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Molecular mechanisms of acquired antifungal drug resistance in principal fungal pathogens and EUCAST guidance for their laboratory detection and clinical implications

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The increasing incidence and changing epidemiology of invasive fungal infections continue to present many challenges to their effective management. The repertoire of antifungal drugs available for treatment is still limited although there are new antifungals on the horizon. Successful treatment of invasive mycoses is dependent on a mix of pathogen-, host- and antifungal drug-related factors. Laboratories need to be adept at detection of fungal pathogens in clinical samples in order to effectively guide treatment by identifying isolates with acquired drug resistance. While there are international guidelines on how to conduct *in vitro* antifungal susceptibility testing, these are not performed as widely as for bacterial pathogens. Furthermore, fungi generally are recovered in cultures more slowly than bacteria, and often cannot be cultured in the laboratory. Therefore, non-culture-based methods, including molecular tests, to detect fungi in clinical specimens are increasingly important in patient management and are becoming more reliable as technology improves. Molecular methods can also be used for detection of target gene mutations or other mechanisms that predict antifungal drug resistance. This review addresses acquired antifungal drug resistance in the principal human fungal pathogens and describes known resistance mechanisms and what in-house and commercial tools are available for their detection. It is emphasized that this approach should be complementary to culture-based susceptibility testing, given the range of mutations, resistance mechanisms and target genes that may be present in clinical isolates, but may not be included in current molecular assays.

Introduction

Amphotericin B was the first antifungal drug for systemic treatment of invasive fungal infections (IFIs), and has been the 'gold standard' of antifungal therapy for nearly 50 years.¹ It has activity against a broad range of human pathogenic fungi, that includes those under review, and extremely few develop resistance following exposure to the drug.

The introduction to the clinic of the triazole antifungals itraconazole and fluconazole in the 1980s offered more therapeutic

options for treating IFIs. Additionally, azoles present fewer class-related side effects compared with amphotericin B.²

Fluconazole resistance in *Candida albicans* was first reported in the 1990s in AIDS patients who were receiving prolonged low-dose treatment courses for recurrent mucosal candidiasis.³ Investigations revealed that multiple molecular mechanisms could be involved in acquired resistance in *C. albicans*.⁴ *Candida krusei* species (new taxonomic name: *Pichia kudriavzevii*) are intrinsically resistant to fluconazole, while *Candida glabrata* has reduced susceptibility. There are increasing reports of acquired

fluconazole resistance emerging in *Candida tropicalis* and *Candida parapsilosis*. The newly identified *Candida auris*, which has spread widely across the globe, is characterized by frequent acquired resistance to fluconazole, amphotericin B and often multi-antifungal drug resistance.⁵

Although most clinical isolates of *Cryptococcus neoformans* are susceptible to fluconazole, the emergence of drug-resistant strains has been reported, usually linked to prior drug exposure.⁶

Subsequent to its introduction for clinical use, acquired resistance to itraconazole emerged in *Aspergillus fumigatus*.^{7,8} The third-generation triazoles, voriconazole, posaconazole and isavuconazole have greater activity against mould fungi than itraconazole. Voriconazole was licensed for the treatment of invasive aspergillosis (IA) in the early 2000s and soon became the drug of choice for that indication. However, within a few years, reports of multi-triazole resistance impacting these triazoles emerged from the Netherlands and the *A. fumigatus* isolates involved were found to have novel molecular changes in the gene encoding the target of the azoles, *cyp51A*.⁹ Additionally, some triazole-resistant *A. fumigatus* isolates have *cyp51*-independent resistance mechanisms.

The echinocandins caspofungin, micafungin and anidulafungin are members of the most recent major class of antifungals to be licensed for clinical use. They are established first-line therapy in patients with candidaemia and other forms of invasive candidiasis.¹⁰ Since their introduction, reports have appeared of acquired echinocandin resistance in *Candida* spp., most notably in *C. glabrata*.¹¹ By contrast, resistance to echinocandins in *Aspergillus* spp. appears to be uncommon¹² but their clinical use as monotherapy in aspergillosis is limited because their *in vivo* activity is lower, a potential consequence of their *in vitro* antifungal effect on *Aspergillus* spp. being only fungistatic.¹³

Flucytosine has its main antifungal activity against *Cryptococcus* and *Candida* spp., but because of the rapid development of fungal resistance during flucytosine monotherapy, its use is mainly limited to combination therapy with amphotericin B. Acquired resistance is associated with mutations affecting cellular drug uptake and the target nucleic acid synthetic pathway. Flucytosine is not recommended for the treatment of aspergillosis because of apparent intrinsic drug resistance, but its efficacy is pH dependent and so it could show *in vitro* antifungal activity at anatomical sites of *Aspergillus* infection where there is an acidic environment.^{14,15}

Terbinafine has fungicidal activity and main indication in dermatophyte infections.¹⁶ It is also occasionally used in combination with another antifungal agent for the treatment of mould infections. This is because of its potential to act synergistically in drug combination where there is shared inhibitory action on ergosterol biosynthesis. There are recent reports of terbinafine resistance in the dermatophytes *Trichophyton mentagrophytes/Trichophyton interdigitale* complex and *Trichophyton rubrum* particularly from India,¹⁷ but this is increasingly being reported in other countries as well.^{18–21} Recently, the taxonomy for the *T. mentagrophytes/T. interdigitale* complex was revised and a new species, *Trichophyton indotineae*, proposed for the highly terbinafine-resistant Indian isolates.^{22,23}

Trimethoprim/sulfamethoxazole, although principally used as an antibacterial agent, is established as first choice to treat *Pneumocystis jirovecii* pneumonia (PcP). While mutations in target fungal genes are well characterized, their

clinical relevance for predicting drug resistance is less clearly established.

The EUCAST sub-committee on Antifungal Susceptibility Testing (as well as the CLSI) has developed susceptibility testing methods for human pathogenic yeasts and moulds, including dermatophytes, with freely available evidence-based break-points for categorizing susceptible versus resistant isolates (www.eucast.org/astoffungi) that correlate with clinical outcomes. For less frequent organisms where MICs are available but MIC-outcome data are not, epidemiological cut-off values (ECOFFs) allow detection of isolates with acquired resistance mechanisms. However, susceptibility testing is dependent on culture of the isolate, which is not always possible or available within a reasonable time frame. When cultured samples yield one of the principal fungal pathogens discussed here, this is usually achieved within 48–72 h, whereas dermatophytes require week(s) of incubation, and culture of *P. jirovecii* is not possible in the diagnostic laboratory.

Recognizing the above limitations, molecular tests have been developed and evaluated for rapid fungal detection in body fluids and tissues, and for fungal identification where growth is detected in clinical samples such as blood cultures; these have been extensively and critically reviewed elsewhere.^{24–26} Non-culture-based molecular tests to detect antifungal drug resistance in fungal pathogens, whether in-house or commercial, are more limited in number and are reviewed here and summarized in Table 1 for *Aspergillus* spp.,^{27–38} *Candida* spp.,^{39–41} *Trichophyton* spp.^{42,43} and *P. jirovecii*.^{44,45} This is accompanied by guidance points on their use in clinical practice (Table 2). Of note, in this review, the term ‘mutation’ is used for non-synonymous changes in resistance genes that are confirmed or suspected to be related to resistance because they occur in phenotypically resistant strains. The consequent amino acid changes are referred to as alterations.

***Aspergillus* spp.**

Background

Acquired antifungal drug resistance has been described in different *Aspergillus* spp. and for various antifungals, but research has focused mainly on triazole resistance in *A. fumigatus*. Triazoles are not mutagenic, but genetic variation (including triazole resistance mutations) may arise through spontaneous mutations, mitotic recombination or meiotic recombination.⁴⁶ When a population of *Aspergillus* conidia is exposed to triazole selection pressure, isolates harbouring a resistance mutation will thrive in comparison with WT isolates and become dominant in the population. This process of resistance selection can take place in a patient who receives prolonged treatment with triazoles (in-host selection) or in the environment where residues of azole fungicides with activity against *A. fumigatus* may provide selection pressure.

Acquired triazole resistance in *A. fumigatus*

Cyp51-mediated triazole resistance

In *A. fumigatus*, triazole resistance mechanisms are mainly associated with alterations in the *cyp51A* gene that encodes the

Table 1. In-house and commercial non-culture methods that detect molecular resistance mechanisms in reviewed fungal pathogens

Fungal pathogen (Specimen types)	Antifungal drug	In-house detection of drug resistance mechanism(s)	Commercially available assays for detection of drug resistance mechanism(s)
<i>Aspergillus</i> spp. (Sputum, bronchoalveolar lavage, serum, or plasma)	Triazoles	Real-time PCR studies ²⁴⁻³⁰ Pyrosequencing ³¹	Aspergenius [®] multiplex real-time PCR detects <i>Aspergillus</i> TR ₃₄ /L98H; TR ₄₆ /T289A; TR ₄₆ /Y121F gene mutations in <i>cyp51A</i> ^{32,33,36-38} Aspergenius [®] multiplex real-time PCR detects G54 and M220 RUO in <i>cyp51A</i> of <i>A. fumigatus</i> ³⁴ MycogenIE [®] detects <i>A. fumigatus</i> TR ₃₄ /L98H gene mutations ³⁵⁻³⁸ FungiplexR [®] Aspergillus Azole-R IVD real-time PCR detects <i>A. fumigatus</i> TR ₃₄ and TR ₄₆ ^{24,38}
<i>Candida</i> spp.	Triazoles	No assays for azole resistance due to multiple mechanisms playing in concert	
	Echinocandins	PCR assay to detect Glucan synthase (<i>FKS</i>) gene(s) for subsequent sequencing to identify mutations in the hotspots of <i>fks1</i> and <i>fks2</i> (<i>C. glabrata</i> only) ^{39,40}	
<i>Cryptococcus</i> spp.	Fluconazole	No assays due to variable mechanisms of resistance	
Dermatophytes	Terbinafine	PCR assay to detect squalene epoxidase (<i>SQLE</i>) gene for subsequent sequencing to identify mutations in <i>Trichophyton mentagrophytes/interdigitale</i> and <i>T. rubrum</i> ^{17,42}	DermaGenius [®] Resistance Multiplex real-time PCR kit. ⁴³ Detects: <i>T. rubrum/Trichophyton soudanense</i> , <i>T. interdigitale/mentagrophytes</i> , <i>T. mentagrophytes (ITS type IV)</i> , <i>T. tonsurans</i> , <i>T. violaceum</i> , <i>Trichophyton quinckeanum/Trichophyton schoenleinii</i> and <i>SQLE</i> alterations: L393F, F397L, L393S, F397I, F397V.
<i>P. jirovecii</i>	Trimethoprim/sulfamethoxazole	Dihydropteroate synthase gene mutations: Detected using RFLP, PCR sequencing, SSCP, MLST, PCR pyrosequencing ⁴⁴ Atovaquone mutations: Sequencing of Cytochrome b substitutions in the Qo region T121I, L123F, T100I, I120V, S125A, P239L and L248F (see Table S1)	PneumoGenius [®] real-time PCR detects mutations at codons 55 and 57 in dihydropteroate synthase (DPHS) gene-encoding sulphonamide resistance ⁴⁵

14 α -demethylase, an enzyme responsible for the final step of the ergosterol biosynthesis pathway. Ergosterol is the major sterol component of fungal membranes and is critical for membrane permeability and fluidity, thereby being essential for fungal growth and survival.⁴⁷ *A. fumigatus* carries one Cyp51A and one Cyp51B protein, where Cyp51B is constitutively expressed and Cyp51A expression is inducible.⁴⁸ Lanosterol 14 α -demethylase point amino acid mutations mainly appear in Cyp51A and can lead to amino acid changes that result in modifications to ligand access channels through which azoles gain access to the enzyme active site and bind to the haem molecule.⁴⁸ Hotspots for amino acid substitutions include G54, G138, M220 and G448, which correspond with specific azole resistance phenotypes (Table 3).⁴⁹⁻⁸² These single resistance mutations are commonly found in patients with prior exposure to triazole therapy. Non-synonymous

substitutions of *A. fumigatus* Cyp51A protein, such as L98H, Y121F and T289A, are commonly accompanied by tandem repeats (TRs) in the gene promoter,³⁰ which up-regulate the expression of the *cyp51A* gene.⁸³ Resistance mutations that involve TRs, such as TR₃₄/L98H and TR₄₆/Y121F/T289A (Table 3), are commonly associated with environmental resistance selection through exposure to azole fungicides.⁸⁴ Molecule similarity between triazole fungicides and medical triazoles is believed to be responsible for cross-resistance.⁸⁵ Single resistance mutations are frequently found in patients treated with triazoles, but they have also been recovered from the environment.⁸⁶ While TR-mediated resistance mutations are associated with environmental resistance selection, in-host selection of a TR₁₂₀ resistance mutation,⁷⁸ and also an in-host selection of a TR variation TR₃₄/L98H, has been reported.⁸⁷ Ultimately, characteristics of

Table 2. Key points to guide the use of molecular tests for detection of antifungal drug resistance (AFDR)

General:	<p><i>The clinical need for molecular testing for AFDR may be considered:</i></p> <p>In patients who fail a course of appropriate antifungal therapy, who have a subsequent relapse of their infection after an appropriate course of therapy, or who develop a new fungal infection after an earlier prolonged course of antifungal therapy.</p> <p>Where local epidemiology suggests that there are high rates of resistance in a particular fungal species, examples include <i>C. glabrata</i> resistance to echinocandins, and <i>A. fumigatus</i> resistance to voriconazole.</p> <p>Where there is limited access to local EUCAST/CLSI compliant phenotypic antifungal susceptibility testing.</p> <p>Where phenotypic testing gives borderline susceptible/resistant result and determining if there is a resistance mechanism present can be helpful to guide clinical decision making.</p>
Pathogen-specific:	<p><i>Aspergillus:</i></p> <p>Molecular tests have mostly been applied to detect triazole resistance mechanisms in <i>A. fumigatus</i>, less data are available for other <i>Aspergillus</i> spp.</p> <p>The analytical sensitivity of PCR tests for triazole resistance in <i>A. fumigatus</i> due to <i>cyp51</i> gene mutation(s) (single copy gene) is lower than sensitivity of PCR to detect presence of the fungus (targets multi-copy gene) in clinical samples.</p> <p>A positive PCR for a resistance mechanism to triazoles in <i>A. fumigatus</i> suggests avoidance of triazole therapy. However, a negative PCR result for resistance does not confirm triazole susceptibility.</p> <p>Acquired resistance to amphotericin B or echinocandins in <i>A. fumigatus</i> is rare.</p> <p><i>Candida:</i></p> <p>For triazoles, and in particular fluconazole, the existence of multiple resistance mechanisms in individual strains of <i>Candida</i> spp. (e.g. combined <i>erg11</i> target gene mutation + efflux mechanism) means that molecular tests to detect AFDR have had limited clinical application; furthermore non-detection of a particular resistance mechanism does not infer triazole susceptibility.</p> <p>For echinocandins PCR protocols have been developed that target the hotspot regions of the <i>FKS</i> genes, but these are technically demanding and species-specific.</p> <p>Acquired echinocandin resistance is most frequent in <i>C. glabrata</i>. It is unlikely to occur in patients not previously exposed to echinocandin therapy unless when part of a nosocomial outbreak.</p> <p>Acquired resistance to amphotericin B is considered rare.</p> <p>There are PCR protocols to detect flucytosine resistance, but as this drug is uncommonly used in candidiasis treatment, phenotypic susceptibility testing is preferred.</p> <p><i>Cryptococcus:</i></p> <p>Because fluconazole and flucytosine resistance mechanisms in <i>Cryptococcus</i> spp. are complex, phenotypic testing is preferred over molecular methods.</p> <p>Dermatophytes:</p> <p>Because of slow growth in laboratory cultures, and limited experience with phenotypic sensitivity testing, the use of molecular tests to detect resistance mechanisms is attractive.</p> <p>Both in-house and, recently, commercial PCR tests are available to detect the most common <i>sqle</i> gene mutations reported to be associated with terbinafine-resistant <i>Trichophyton</i> spp.</p> <p>Molecular tests to detect resistance mechanisms for triazoles, in particular itraconazole, are less well developed and not recommended for routine diagnostic use.</p> <p><i>P. jirovecii:</i></p> <p>Because of inability to culture <i>P. jirovecii</i> in the diagnostic laboratory there are no correlates between phenotypic sensitivity and putative resistance mutations in therapeutic drug target genes.</p> <p>The detection of mutations in drug target genes is not always associated with treatment failure, as this is likely also associated with initial disease severity, but mutations may be related to diminished efficacy of trimethoprim/sulfamethoxazole for PCP prophylaxis.</p> <p>Molecular tests may be warranted for clinical decision making in cases of PCP considered refractory to treatment (no improvement post > 7 days treatment).</p>

the fungus and its (azole) environment will determine the supply of mutations and subsequent selection rather than whether resistance was selected in a host or in the environment.^{86,88}

Other triazole resistance mechanisms in *A. fumigatus*

In 10%–50% of triazole-resistant *A. fumigatus* isolates, a WT *cyp51A* gene sequence is found, indicating that other pathways or resistance mutations are likely to be present.⁵⁴ The *hap* gene complex member *hapE* has been shown to be associated with an azole-resistant phenotype.^{89,90} Another target involves the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase-encoding gene (*hmg1*), which represents a rate-limiting enzyme in the ergosterol biosynthetic pathway.⁹¹ *Hmg1* mutations have been proposed as an underlying mechanism in azole-resistant isolates lacking *cyp51A* mutations.^{91–94} Other mechanisms include the overexpression of *cyp51B*,⁹⁵ the overexpression of efflux pumps (e.g. Cdr113 and AtrF),^{96,97} and mutations in regulators and the transcriptional network (e.g. SrbA).⁹⁸ The negative cofactor two A and B (NCT) complex was recently identified as a key regulator of triazole resistance by modulating the expression levels of the transcription factors associated with ergosterol biosynthesis and triazole resistance.⁹⁹

Trends in triazole resistance phenotypes and genotypes in *A. fumigatus*

SNPs are commonly found in the *cyp51A* gene of *A. fumigatus* isolates of which the significance for the resistance phenotype is unknown. In a recent resistance survey involving 640 clinical *A. fumigatus* isolates, 445 isolates harboured TR₃₄/L98H of which 24 (5%) exhibited one or more additional mutations, including F495I (9 isolates), Q259H (5), S297T (4), D262N (1), N326H (1), P337L (1), Y341H (1), I364V (1), G328A (1) and L399V (1).⁴⁹ These SNPs might or might not impact on the triazole resistance phenotype. F495I was recently shown to be associated with resistance to imidazole fungicides, such as imazalil and prochloraz, which was confirmed by recombination experiments.⁵⁸ However, TR₃₄/L98H with F495I showed lower voriconazole MICs compared with TR₃₄/L98H without F495I, indicating an effect of this SNP on the activity of voriconazole.⁵⁸ *Hmg1* gene mutations have been found in isolates harbouring TR₃₄/L98H and TR₄₆/Y121F/T289A and have been suggested to alter the triazole-resistant phenotype.⁹¹ However, although *hmg1* mutations were found in 24% of *A. fumigatus* isolates with a triazole-resistant phenotype, 8% of isolates with a WT phenotype also harboured SNPs in this gene.¹⁰⁰ The location and type of SNP may determine their significance for the triazole-resistant phenotype, but this requires further studies.

In addition to SNPs, an increased number of TRs have been observed in triazole-resistant *A. fumigatus* isolates, including three or four copies of TR₄₆.⁷⁶ However, no specific phenotype change could be demonstrated in these isolates.

Finally, a significant trend towards decreased voriconazole resistance was noted in TR₃₄/L98H isolates in the above-mentioned resistance survey.⁴⁹ In 2013 96% (44 of 46) of TR₃₄/L98H isolates were classified as voriconazole resistant, while in 2018 only 55% (59 of 108) of TR₃₄/L98H isolates exhibited a voriconazole-resistant phenotype ($P=0.0001$).⁴⁹ However, no underlying mechanism was found that could explain the voriconazole phenotype shift. Through known and unknown resistance

mechanisms, an increasing diversity in azole-resistant phenotypes and genotypes is emerging, which is likely to result from the dynamic environments *A. fumigatus* is exposed to and its ability to adapt to these changes.

Resistance testing of *A. fumigatus* and clinical implications

Although susceptibility testing remains the cornerstone for guidance of antifungal therapy choices, there are increasing challenges with respect to *A. fumigatus* resistance. Firstly, many cases of IA are diagnosed in the absence of a positive *Aspergillus* culture. Mixed triazole-susceptible and triazole-resistant isolates causing infection in patients with IA have been reported and testing of multiple *A. fumigatus* colonies is therefore recommended.¹⁰¹ As susceptibility testing of multiple colonies is very laborious, an agar-based screening strategy has proven useful to detect resistant colonies.¹⁰² Clinical breakpoints are available for triazoles and *A. fumigatus*, although it remains unclear if susceptible isolates can be safely treated with a triazole if the isolate is resistant for one or more other triazoles.¹⁰³ In culture-negative patients, commercial resistance PCR assays are available to detect TR₃₄/L98H and/or TR₄₆/Y121F/T289A alterations directly in clinical specimens (Table 1).^{38,104} As these assays rely on the amplification of specific targets, which are confirmed as the cause of azole resistance, additional/alternative resistance mutations and single resistance mutations are not detected. With increasing variation in resistance phenotypes, and thus of the proportion of resistance due to the mechanisms not targeted by the PCR, the correlation between resistance PCR and resistance phenotype will become less well defined. Furthermore, the analytical sensitivity of resistance PCRs in bronchoalveolar lavage (BAL) (which target the single copy *cyp51A* gene) is lower than the *Aspergillus* PCR (where a multi-copy gene is amplified), resulting in the inability to obtain resistance target amplification in 30% of patients despite resistant infection.¹⁰⁵ Thus, although resistance PCR positivity documents resistance, and triazole therapy thereby can be avoided if a resistance mutation is detected, PCR negativity does not confirm azole susceptibility, which in some circumstances, such as CNS aspergillosis, is critical. New approaches are warranted to enable detection of a broad range of resistance mutations, such as sequence-based strategies.³¹

Acquired triazole resistance in other *Aspergillus* species

Much less is known about the prevalence and underlying mechanisms of acquired triazole resistance in other *Aspergillus* spp. This is probably in part because they are less often causes of invasive disease, but also because acquired resistance is less frequently researched. *Cyp51A* alterations have been reported in azole-resistant *Aspergillus terreus* including M217I and G51A,⁸⁴ D344N and M217I,¹⁰⁶ and M217T and M217V.¹⁰⁷ The codons G51 and M217 in *A. terreus* correspond, respectively, to codons G54 and M220 in *A. fumigatus* which, as described above, are also linked to triazole resistance. Molecular resistance mechanisms have also been investigated in *Aspergillus flavus*. P214L has been found in *Cyp51A*, and S240A and H349R in *Cyp51C* in triazole-resistant isolates, but not in susceptible counterparts.^{108,109} Moreover, MDR2, atrF and *mfs1* up-regulation

Table 3. *Cyp51* gene-related mutations and corresponding phenotype (EUCAST methodology) in triazole-resistant clinical *A. fumigatus* isolates

Resistance mutation	Phenotype (MIC mg/L) ^a			Comment	References
	Itraconazole	Voriconazole	Posaconazole		
TR _{3,4} /L98H	2->16	0.5->16	0.25-2	A significant trend towards lower VRC MICs was observed between 2013 and 2018 in a national surveillance program ⁴⁹	49-68
TR _{3,4} /R65K/L98H	>16	8	4		69
TR _{3,4} /L98H/S297T	>16	2	0.5	S297T not considered relevant for azole resistance ²⁹	51
TR _{3,4} /L98H/S297T/ F495I	>16	1-8	0.5->8	S297T not considered relevant for azole resistance; ²⁹ F495I associated with imidazole resistance ⁵⁸	51, 54, 58, 60, 67, 71
TR _{3,4} ³ /L98H	>16	4 (-8)	1		87
TR _{4,6} /Y121F/T289A	0.5->16	>16	0.125-2		56, 58-61, 64-68, 72-74
TR _{4,6} /Y121F/M172I/ T289A	1	>16	0.5	M172I is also found in WT isolates ²⁹	75
TR _{4,6} ³ /Y121F/M172I/ T289A/G448S	>16	>16	1	M172I is also found in WT isolates ²⁹	74, 76
TR ₅₃	>16	16	0.25		77
TR ₁₂₀ /F46Y/M172V/ E427K	16->16	4	0.5	In-host selection of TR ₁₂₀ in an isolate harbouring F46Y/M172V/E427K with a triazole WT phenotype	78
F46Y/M172V/E427K	4	0.5-8	1-4	F46Y, M172V and E427K have also been found in WT isolates ²⁹	50, 57, 79
F46Y/M172V/N248T/ D255E/E427K	>8	2	0.5	F46Y, M172V, N248T, D255E and E427K have also been found in WT isolates ²⁹	53, 57, 71
G54W,E,R,V	>16	0.06-2	0.5->8		50, 52, 53, 55, 63
G138C	>8	8->8	1->8		53, 80, 81
P216L	>16	0.5-2	0.25-0.5	POS MIC >16 described in one study, ⁶¹ possibly suggesting accumulation of additional resistance mutations	52, 53, 61, 63, 67
F219L	>16	0.25	0.25		52
M220I,K,L,R,T,V	>16	0.5-4	0.5->8		50, 51, 53, 59, 63
Y431C	>8	2-4	1-2		81
G448S	>8	2-8	0.25-1		53

VRC, voriconazole; POS, posaconazole.

^aMIC ranges may represent a single isolate or the accumulation of MICs of multiple isolates from different studies. Broad MIC ranges could reflect technical variation in MIC determination, factors related to the resistance mechanism detected or the presence of (undetected) additional resistance mutations. Isavuconazole resistance classification is highly similar to that of voriconazole.⁸²

resulting in efflux were reported in azole-resistant *A. flavus* from South Korea.¹¹⁰ Thus, as for *A. fumigatus*, target gene sequencing can help detect acquired resistance, but it is not enough to rule out additional mechanisms that cause triazole resistance in these species.

Acquired echinocandin and amphotericin B resistance in *A. fumigatus*

Although echinocandin resistance was successfully promoted in laboratory strains of *A. fumigatus* with *fkp* gene mutations as early as in 2005, and this mechanism is common in *Candida*, echinocandin resistance has rarely been reported in *A. fumigatus*. The first example of echinocandin resistance in clinical *A. fumigatus* isolates was reported in 2008 in a clinical isolate displaying overexpression of the *fkp1* gene¹¹¹ whereas the second involved a point mutation in *fkp1* hotspot 1.¹² Recently, it was shown that caspofungin may induce cellular stress, promoting formation of mitochondrion-derived

reactive oxygen species and triggering an alteration in the composition of plasma membrane lipids surrounding glucan synthase, rendering it non-susceptible to echinocandins.¹¹² None of these mechanisms is easily detectable in clinical microbiology laboratories. Finally, we are unaware of any documented clinically relevant acquired amphotericin B resistance mechanisms in *A. fumigatus*.

Candida species

Background

Acquired azole drug resistance rates in *C. albicans* are relatively low worldwide ($\leq 1\%$).^{113,114} In a 20 year global surveillance study, using CLSI criteria, azole resistance in *C. glabrata* isolates ranged from 5.6% to 10.1%; however, these rates were much higher in North America (10.6%) than in Asia-Pacific (6.8%), Europe (4.9%), or Latin America (2.6%).¹¹⁵ Resistance rates in *C. parapsilosis* and *C. tropicalis* were as high as 5.4% and 4.9%,

respectively, with remarkable differences between continents.¹¹⁵ *C. krusei* is intrinsically resistant to fluconazole with MIC values usually >32 mg/L.¹¹³⁻¹¹⁵ Other species with intrinsically elevated fluconazole MIC values include *C. glabrata*, *Candida inconspicua*, *Candida lipolytica*, *Candida norvegensis*, *Candida rugosa*, *Candida pelliculosa* and *Candida guilliermondii*.¹¹⁴

Acquired azole resistance in *Candida* spp.

The gene encoding the target enzyme for azoles in *Candida* is the *erg11* (equivalent to the *cyp51A* gene in *Aspergillus*). Acquired azole resistance in *Candida* spp. is quite uncommon and when found is often preceded by months of therapy.¹¹⁶ It can be caused by a variety of resistance mechanisms¹¹⁷⁻¹¹⁹ that often work concurrently in clinical isolates and consequently the underlying mechanisms in isolates with azole resistance are rarely dissected. Elevated non-WT fluconazole MICs are most commonly observed in *C. glabrata* and *C. auris*.

Alterations in *erg11*

Amino acid substitutions in Erg11 can affect the optimal binding of the azoles to their target. Erg11 amino acid substitutions have been reported in *C. albicans* by Morio *et al.*¹²⁰

A study looking at 63 fluconazole-resistant *C. albicans* clinical isolates observed that 55 carried at least one mutation in *erg11*.¹²¹ When these mutations are introduced into an azole-susceptible *C. albicans* strain, an increase in fluconazole MIC values is observed and is most prominent if the mutations are homozygous.^{121,122}

C. tropicalis isolates displaying azole resistance alone, or combined with amphotericin B, carried *erg11* mutations, which lead to alterations at G464D and Y132F among isolates displaying fluconazole MIC values >64 mg/L and voriconazole MIC values of >8 mg/L.¹²³ Beyond the Erg11 alterations, these isolates displayed Erg3 substitutions S258F and S113G. In a study evaluating azole resistance among 431 *C. parapsilosis* and 227 *C. tropicalis* isolates collected worldwide, 38 of 46 *C. parapsilosis* and 3 of 6 *C. tropicalis* isolates had the Erg11 alteration Y132F.¹¹⁴

In *C. auris*, a small number of *erg11* gene mutations cause azole resistance and these are usually clade specific.^{124,125} Y132F causes high resistance rates to fluconazole and voriconazole with CLSI MIC values of 1 or 2 mg/L, while K143R and F126T increase fluconazole MIC values, but voriconazole values remain below 0.5 mg/L.¹²⁴

Up-regulation of *erg11*

Up-regulation of *erg11* is not commonly noted in clinical isolates and seems to only have a modest effect on azole MIC values.^{113,114}

Alterations in *erg3*

Missense or nonsense mutations in *erg3* have been reported to enable fungal cells to develop resistance to polyenes and azoles¹²⁶ but these are uncommon in clinical isolates.

Efflux up-regulation

Up-regulation of efflux systems from the ATP-binding cassette (ABC) transporters and the major facilitator superfamily (MFS)

can cause lower intracellular accumulation of the azoles, leading to resistance.¹¹⁹ The ABC transporters involved in azole resistance in *C. albicans* are Cdr1 and Cdr2. Functional homologues of these are noted in other *Candida* spp. The most important MFS transporter in *Candida* spp. is the Mdr1 pump, previously named BenR. In contrast to Cdr1 and Cdr2, which have all azoles as substrates, Mdr1 only extrudes fluconazole. Their substrate specificity in *C. albicans* and *C. glabrata* was confirmed in a study by Sanglard and Coste¹²⁷ who evaluated the Cdr1, Cdr2 and CgCdr1 from *C. glabrata*, as well as Mdr1 and Erg11 alterations. They demonstrated that fluconazole MIC values were affected by all resistance mechanisms, whereas the MIC values of itraconazole, isavuconazole, posaconazole and voriconazole were unchanged when only Mdr1 was overexpressed.

Alterations in various zinc cluster transcription factors (ZCFs) have been identified as being responsible for the up-regulation of the efflux systems in *Candida* spp. In *C. albicans*, the promoter Tac1 is known to up-regulate Cdr1 and Cdr2.^{128,129} Mutations in multidrug-resistant regulator (*mrr*) 2 increasing the expression of Cdr1 have also been described.¹³⁰ In *C. glabrata*, resistance to azoles is mediated by the overexpression of the ABC multidrug transporter regulated by the ZCF CgPdr1.¹³¹ This regulator is important for fungal-host interactions. Mutations in this transcription factor have been demonstrated to increase azole resistance *in vitro* and *in vivo*.¹³¹

Overexpression of Mdr1 in *C. albicans* has been associated with a gain-of-function mutation in the promoter *mrr1*.¹³² *C. albicans* isolates that became homozygous to *mrr1* with a single nucleotide substitution, resulting in P683S and G997V, demonstrated elevated fluconazole MIC values. In a recent global surveillance study, overexpression of Mdr1 was detected in 38 of 46 azole-resistant *C. parapsilosis* isolates exhibiting azole non-susceptible or non-WT phenotypes.¹¹⁴

Combinations of resistance mechanisms

In most *Candida* spp. azole resistance is the result of a combination of mechanisms. In *C. albicans* laboratory isolates, resistance usually occurs through the gain-of-function alterations in the transcription factors Mrr1, Tac1 and Upc2, which results in the up-regulation of efflux pumps and ergosterol biosynthesis genes concomitantly. The combination of these mechanisms generated a 500-fold increase in fluconazole MIC values.¹³³ In addition to up-regulating genes encoding efflux transporters and *erg11*, clinical isolates had Erg11 amino acid substitutions known to cause resistance.¹¹⁶

Molecular detection of azole resistance in *Candida*

Due to this plethora of resistance mechanisms that can co-exist, susceptibility testing is a better tool for clinical laboratories to detect resistance to azole agents than genetic methods. Molecular detection of *erg11* gene mutations known to cause azole resistance could be used to detect or confirm resistance.¹³⁴ However, resistance could not be ruled out in the absence of these alterations.

Echinocandin resistance

The 1,3- β -D-glucan synthase (GS) complex mainly comprises two subunits Fks and Rho, of which Fks1p is the main target of the

	Anidulafungin EUCAST ECOFF (mg/L)	Fks1				Fks2			
		Hotspot 1		Hotspot 2		Hotspot 1		Hotspot 2	
		1st AA no.	AA sequence	1st AA no.	AA sequence	1st AA no.	AA sequence	1st AA no.	AA sequence
<i>C. albicans</i>	0.03	641	FL <u>T</u> <u>S</u> LRDP	1357	D W <u>I</u> <u>R</u> RYTL				
<i>C. auris</i>	NA	635	FL <u>T</u> <u>S</u> LRDP	1350	DWIRRYTL				
<i>C. dubliniensis</i>	0.03	641	FL <u>T</u> <u>S</u> LRDP	1357	DWIRRYTL				
<i>C. glabrata</i> ^a	0.06	625	FL <u>I</u> <u>L</u> <u>S</u> LRDP	1340	D W <u>V</u> RRYTL	659	L <u>I</u> <u>L</u> <u>S</u> LRDP	1374	D W <u>T</u> ^R RYTL
<i>C. kefyr</i>	(0.03)	651	L <u>T</u> <u>S</u> LRDP	1366	DWVRRYTL				
<i>C. krusei</i>	0.06 ^c	655	FL <u>I</u> <u>L</u> <u>S</u> LRDP	1364	DWIRRYTL				
<i>C. lusitanae</i>	(0.06)	634	FL <u>T</u> <u>S</u> LRDP	1348	DWIRRYTL				
<i>C. tropicalis</i>	0.06	650	FL <u>T</u> <u>S</u> LRDP	1366	DWIRRYTL				
<i>C. parapsilosis</i>	4	652	FLTL <u>S</u> LRD A	1369	DWIRRYTL				
<i>C. metapsilosis</i>	(4)	104 [*]	FLTL <u>S</u> LRD A	821 [*]	DWIRRYTL				
<i>C. orthopsilosis</i>	(4)	645	FLTL <u>S</u> LRD A	1362	DWVRRYTL				
<i>C. guilliermondii</i>	(4)	632	F <u>M</u> <u>A</u> SLRDP	1347	DWIRRYTL				
<i>C. lipolytica</i>	NA	662	FL <u>I</u> <u>L</u> SLRDP	1387	DWIR <u>C</u> YL				
<i>S. cerevisiae</i>	(1)	639	FL <u>V</u> SLRDP	1353	DWVRRYTL	658	FL <u>I</u> <u>L</u> SLRDP	1372	DWVRRYTL

Figure 1. Amino acid (AA) sequences of Fks1 and Fks2 in 10 WT *Candida* species. Amino acid codons associated with increased MIC are underlined and in bold font. In the online version a colour indication is applied to inform origin (naturally occurring or acquired) and impact on the MIC (strong, weak or silent). **Red:** ‘strong R’ mutation, subscript at codons involving a mutation or deletion; superscript at codon involving a mutation or stop codon. **Yellow:** ‘weak R’ mutation. **Blue:** inherent AA difference with proven or possible relation to intrinsic lower susceptibility. **Grey:** inherent AA difference of unknown importance. **Green:** inherent AA difference, probably with no effect. ^aOf note: combination of the following alterations outside the defined hotspots has also been confirmed as cause of echinocandin resistance: Fks1 W508stop combined with Fks2 E655K. ECOFFs indicated in () are estimated WT upper limits (peak MIC + 2 dilutions) based upon the MICs of Danish blood isolates. ^{*}Inaccurate annotation, sequencing of entire gene-sequence required. [#]The micafungin (but not anidulafungin) ECOFF for *C. krusei* is noticeably higher (0.25 mg/L) than for *C. albicans* (0.015 mg/L) and *C. glabrata* (0.03 mg/L). NA, not available. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

echinocandins.^{135,136} Fks1, Fks2 and Fks3 are encoded by the genes *fks1*, *fks2* and *fks3*, respectively. Resistance in *Candida* spp. is caused by mutations in *fks1* for most *Candida* spp. and also in *fks2* in *C. glabrata*.¹³⁷ These occur within two specific regions, known as hotspots (HS) 1 and 2 (Figure 1).¹³⁶ These regions are highly conserved within each species, but have different amino acid sequences. Intrinsically elevated echinocandin MIC values have been associated with inherent genetic polymorphisms within the *fks* sequence among certain species, notably including *C. parapsilosis*.¹³⁸

Echinocandin resistance rates are low for most *Candida* spp., with the exception of *C. glabrata*. Results for 20 years of the SENTRY Antifungal Surveillance Program demonstrated that echinocandin resistance did not show dramatic changes for the five most common *Candida* spp.¹¹⁵ This is different to the findings of Alexander *et al.*¹³⁹ who showed an increase in echinocandin resistance in *C. glabrata* of 7.3%. However, a large survey performed by the CDC, Atlanta, USA, found that the rate of echinocandin non-susceptible *C. glabrata* isolates increased from 4.2% to 7.8% between 2008 and 2014.¹⁴⁰ Recent data from a global surveillance study demonstrate a decrease in caspofungin resistance rates among *C. glabrata*. From 2017 to 2020, only 1.9% of the 1448 *C. glabrata* collected in 29 countries exhibited caspofungin resistance and 2.7% displayed resistance to any echinocandin tested (M. Castanheira, unpublished). In a

nationwide Danish survey of *Candida* spp. isolates from cases of candidaemia (2004–18), the proportion of *Candida* spp. susceptible to fluconazole decreased while there was a slight increase in echinocandin resistance and this was associated with a proportional decrease in *C. albicans* isolates and an increase in *C. glabrata* isolates.¹⁴¹ In one single-centre survey of *Candida* spp. isolates, also from candidaemia cases collected between 2002–19, there was an increase in fluconazole resistance (3.5%–6.8%) while echinocandin resistance remained stable at around 3%.¹⁴²

C. albicans is the second most common *Candida* sp. to display echinocandin resistance, most likely due to its high prevalence in clinical settings. Heterozygous and homozygous mutations in *C. albicans* may generate different phenotypes.¹⁴³

In a study of *C. glabrata* isolates over a 10 year period, 119 isolates displaying non-WT MIC values for echinocandins were screened for *fks* gene mutations.¹⁴⁴ A total of 28 had alterations in *fks* hotspots. The most common alterations were *fks2* HS1 S663P or F659S/V/Y, followed by *fks1* HS1 S629P. This is also true for the emerging species *C. auris*, which is a major problem in certain countries.^{145–147}

Candida spp. isolates from recent studies have mostly demonstrated mutations of the corresponding codons in both *C. glabrata* and *C. albicans*, and other species, confirming that these are the dominant amino acid alterations (Figure 1).^{113,114,136}

Isolates with double mutations have higher MIC values. Lackner *et al.*¹⁴³ demonstrated that *C. albicans* laboratory mutants with homozygous double mutations significantly enhance resistance in an *in vivo* model when compared with heterozygous single mutations.

Although alterations in Fks2p and Fks3p are deemed unimportant for most *Candida* spp., homozygous deletions of *fks2* and *fks3* in laboratory-engineered strains of *C. albicans* decrease their susceptibility to echinocandins since they result in a compensatory overexpression of Fks1 and increase in cell wall glucan.¹⁴⁸

Interestingly, the MIC values for isolates with the same *fks* gene mutations are not always the same. This finding suggests the presence of compensatory mutations that could lead to changes that would improve the binding of the echinocandins—although not to the same level as a WT enzyme.¹⁴⁹ Unlike with azoles, drug efflux and biosynthesis pathway modulation of the fungal cell wall does not seem to affect *Candida* spp. susceptibility to echinocandin agents.

Clinical aspects of echinocandin resistance

Candida spp. isolates can develop resistance to echinocandins after short therapy courses or long-term treatment (median ~1 month),¹⁵⁰ and resistance has not been described in drug-naïve patients except where it has been acquired nosocomially.¹⁴⁹ This means that echinocandin use is the main driver of mutations and resistance. Thus, isolates from breakthrough infection have elevated MIC values and amino acid substitutions in the Fks hotspots.¹⁵¹

In a study evaluating the risk factors for patients having *C. glabrata* candidaemia with an isolate harbouring an *fks* mutation, patients who received 3 or more days of echinocandin therapy were more likely to have isolates that developed echinocandin resistance and to carry *fks* mutations.¹⁵²

Molecular detection of echinocandin resistance in *Candida* spp.

Molecular methods for detection of echinocandin resistance have been developed.^{153–155} The most commonly used was PCR targeting the hotspot regions for the *fks* sequences, followed by conventional sequencing methods. However, *fks* genes are over 3 kb in length and sequencing the entire gene can be cumbersome. Furthermore, primers for *fks* gene amplification are species specific, and this increases both the need for expertise and added laboratory workload. A pyrosequencing method was described for detecting *fks* mutations¹⁵⁶ and, more recently, next-generation sequencing has been used to evaluate several different traits in fungal isolates, including echinocandin resistance in *Candida* spp.^{113,114,157}

Amphotericin B and flucytosine resistance

Acquisition of amphotericin B resistance in *Candida* spp. is apparently rare,¹⁵⁸ but current MIC-based methods may fail to detect resistant isolates. Overall, low rates of breakthrough candidemia in patients treated with amphotericin B have been described.¹⁵⁹

Resistance to flucytosine in *Candida* spp. is primarily related to impaired drug uptake by the cytosine permease encoded by *fcy2*,

and alterations in enzymes involved in the conversion of flucytosine to the active compound 5-fluorouracil (Fcy1 and Fur1).¹⁶⁰

Molecular detection of amphotericin B and flucytosine resistance in *Candida*

Amphotericin B resistance in *Candida* spp. is mainly due to changes in the ergosterol biosynthesis pathway.¹⁶¹ In contrast to azole resistance, where *erg11* mutations have been directly associated with a resistance phenotype, there is no clear marker for detection of amphotericin B resistance.¹⁶² Mutations in *fcy2* or *fur1* that are associated with flucytosine resistance can be detected by PCR.^{163,164} However, as flucytosine is rarely used for *Candida* infections knowledge on resistance mutations is still limited and phenotypic testing remains the more attractive method to detect resistance.

Cryptococcus spp.

Background

Cryptococcal meningitis occurs mainly in people living with HIV and represents an important fungal cause of mortality, particularly in sub-Saharan Africa.¹⁶⁵ Recommended treatment for cryptococcal meningitis is an induction regimen comprising amphotericin B combined with flucytosine, followed by a consolidation phase with fluconazole.^{166,167} Regimens may vary depending on drug availability.¹⁶⁷ For maintenance therapy, fluconazole is the treatment of choice. Indeed, amphotericin B, flucytosine and the azoles show good *in vitro* activity against *Cryptococcus* spp.¹⁶⁸ In contrast, *Cryptococcus* spp. are intrinsically resistant to echinocandins.^{169,170}

Acquired resistance to amphotericin B in *Cryptococcus* spp. seems very uncommon.¹⁶⁸ Resistance rates to fluconazole and flucytosine remained low, at least in Europe and the USA, while higher rates of fluconazole resistance have been reported from other parts of the world such as Cambodia, South Africa and Taiwan.^{171–173} In a comprehensive review of 29 studies published between 1988 and 2017, the mean fluconazole resistance rate was 10.6% for incident isolates, while it rose to 24.1% for relapse isolates.¹⁷³ Of note, in Africa specifically, 70% of isolates from cases of relapse were resistant.¹⁷² Exposure to fluconazole during therapy is probably the main driving force for emergence of resistance.¹⁶⁸ However, exposure to fungicides in the environment may also be responsible for fluconazole resistance.^{174–176} Indeed, it has been shown that *in vitro* exposure to both azole or even non-azole fungicides used in agriculture can select fluconazole cross-resistant isolates of *C. neoformans* and *Cryptococcus gattii*.^{174,175} Although primary flucytosine resistance is rare in *C. neoformans*,¹⁶⁸ acquired resistance is common when the drug is used as monotherapy.^{177,178}

In vitro antifungal susceptibility testing

Although there are reference methods for antifungal susceptibility testing, clinical breakpoints are currently not available for *Cryptococcus* with the exception of 1 mg/L for amphotericin B for EUCAST (www.eucast.org).¹⁷³ Nevertheless, the values of >32 and >16 mg/L for fluconazole and flucytosine, respectively, and mainly obtained by the CLSI methodology, are often used as

Table 4. Summary of mechanisms of resistance in *Cryptococcus* spp. to the main antifungal drug classes^a

Drug class	Mode of resistance ^b	Molecular mechanisms	References
Azoles ^c	Decreased affinity of 14 α -demethylase, mutations in ERG11	G484S, G470R, Y145F, (G344S)	182–188
	ERG11 overexpression		182, 189
	Decreased intracellular concentration of azoles and efflux pumps	Up-regulation of ABC transporter CnAFR1	179, 182, 183, 190–192
	Heteroresistance, chromosome duplication	Chromosome 1	193–200
Amphotericin B	Ergosterol depletion	Defect in delta 8-7 isomerase	183, 205
	Unknown		190, 204
Flucytosine	Decreased uptake or metabolism	Alterations of cytosine permease (FCY2), cytosine deaminase (FCY1), uracil phosphoribosyltransferase (FUR1)	177, 209
	Unknown	Unknown	208

^a*Cryptococcus* spp. are inherently resistant to echinocandin drug class.

^bSeveral mechanisms of resistance may be present in a single isolate.

^cApplies mainly to fluconazole.

breakpoints in the literature.¹⁶⁸ For fluconazole specifically, heteroresistance, where clinical resistance is not related to the MIC (see below), further complicates MIC interpretation. Despite these technical issues, there are many supportive data that correlated the high *in vitro* MICs to fluconazole, flucytosine, or amphotericin B, with clinical failure both in experimental animal models^{179–181} and in patients.¹⁶⁸

Acquired azole resistance

Mechanisms of fluconazole resistance in *Cryptococcus* are varied and include mutations in *erg11*,^{182–188} overexpression of *erg11*,^{182,189} efflux pumps^{179,182,183,190–192} and heteroresistance.^{193–200} (Table 4).

Alteration of the target enzyme, 14 α -demethylase has been reported in several studies (Table 4). A reduced affinity of the enzyme for azoles, associated with other mechanisms of resistance, was demonstrated in fluconazole-resistant clinical isolates of *C. neoformans*.^{182,183} More recently, several mutations in *erg11* associated with azole resistance have been reported, including G484S (corresponding to G464S in *C. albicans*),^{184,186} G470R¹⁸⁷ and Y145F (equivalent to Y132F in *C. albicans*).¹⁸⁵ A G344S mutation in *erg11* has also been observed in a multi-azole-resistant laboratory mutant.¹⁸⁸

Overexpression of *erg11* is also a common mechanism of azole resistance in yeasts. In an early study, it was shown that there was an increased level of 14 α -demethylase within fluconazole-resistant *C. neoformans* isolates from AIDS patients.¹⁸² Similarly, overexpression of *erg11* in the presence of fluconazole, associated with overexpression of an efflux pump, was observed in an animal strain of *C. neoformans*.¹⁸⁷

Several studies demonstrated a decreased intracellular content of fluconazole in fluconazole-resistant isolates of *C. neoformans* supporting the presence of active multi-drug efflux mechanisms.^{182,183,190} Subsequently, genes encoding multi-drug transporters have been demonstrated in *C. neoformans*,²⁰¹ and up-regulation of the ABC transporter CnAFR1 was shown to be involved in fluconazole resistance in laboratory mutants,

confirmed *in vivo* in a murine model of cryptococcosis and observed (in association with overexpression of *erg11*) in an isolate of *C. neoformans* from a case of feline cryptococcosis.^{179,189,192} Efflux pumps have been demonstrated in *C. gattii* and shown to confer fluconazole resistance when expressed in *Saccharomyces cerevisiae* although their contributions to azole resistance in clinical strains remain to be confirmed.¹⁹¹

Heteroresistance is another mechanism that could be associated with treatment failure in *Cryptococcus* spp. Heteroresistance is defined by the presence, in a single isolate, of different populations with differing susceptibility to a drug.^{196,200–203} Heteroresistance, first described in 1999 in *C. neoformans*,¹⁹³ is a dynamic and heterogeneous trait, not related to initial MIC¹⁹⁴ but temperature dependent.^{193,195} It is an intrinsic phenomenon, as it was demonstrated in strains isolated before the discovery and use of azole drugs.^{196,200} It has been reported both in *C. neoformans* and *C. gattii* and seems to be more frequent in the latter species.²⁰⁰ Heteroresistance has been linked to chromosome duplication, in particular a disomy of chromosome 1, which carries the genes coding for *Afr1* and *Erg11*.^{197,199} It is strain dependent and more frequent after fluconazole treatment both in an animal model¹⁹⁸ and in patients.¹⁹⁹

Amphotericin B resistance

Laboratory-generated amphotericin B-resistant mutants have been characterized and showed no alteration in ergosterol content.^{190,204} In contrast, in amphotericin B-resistant clinical isolates, a low content of ergosterol was observed and could be attributed to a defect in the delta 8-7 isomerase, which plays a role in ergosterol synthesis.^{183,205}

Flucytosine resistance

Mechanisms of resistance to flucytosine in *C. neoformans* may be due to mutation in cytosine permease (*fcy2*), cytosine deaminase (*fcy1*), or uracil phosphoribosyltransferase (*fur1*).^{206,207} Flucytosine resistance has also been reported in clinical isolates of *C. gattii*, but with an as yet unknown mechanism(s).²⁰⁸

Nevertheless, *in vitro* experiments showed that defects in DNA mismatch repair (MSH2) promote mutations responsible for flucytosine resistance in *Cryptococcus deuterogattii*.²⁰⁹ It should be pointed out that combination therapy with amphotericin B and flucytosine may still be effective even in a case of flucytosine resistance, depending on the mechanism of resistance involved.²¹⁰

Molecular detection of drug resistance in *Cryptococcus*

Although antifungal drug resistance has been clearly demonstrated in *Cryptococcus* spp., the mechanisms involved are diverse and not fully understood. There are currently no commercialized nor easy diagnostic methods for the detection of the molecular mechanisms of antifungal resistance in *Cryptococcus* spp. in clinical microbiology laboratories.

Dermatophytes

Background

The majority of dermatophyte infections are caused by three genera: *Epidermophyton*, *Microsporum* and *Trichophyton*. Mild infections are treated topically with terbinafine, azoles (ketoconazole, miconazole, clotrimazole, luliconazole, sertaconazole, eberconazole), amorolfine or ciclopirox. Serious infections, as well as scalp and nail infections are treated systemically with terbinafine, triazoles (itraconazole or fluconazole) or griseofulvin and often in combination with topical treatment. Newer systemic triazoles including voriconazole and posaconazole are increasingly used off label in failing case settings.^{211,212} Resistance in dermatophytes was first reported shortly after the turn of the millennium and as of today has been found in *Trichophyton* and *Microsporum* spp. but not in *Epidermophyton*.²¹³ Although drug resistance in dermatophytes is not routinely investigated, resistance in *Trichophyton* is increasingly reported worldwide.^{42,214–217} The highest rates are observed in India, i.e. 36% for terbinafine (MIC ≥ 4 mg/L) and 68% for fluconazole (MICs ≥ 16 mg/L) and apparently involve the spread of an early diverging unique clade referred to as *T. indotineae* and previously reported as *T. mentagrophytes/T. interdigitale* complex and *T. mentagrophytes* genotype VIII.²³ Recently, a study carried out under the auspices of the European Academy of Dermatology and Venereology task force of Mycology reported that among 20 European countries, only one country reported no known resistance although susceptibility testing is not a routine test in clinical laboratories.²¹ A total of 126 cases were reported as having either clinical and/or microbiological-confirmed antifungal resistance with infections located in the scalp, body (some very widespread), groin, palm, soles (and co-infection of nails) and genital areas. In Denmark, an increasing number of resistant cases are reported.²¹⁸ Finally, terbinafine resistance was also found in two isolates from wild hedgehogs in Poland, illustrating that resistant isolates may be shed in the environment.²¹⁹

In vitro antifungal susceptibility testing

A number of studies have reported terbinafine MICs for *Trichophyton* by the CLSI M38-A2 method.^{213,220–226} In these studies, MIC ranges vary from <0.007 – 0.031 to 0.125 – ≥ 32 mg/L and 0.004 – 0.06 to 0.06 – 0.06 mg/L for *T. interdigitale* and *T. rubrum*, respectively,

suggesting some interlaboratory variation.^{220–223} Moreover, differential criteria have been adopted for identification of resistant isolates, e.g. >0.25 and >2 mg/L, suggesting a need for standardization.^{221,226} No formal CLSI clinical breakpoints are established but it is stated that most *Trichophyton* MICs are ≤ 0.25 mg/L but some *T. rubrum* have MICs of >0.5 mg/L.²²⁶ A EUCAST method (E.Def 11.0) has been developed and validated in a multicentre study.²²⁷ This method adopted an objective spectrophotometric endpoint reading to improve reproducibility and facilitates a broader implementation of antifungal susceptibility testing for dermatophytes. Tentative ECOFFs have been established but no breakpoints so far. These, as well as tentative MIC targets and ranges for two Quality Control strains, can be found at the EUCAST website (www.eucast.org).

Terbinafine resistance

Terbinafine resistance has been linked to hotspot mutations in the squalene epoxidase (SQLE) target gene of *Trichophyton* spp.^{20,42,214,221,228} The corresponding amino acid alterations detected so far are summarized in Table 5. High-level resistance (≥ 5 2-fold dilution elevation of the modal MIC) is associated with alterations involving L393 or F397 and these two codons are the most common ones involved in terbinafine resistance in both *T. mentagrophytes*, *T. interdigitale*, *T. indotineae*, *T. rubrum* and *Trichophyton tonsurans*. Q408L is a novel alteration recently described in highly resistant *T. mentagrophytes* isolates causing refractory infection in a married couple and L437P has recently been found in a Danish *T. rubrum* isolate with high-level resistance.^{218,229} Less prominent MIC elevation (2–3 2-fold dilution elevation of the modal MIC) has been associated with Q408L, F415S, H440Y, S443P and combined I121M and V237I alterations.^{42,214,218} I121M combined with V237I is a novel alteration, possibly associated with resistance given both codons are in close proximity to the terbinafine binding site and are also situated in a region that is conserved across several fungal species.⁴² Importantly, however, although most terbinafine-resistant *Trichophyton* isolates harbour SQLE mutations, resistant isolates without such mutations have also been described, suggesting additional mechanisms may play a role.²³⁰ Indeed, ABC transporters and MDR2, in particular, appear to be involved in resistance to terbinafine in *Trichophyton* and also to other antifungal compounds.^{231,232}

Terbinafine resistance in *Microsporum* is to our knowledge only described in a single isolate from a feline patient treated unsuccessfully with topical terbinafine for 3 months for severe skin infection.²¹⁷ In this isolate, the underlying mechanism involved overexpression of the *pdr1*, *mdr1*, *mdr2* and *mdr4* genes encoding ABC transporter proteins.²³³

Azole resistance

Within the *T. rubrum* genome, four ATP-binding cassette (ABC) transporters (TruMdr1, TruMdr2, TruMdr3 and TruMdr5) and TruMfs2 transporter belonging to the MFS have been shown to be able to operate as azole efflux pumps.²³⁴ TruMdr3 and TruMfs1 can act with all azole compounds, while TruMdr1 and TruMfs2 only export fluconazole and voriconazole, and TruMdr2 and TruMdr5 are specific for itraconazole.²¹⁶ As mentioned above, efflux pumps can also transport terbinafine and can account for azole–terbinafine cross resistance.²³⁴ Similarly,

Table 5. Overview of mutations in the squalene epoxidase (SQLE) target gene of *T. rubrum* and *T. interdigitale* and their implication for terbinafine susceptibility (for references, see text)

	High-level resistance	Low-level resistance	Unknown impact (Direct sequencing, unable to grow in EUCAST AFST)	Unrelated to resistance
<i>T. rubrum</i>	L437P, L393F, L393S, F397L, F397I	I121M/V237I, F415S, H440Y/F484Y, S443P	Y414C/L438C, F415V	F484Y, I479V
<i>T. indotineae</i> (<i>T. mentagrophytes</i> / <i>T. interdigitale</i>)	L393F, L393S, S395P, F397L, Q408L, H440T	S443P		L335F, A448T

itraconazole and fluconazole resistance in *Microsporum canis* has been linked to efflux pumps.²³⁵

Molecular detection of drug resistance in dermatophytes

Molecular detection of recognized resistance mutations provides a possibility for rapid detection of resistance and particularly so when compared with susceptibility testing methods for the slow-growing dermatophytes. It also provides an option to detect resistance when the isolate is not growing sufficiently well in the susceptibility testing medium, and potentially also for direct detection of resistance in clinical material. SQLE target gene amplification and sequencing allow detection of mutations conferring high- and low-level resistance (Table 5 and described above). Moreover, a PCR test for the detection of the two most common alterations T1189C and C1191A conferring alterations in L393F and F397L, respectively, was recently published,²³⁶ and a commercial PCR kit detecting alterations L393F, F397L, L393S, F397I and F397V (Table 1).⁴³

P. jirovecii

Background

Potentially drug-resistant PcP was first documented in the 1990s, but it continues to be difficult to determine its current scale.^{237,238} Polymorphisms in the genes encoding targets for anti-PcP therapies are recognized as a potential reason for resistant disease. Trimethoprim/sulfamethoxazole is recommended as agent of first choice for treatment and prophylaxis of PcP. Certain aspects of the disease itself might represent a problem for molecular diagnostic testing. Low fungal burdens in HIV-negative patients can limit the applicability of PCR-based detection. Second, many cases of PcP are caused by multiple co-infecting strains, which include both WT and mutant genotypes.^{239,240} Finally, unlike most fungal diseases, laboratory culture plays no role in the diagnosis and management of PcP.

In vitro antifungal susceptibility testing

The difficulty in culturing *Pneumocystis* has hindered both PcP diagnosis and research and precludes the possibility of *in vitro* susceptibility testing.²⁴¹

Trimethoprim/sulfamethoxazole and atovaquone resistance

A wide range of mutations have been identified in dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) genes,

although even the presence of the most common polymorphisms (DHPS codons T55A and S57P) associated with sulfamethoxazole-based resistance are not necessarily related to treatment failure²⁴² (Table S1, available as [Supplementary data](#) at JAC Online). Mutations in cytochrome B gene have been associated with potential atovaquone resistance, but the impact on patient survival is unclear.^{243,244}

It is very difficult to determine the prognostic impact of PcP caused by a potentially resistant strain, irrespective of the type of anti-PcP therapy, but in patients infected with strains possessing *dhps* gene mutants an increased duration of hospital stay and the need for mechanical ventilation have been noted.²⁴⁵ The impact of drug-resistant disease in the non-HIV cohort remains difficult to determine, due to the fulminant nature of the infection in this broad population. Some studies describe prior drug exposure as the factor behind resistance, yet others describe resistant disease in sulfamethoxazole-naïve patients.^{246,247} This could reflect the nature of the infection, and the need for infection control measures, which are currently not widely applied.^{246,247}

The geographical distribution of mutations potentially associated with PcP resistance is broad, with the studies originating from all continents with the exception of Antarctica^{248–270} (Table S1). The number of documented cases is likely limited by the number of studies investigating this issue, and evidence would suggest that *Pneumocystis* strains with potential resistance to therapy will be widespread. Of the available studies, mutation rates associated with sulfamethoxazole treatment failure vary between 0% and 100% (Table S1), with the higher mutation rates documented in the USA compared with Europe and the lowest rates documented in resource-limited countries.⁴⁴

Various routes of acquisition of resistant PcP have been described, including human-to-human transmission of a resistant strain, *de novo* development of resistance due to use of (possibly sub-optimal) therapy, infection with resistant strains from an environmental source and reactivation of latent disease.⁴⁴

Molecular detection of drug resistance

Of the novel diagnostic approaches, only molecular testing has the potential to detect drug resistance. A variety of methods (RFLP, DNA sequencing, SSCP, MLST and real-time PCR) have been used, but DNA sequencing of target genes is usually applied.⁴⁴ A commercial real-time PcP PCR assay capable of detecting mutations at codons 55 and 57 in the *dhps* gene potentially associated with sulphonamide treatment failure

is available,⁴⁵ but clinical validation is limited. One of the major drawbacks of molecular testing for PcP is the lack of knowledge regarding which target gene alterations affect drug susceptibility. Indeed, it may be that other molecular mechanisms are involved in combination with the polymorphisms already identified and persistent PcP PCR positivity where Ct values indicate a consistent or increasing burden despite therapy could be associated with treatment failure. Therefore, molecular detection of drug resistance in PcP is yet not ready for clinical use.⁴⁴

Conclusions

Molecular detection of antifungal drug resistance is, from a clinical perspective, still in its infancy, but technically feasible and potentially helpful, at least for the rapid detection of azole resistance in *A. fumigatus*. Other important applications include detection of echinocandin resistance in *C. glabrata* and terbinafine resistance in *Trichophyton* spp. New technologies, including next-generation and metagenomic sequencing, have the potential to enable screening of pathogens and clinical specimens for the presence of known and yet to be identified resistance mechanisms.^{24,271} However, it is important to be aware that molecular testing alone cannot determine an organism's antifungal drug susceptibility.

Not all resistance mechanisms are suitable for molecular detection, as diagnostic accuracy may be unknown, and new mechanisms of resistance continue to arise. Moreover, interpretation of the clinical impact of a given mutation requires expertise about the level and spectrum of resistance dependent on the codon involved as well as the specific amino acid substitution.

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Supplementary data

Table S1 is available as [Supplementary data](#) at JAC Online.

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