less frequently in resistant isolates^b</i>			
N22	D	Itraconazole resistant, other azole susceptibilities not reported	[49]
S52	T	Pan-azole resistant	[45]
G138	C, R	Pan-azole resistant	[2]
Q141	H	Pan-azole resistant	[45]
H147	Y	Pan-azole resistant	[2]
P216	L	Itraconazole and posaconazole resistant, voriconazole susceptible	[2]
M236	K, T, V	Itraconazole resistant	[50]
S297	T	Itraconazole and posaconazole resistant, voriconazole susceptible	[9,30]
P394	L	Itraconazole resistant, other azole susceptibilities not reported	[49]
Y431	C	Pan-azole resistant	[2]
G434	C	Pan-azole resistant	[2]
T440	A	Itraconazole resistant, other azole susceptibilities not reported	[49]
G448	S	Pan-azole resistant	[2]
Y491	H	Itraconazole resistant, other azole susceptibilities not reported	[49]
F495	I	Itraconazole and posaconazole resistant, voriconazole susceptible	[9,30]
<i>Mutations found in azole susceptible and resistant isolates</i>			
F46	Y	Azole susceptible and resistant	[2,17]
M172	V	Azole susceptible and resistant	[2,17]
N248	T	Azole susceptible and resistant	[2,17]
D255	E	Azole susceptible and resistant	[2,17]
E427	G, K	Azole susceptible and resistant	[2,17]

^aMutations confirmed to be associated with azole resistance by transformation (see text).

^bMutations which have yet to be confirmed associated with azole resistance.

Note: Some were found in combination with other *cyp51A* mutations.

far been proven to confer resistance. Mutations at codons 54, 98 and 220 are reported with much greater frequency, plus their link with azole resistance has been confirmed by transformation of the altered gene into wild-type strains, so these codons are known as hot spots. Mutations in codons 46, 172, 248, 255, and 427 have also been found

in azole susceptible strains and so are unlikely to be associated with resistance [2].

Cyp51A sequences have been studied in three entire clinical culture collections. In the azole resistant isolates from The Netherlands, alterations at only two different *cyp51A* codons were revealed, with a predominance (94%) of alterations at codon 98 [9]. Similarly, Rodriguez-Tudela *et al.* reported three affected mutational sites in the Spanish report, again the majority (53%) at L98 [17]. Whereas in the Manchester (UK) collection, a greater variety of mutations (18 in total) in the *cyp51A* gene was found, and there was no prevalence of any one alteration [2].

Serial sampling of *Candida* isolates from the same patient over time suggests continuous rather than independent evolution, as mechanisms/mutations are accumulated [26–28]. The converse may be the case for *Aspergillus*, although the sample size is small, as in two reports same-strain isolates have revealed different mutations without accrual [2,29]. This could indicate replacement with a more dominant mutant, or could be a sampling issue.

In addition to target mutation alterations, resistance may also be associated with an increase in target concentration. For example those bearing a mutation at codon 98 also contain a 34-base tandem repeat in the promoter region, which causes an eight-fold increase in expression of the *cyp51A* gene [30]. Furthermore a recent report described a four- to five-fold increase expression of the *cyp51A* gene in a resistant isolate without mutations in the target gene [31]. Upregulation of efflux pumps, decreasing the intracellular antifungal concentration, is one of the most frequently characterized azole resistance mechanisms in yeasts [32]. Yet relatively few *Aspergillus* strains have been investigated for efflux pump expression to date, so the extent of their contribution is unknown, but has been proposed in some *A. fumigatus* [33,34, Mowat E, Warn P, Denning D, *et al.* The potential role of *Afumdr4* in azole resistance during *Aspergillus fumigatus* multicellular growth. Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington USA, 2008, Abstract M2190].

Multiple mechanisms occurring simultaneously are common in yeasts [28,32,35], so it would not be entirely surprising that the picture might also be more complex in *Aspergillus* than originally believed. Furthermore, a recent study suggests that mechanisms other than *cyp51A* mutations may be becoming more common, i.e., 39% of resistant *A. fumigatus* from 2006–2009 demonstrated a wild-type *cyp51A* sequence compared to 0% prior to 2006 (Harrison E, Howard SJ, Buied A, *et al.* The changing prevalence of azole resistance mechanisms in *Aspergillus fumigatus*. Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco USA, September 2009, Abstract M1720).

Azole cross-resistance

The risk of cross-resistance between the azole compounds is high, in one report 74% and 65% of itraconazole resistant isolates were cross-resistant to posaconazole and voriconazole respectively [2].

Cross-resistance patterns are closely linked with the position of the mutation in the *cyp51A* gene [2,17]. Isolates with alterations at codons 98, 138, 431, 434 and 448 generally demonstrate a pan-azole resistance phenotype. Isolates with mutations at codon 54 remain voriconazole susceptible although cross-resistant to posaconazole. Cross-resistance patterns in isolates with M220 alterations appear to be unpredictable, particularly with respect to voriconazole.

With the high risk of cross-resistance, the likelihood of different mutations occurring over time, and the potential contribution from other mechanisms of resistance, susceptibility should be routinely monitored as the resulting pattern of cross-resistance can change.

Mechanisms of echinocandin resistance

Manipulated or laboratory-selected strains with various degrees of caspofungin resistance have been described [36–38]. Some of these strains have been found to have mutations in the *ECM33* gene (*AfuEcm33*), encoding cell wall proteins important for fungal cell wall organization. In addition to possessing caspofungin resistance, they were also hypervirulent in animal models [37]. Laboratory engineered strains with mutations in the *FKS1* gene encoding a subunit of the β -1,3-D-glucan synthase enzyme involved in cell wall synthesis and decreased susceptibility to caspofungin have been generated [36]. This mechanism has also been detected in clinical *Candida* isolates with reduced susceptibility to caspofungin [10,39–44]. In other resistant laboratory-selected *Aspergillus* mutants with unknown mechanism, the glucan synthase exhibited a wild-type *AfFKS1* gene sequence, function, level, and the enzyme itself was susceptible to caspofungin [36].

However, only very few clinical isolates associated with treatment failure have been investigated [10–12]. In two such isolates, sequencing of the *FKS1* target gene was performed without detection of mutations, but in one of the cases expression of the *FKS1* gene was found to be upregulated [10,12]. Although there are limited data currently, these observations suggest that while mutations in the hot spot regions of the *FKS* gene is the predominant resistance mechanism in *Candida*, it is possible that mechanisms outside the target gene may be more important in *Aspergillus*.

Development of resistance

Current data suggests resistant infections can develop by two routes, i.e., either *in situ* within the lung [2,31], or acquisition of a resistant *A. fumigatus* from the environment potentially driven by agricultural use of azole compounds [45]. Clinical data is scarce, but risk factors associated with the development of azole resistance during treatment include chronic *Aspergillus* infections, the presence of aspergillomas (high fungal burden) and azole exposure [2]. To back up the drug exposure theory, environmental azole resistant strains have been found in The Netherlands [45] and Denmark (Mortensen KL, Mellado E, Arendrup MC. Azole resistant environmental *A. fumigatus* in Denmark. Advances Against Aspergillosis, Rome Italy, 2010, Abstract #103) and resistant isolates with the same resistance mechanism as in environmental isolates have been found in azole naïve patients.

Conclusion and perspective

Resistance in *Aspergillus* is emerging. Echinocandin resistance has been detected in only a few clinical cases involving echinocandin exposed patients but may well be under-diagnosed due to lack of susceptibility testing and to methodological issues hampering the ability to correctly identify resistant isolates. Azole resistant isolates have been detected in azole exposed patients, in azole naïve patients, as well as in the environment of at least selected areas of Europe. We are now able to have confidence in the detection of azole resistance *in vitro* with advances in susceptibility testing for filamentous fungi, and with the ability to readily detect some mechanisms known to confer resistance. However, it is well recognized that aspergilli are frequently not isolated from primary samples in aspergillosis patients. In these cases *in vitro* susceptibility testing is not possible. A technique which may be of benefit in these cases is detection of *cyp51A* mutations in primary samples using beacon technology, and several assays have been published [46–48]. The advantages of this type of assay are not only the ability to detect resistance in culture negative samples, but also potentially quicker resistance detection in culture positive samples. However what the assays lack is the ability to detect the influence from other mechanisms of resistance. With concerns that resistance cannot consistently be detected using molecule techniques, and due to the increasing number of resistant cases, susceptibility testing of *Aspergillus* isolates before and during treatment is important.

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