Epidemiology and molecular mechanisms of antifungal resistance in *Candida* and *Aspergillus*

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Summary

The significant increase in the use of antifungal agents, both for the treatment of candidiasis and invasive aspergillosis and as azole fungicides in agricultural crop protection has resulted in the emergence of resistant clinical isolates, particularly to triazoles and echinocandins. Notably, among isolates that were primarily sensitive to fluconazole such as *Candida parapsilosis* and *Candida tropicalis* have witnessed an emerging resistance development. Also for echinocandins, the occurrence of *Candida* isolates with lower susceptibility to these drugs has been reported, which is possibly due to its broad clinical use. Triazole resistance among *Aspergillus fumigatus* and other *Aspergillus* species is commonly found in European and Asian countries. Specific mutations are associated with azole resistance in *A. fumigatus* and these mutations are now reported globally from six continents. Therefore, we highlight the need to conduct antifungal resistance surveillance studies using clinical isolates of *Candida* and *Aspergillus* in different geographical regions and monitoring of the infection rates in distinct population groups for early detection of resistance to these drugs and implementation of efficient policies for infection control and treatment.

Key words: Antifungal resistance, *Candida* spp., *Aspergillus* spp., azoles, echinocandins, amphotericin B.

Introduction

In recent decades, there has been a substantial increase in the occurrence of invasive fungal infections (IFIs) due to *Candida* and *Aspergillus* in tertiary hospitals throughout the world.1–3 Despite the large geographical variability in their incidence rates, IFIs have undoubtedly become very important worldwide, particularly among critically ill patients, those with degenerative or neoplastic diseases and in patients with organ transplantation.3–5 Given the difficulties in diagnosing IFIs due to *Candida* and *Aspergillus* and their high mortality rates, prevention strategies and empirical antifungal therapies have been increasingly used in different risk groups.2,6–9

In addition the use of antifungal drugs has drastically increased in medical centres throughout the world although this increase is not always compatible with good clinical practice. In fact, some authors have reported a large number of inappropriate prescriptions of antifungal drugs even in teaching hospitals, a factor that increases not only cost and the risk of toxicity but also the possibility of resistance development to antifungal agents.10–13 Concurrent with the global increased consumption of antifungal drugs, there has been an increase in the number of reported cases of resistance to different therapeutic antifungal classes among *Candida* and *Aspergillus* species. The present...
review will discuss various antifungal resistance patterns that have emerged in these two most important fungal pathogens and will also update about the risk factors and epidemiology of associated fungal infections.

**Epidemiology of triazole resistance in Candida spp. and Aspergillus spp.**

In IFI involving Candida, the gradual use of fluconazole and other triazoles in therapeutic regimens for prophylaxis, empirical therapy and diagnostic-driven therapy has led to the development of selective pressure with the emergence of less sensitive species and secondary resistance among isolates primarily sensitive to these drugs. At first, different medical centres worldwide reported the increase in infections due to Candida glabrata and C. krusei among patients previously exposed to triazoles. At present, C. glabrata accounts for 18.1%–40.7% of the cases of candidaemia in the US and 8.5%–31.0% in Europe. Until 2005, epidemiological studies conducted in Brazil reported a low frequency (<5%) of candidaemia due to C. glabrata. Recently, consistent with the epidemiology in the northern hemisphere, a substantial increase in cases of fungaemia due to C. glabrata has also been observed in Brazil. In fact, C. glabrata has accounted for 11.2%–13.1% of the reported cases of candidaemia in different regions of Brazil. The same phenomenon was recently reported in Saudi Arabia by Omrani et al. According to these authors, the proportion of C. glabrata has significantly increased between January 2003 and December 2012.

Candidaemia surveillance in six countries in Asia namely China, Hong Kong, India, Singapore, Taiwan and Thailand reported C. albicans (41.3%) as the most common species. Among non-albicans Candida species C. tropicalis (25.4%) was followed by C. glabrata (13.9%) and C. parapsilosis (12.1%). Although the relative distributions of Candida species varied among the countries, the proportion of C. tropicalis among blood isolates was higher in tropical areas (India, Thailand and Singapore) than other geographical regions. Candida parapsilosis accounted for 33% of candidaemia cases in one Indian hospital, 26% in a Chinese hospital and 14% in a Taiwanese hospital. However C. glabrata and C. krusei contributed to 26% and 12.2% candidaemia cases in tropical countries (China, India and Singapore). Although, most of the Indian studies demonstrate C. albicans as a predominant yeast causing invasive candidiasis, nosocomial C. tropicalis candidaemia ranged from 67% to 90% in some Indian hospitals. In Qatar, a retrospective analysis covering the period from January 2004 to December 2010 identified 201 episodes of candidaemia, of which 66.2% was due to non-albicans Candida species. Similar data were obtained in the United Arab Emirates. In this study, non-albicans Candida species occurred more frequently than C. albicans in adults (67%), haematological malignancy patients (58%) and in cases with break through candidaemia (83%).

First described in Japan in 2009, C. auris is increasingly encountered from cases of candidaemia especially from the Indian subcontinent. These findings represent the beginning of an epidemiological shift which is different from Western countries where the increase in uncommon Candida species, as C. kefyr and C. lusitaniae, is likely due to broad use of echinocandins.

A second epidemiological change, first described in developed countries, was the increasing occurrence of fluconazole resistance among isolates of the C. parapsilosis complex and C. tropicalis. Recent studies that performed susceptibility tests using reference methods reported variations in resistance rates among C. parapsilosis isolates of 3.4%–7.5% in the US and 0%–6% in Europe. For C. tropicalis, these rates were 2.4%–9% in the US and 1.7%–22.0% in Europe. Although infrequent, this phenomenon has been documented in some South American countries, as shown in Table 1. In Brazil, the rates of fluconazole dose-dependent susceptibility and/or resistance in C. tropicalis and C. parapsilosis are 0%–26.8% and 0%–26.9% respectively. Recently, Pinhati et al. [44] reported a candidaemia outbreak due to fluconazole-resistant C. parapsilosis in patients admitted to intensive care units (ICUs) and Souza et al. [45] reported mutations associated with this resistance.

Conceptually, the azole resistance phenotype in Candida spp. results from the combination of more than one resistance mechanism. Table 2 summarises the major mechanisms of resistance to this class of antifungal drugs.

In IFI involving Aspergillus, two phenomena associated with resistance to antifungal agents were described: (i) increase in the number of infections due to non-Aspergillus fumigatus, including emerging species primarily resistant to triazoles and eventually to amphotericin B; and (ii) occurrence of secondary azole resistance in A. fumigatus isolates. Table 3 summarises the major species of non-A. fumigatus and those belonging to the section Fumi gati associated with the phenotype of resistance to the main antifungal agents.
commonly used in clinical practice. The rate of infection due to emerging species of *Aspergillus* is not fully known because only a few case series have performed accurate molecular identification, such as DNA sequencing of *Aspergillus* at the species level. However, there are a substantial number of case series on *A. flavus*, *A. lentulus* and *A. terreus*. Primary resistance to amphotericin B (AMB) is well known in *A. terreus*, however, in a recent study by Kathuria et al. [52] low AMB minimum inhibitory concentration (MIC) (range 0.5–1 mg/L) in 8% of Indian *A. terreus* isolates were observed. Furthermore, in some isolates of *A. flavus* and *A. ustus* also amphotericin B resistance was reported. More recently, this phenomenon has been demonstrated in several species of the section *Fumigati*, including *A. fumigatiaffinis* and *A. lentulus*. Secondary azole resistance in *Aspergillus* has been well documented among isolates of *A. fumigatus*. Acquired azole resistance has been reported also in *A. flavus* and *A. terreus*, although there is no evidence of developing acquired resistance to other antifungal drugs in other species of this genus.

Table 4 summarises the key studies that determined the rates of azole resistance in clinical isolates of *A. fumigatus*. The resistance rate varied between 0.6%...
and 29.6%, and the lowest rate was observed in isolates from the US, where resistance was <1% and mutations in the CYP51A are rare.\textsuperscript{12,60,61} Although triazole resistance is still considered unusual, with rates of <5% in many countries, azole resistance in \textit{A. fumigatus} seems to be increasing in several European countries, and high rates have been observed primarily in medical centres in Denmark (4.5%), France (0.8%-8%) and UK (6%–27%).\textsuperscript{47,62–67} In these countries, particularly in the UK (specifically in Manchester), high resistance rates (6%–27%) have been reported among patients with chronic cavitary aspergillosis who used triazoles for long periods.\textsuperscript{68,69} To find the proper empirical treatment of invasive aspergillosis in areas with a high rate of azole resistance is challenging especially in patients with chronic pulmonary aspergillosis.\textsuperscript{12,70}

A second epidemiological model of the development of triazole resistance in \textit{A. fumigatus} has been described in the Netherlands, where the rates of resistance to these drugs vary between 1.7% and 30% in patients not previously exposed to antifungal therapy, thus suggesting a possible environmental route for the development of triazole resistance.\textsuperscript{67,71–75} An epidemiological study conducted in Japan involving 196 clinical isolates reported that 11.2% of the isolates were resistant to triazoles.\textsuperscript{76}

In Brazil, only two studies reported the prevalence and rates of triazole resistance among emerging species of non-\textit{A. fumigatus}. These studies reported higher MIC values to these drugs among isolates of \textit{A. calidoustus}, \textit{A. clavatus}, \textit{A. tamarii}, \textit{A. nomius}, \textit{A. ochraceus} and \textit{A. terreus}.\textsuperscript{54,77}

The rate of azole resistance in clinical \textit{A. fumigatus} isolates in India was found to be 1.7%–1.9%.\textsuperscript{57,78–80} However, in China the ARTEMIS global sentinel surveillance programme demonstrated a pan-azole-resistant phenotype in 27.5% of \textit{A. fumigatus} isolates.\textsuperscript{81} In contrast, Japan reported 5.2% azole-resistant \textit{A. fumigatus} isolates.\textsuperscript{76,82} A similar range of resistance prevalence of 3.2% and 12.5% \textit{A. fumigatus} isolates was observed in clinical samples from chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA) patients from Iran and Kuwait respectively.\textsuperscript{83,84}

### Molecular mechanisms of azole resistance in \textit{Candida} spp. and \textit{Aspergillus} spp.

Azoles inhibit enzymes involved in the final stages of ergosterol biosynthesis, particularly lanosterol 14α-demethylase. Therefore, sterol precursors – including methylated sterols – accumulate in the cell, resulting in cell membrane instability and consequent fungal growth impairment.\textsuperscript{85–87}

In \textit{Candida} and \textit{Aspergillus}, azole resistance appears to be associated with three major molecular mechanisms, which will be discussed in this review.

<table>
<thead>
<tr>
<th>Table 3 Primary resistance among \textit{Aspergillus} species.</th>
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<tbody>
<tr>
<td>Species</td>
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<tr>
<td>----------------------</td>
</tr>
<tr>
<td>\textit{A. lentulus}</td>
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<tr>
<td>\textit{A. udagawae}</td>
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<tr>
<td>\textit{N. pseudofisheri}</td>
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<td>\textit{A. fumigatiaffinis}</td>
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<td>\textit{A. allilaceus}</td>
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<td>\textit{A. terreus}</td>
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<tr>
<td>\textit{A. alabamensis}</td>
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<tr>
<td>\textit{A. niger}</td>
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<tr>
<td>\textit{A. calidoustus}</td>
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<tr>
<td>\textit{A. versicolor}</td>
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<tr>
<td>\textit{A. sydowii}</td>
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</tbody>
</table>

AMB, amphotericin B; CFG, caspofungin; IA, invasive aspergillosis; VRC, voriconazole.
Decrease in the intracellular drug concentration

The activation of efflux pumps located in the cell membrane has already been described as an important mechanism of resistance to antifungal agents in *Candida* spp. Two different drug efflux systems have been associated with azole resistance: (i) the ATP-binding cassette (ABC) protein superfamily, and (ii) the major facilitator superfamily (MFS) of transporters.

Proteins belonging to the ABC superfamily are primary transporters that hydrolyse ATP molecules and are essential for substrate transport. Many ABC transporters with different topologies have been described in fungal species. In *C. albicans*, it is believed that there are 28 types of transporters, and although their role in fungal species, functional homologues of *C. albicans* ABC1, *CgCDR1*, and other genes encoding ABC transporters have been described and were associated with drug resistance, including *CdCDR1*, *CdCDR2* (also known as *PDH1*) and *CgSNQ2* in *C. glabrata*, *CdCDR1* and *CdCDR2* in *C. dubliniensis* and ABC1 in *C. krusei*. In *C. tropicalis*, isolates with in vitro-induced resistance showed overexpression of *CtCDR1*. However, this profile was not observed in clinical isolates of *C. tropicalis*. In *C. parapsilosis*, Silva et al. [106] reported the increased expression of *CtCDR1* in isolates with induced resistance to fluconazole due to the expression of the transcription factor *Ndt80*. In *C. albicans*, *Ndt80* is involved in microsomal drug transport regulation. In addition, this transcription factor binds to a wide range of genes with diverse biological functions, including other transporter factors, as well as genes that encode ergosterol biosynthesis enzymes.

The second efflux system associated withazole resistance involves MFS transporters, which transport various substrates using the proton gradient generated in the plasma membrane. On the basis of *in silico* analysis, 95 MFS transporters are believed to occur in *C. albicans*. Of these, only the product of the multidrug resistance gene *CDR1* and *CDR2* resulted in increased susceptibility to azoles. Other studies in agreement with these findings have shown that *CDR1* and *CDR2* overexpression plays an important role in the azole resistance phenotype observed in clinical isolates of *C. albicans*.

### Table 4

<table>
<thead>
<tr>
<th>Countries</th>
<th>Study period</th>
<th>Number of samples</th>
<th>Resistance rate (%)</th>
<th>Methodology</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK (single centre)</td>
<td>1997–2007</td>
<td>519</td>
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<td>[68]</td>
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<td>The Netherlands (multicentre)</td>
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<td>1.7</td>
<td>CLSI M38-P</td>
<td>[71]</td>
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<td>The Netherlands (multicentre)</td>
<td>2007–2009</td>
<td>1792</td>
<td>4.6</td>
<td>ITZ agar</td>
<td>[72]</td>
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<tr>
<td>The Netherlands (multicentre, 8 centres)</td>
<td>2009–2011</td>
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<td>6.8</td>
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<td>The Netherlands (multicentre)</td>
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<td>EUCAST</td>
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<td>Spain (multicentre, 29 centres)</td>
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<td>103</td>
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1Triazoles diluted in agar; CLSI, Clinical Laboratory Standard Institute; ITZ, itraconazole; EUCAST, European Committee on Antimicrobial Susceptibility Testing.

Sanglard et al. [90] reported that the deletion of both alleles in the *CDR1* gene in *C. albicans* resulted in increased intracellular levels of fluconazole. Moreover, this mutant is hypersensitive to azoles, terbinafine, amorpholine, and several other metabolic inhibitors, including cycloheximide, brefeldin A and fluphenazine. Azole-resistant *C. albicans* isolates showed high *CDR2* gene expression levels, even higher than the *CDR1* expression levels. Furthermore, the concomitant deletion of *CDR1* and *CDR2* resulted in increased susceptibility to azoles. Other studies in agreement with these findings have shown that *CDR1* and *CDR2* overexpression plays an important role in the azole resistance phenotype observed in clinical isolates of *C. albicans*. In *C. tropicalis*, isolates with in vitro-induced resistance showed overexpression of *CtCDR1*. However, this profile was not observed in clinical isolates of *C. tropicalis*. In *C. parapsilosis*, Silva et al. [106] reported the increased expression of *CtCDR1* in isolates with induced resistance to fluconazole due to the expression of the transcription factor *Ndt80*. In *C. albicans*, *Ndt80* is involved in microsomal drug transport regulation. In addition, this transcription factor binds to a wide range of genes with diverse biological functions, including other transporter factors, as well as genes that encode ergosterol biosynthesis enzymes.

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**Table 4 Rates of triazole resistance in clinical isolates of *Aspergillus fumigatus***

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resistance (MDR1) gene has been associated with azole resistance in clinical isolates.\textsuperscript{15} Importantly, unlike CDR1 and CDR2 overexpression, which culminates in resistance to various azoles, MDR1 overexpression leads to resistance to fluconazole alone and does not appear to be associated with cross-resistance among the azoles.\textsuperscript{15,89} Different mechanisms are involved in MDR1 regulation, including the transcription factor Mrr1 (multidrug-resistance regulator 1).\textsuperscript{14} Inactivation of MRR1 in clinical C. albicans strains blocked MDR1 expression.\textsuperscript{110} Gain-of-function mutations in MRR1 causing constitutive up-regulation of MDR1 have been reported in different Candida species.\textsuperscript{111–113}

Wirsching et al.\textsuperscript{[114]} using gene deletion techniques, found that C. albicans isolates without the MDR1 gene were extremely susceptible to fluconazole. In fact, the correlation between MDR1 overexpression and the fluconazole resistance phenotype has been previously demonstrated.\textsuperscript{93,94,115,116}

MDR1 homologues genes have been found in different species of Candida. Although the CgFLR1 gene confers resistance to fluconazole when expressed in Saccharomyces cerevisiae, the actual role of this gene in the resistance phenotype in C. glabrata is not known.\textsuperscript{89,97} In C. dubliniensis, the MDR1 gene appears to play an important role inazole resistance.\textsuperscript{95,115,117} In C. tropicalis, no association was found between drug resistance of clinical isolates and MDR1 overexpression.\textsuperscript{104,105} In C. parapsilosis, MDR1 expression has been associated with a fluconazole resistance phenotype.\textsuperscript{106,113}

Much less is known about the role of efflux pumps in resistance development in Aspergillus species compared with the number of studies on resistance in Candida. Most studies used A. fumigatus as a reference, and genes coding for efflux transporters are abundant in this species. On the basis of in silico analysis, at least 50 genes encoding ABC transporters and 300 genes encoding MFS transporters were predicted in the genome of this fungal species.\textsuperscript{118,119} Despite the large number of genes encoding these transporters, little is known about the association between gene overexpression and triazole resistance in A. fumigatus. In this species, the overexpression of the transporter genes AfuMDR1, AfuMDR2, AfuMDR3, AfuMDR4 and ATRF is associated with triazole resistance.\textsuperscript{119,120} The ATRF gene product (AtrF) is 1547 amino acids sequence and has characteristic MDR motifs.\textsuperscript{121} The AfuMDR4 gene is also involved in drug resistance of biofilms produced by A. fumigatus.\textsuperscript{118} Da Silva Ferreira et al.\textsuperscript{[121]} reported the expression of five ABC transporters (abcA-E) and three MFS transporters (mfsA-C) in response to the in vitro induction of voriconazole resistance in a clinical, azole susceptible, isolate. However, to date, the association between these findings and clinical resistance to azoles has not been fully elucidated.

Among the transporters characterised in drug-resistant clinical isolates of A. fumigatus, the levels of CDR1B are most consistent and prominently overexpressed. In fact, the mRNA expression levels for CDR1B were 5- to 30-fold higher in these isolates. CDR1B deletion also resulted in a 4-fold increase in the susceptibility to itraconazole in isolates both resistant and susceptible to this drug.\textsuperscript{122} Of note, the data available on the effect of efflux pumps on the decrease in the susceptibility of A. fumigatus to azoles were obtained from studies on resistance induced in vitro and not from clinical isolates for which resistance was acquired during infection of human hosts.\textsuperscript{122}

Changes in the target site of the drug (ERG11/CYP51)

The inhibitory activity of triazoles via changes in the target site has been described as a frequent cause of resistance. In this setting, non-synonymous mutations in ERG11 (CYP51 for Aspergillus) can change the three-dimensional structure of lanosterol 14α-demethylase, resulting in decreased affinity of this molecule to azoles and, consequently, decreased inhibition of ergosterol biosynthesis.\textsuperscript{15,123,124} According to Morio et al.\textsuperscript{[125]} more than 140 types of substitutions in the amino acid sequence of ERG11 have been described in C. albicans. However, few of these substitutions have been associated with azole resistance and, when present, may not contribute to the same extent to the development of the phenotype of resistance to this drug class. To address this hypothesis, some of these substitutions, including K143R, S405F, G464S, I471T and R467K, were described only in azole-resistant isolates, whereas others, including E266D and V488I, were observed in isolates both resistant and susceptible to azoles and, therefore, do not appear to contribute to the resistance phenotype.\textsuperscript{125–130}

Mutations in ERG11 have also been described in other species of Candida, including C. dubliniensis, C. krusei, C. tropicalis and, more recently, C. kefyr and C. parapsilosis.\textsuperscript{104,105,113,111–134} Although the primary mechanism involved in azole resistance appears to be the expression of efflux pumps, Hull et al.\textsuperscript{[135]} reported the presence of a non-synonymous mutation in ERG11 in a clinical C. glabrata isolate with cross-resistance between azoles and amphotericin B.
Unlike yeasts, Aspergillus has two distinct genes – CYP51A and CYP51B – that encode Cyp51. Most studies indicate that point mutations in the CYP51A gene in A. fumigatus confer resistance to triazoles and innate resistance to fluconazole. In contrast, CYP51B is associated with the growth rate and maintenance of the fungal cell, but its role in the susceptibility to azoles is still unclear. Clinical data suggest that CYP51A has an important role in regulating the activity of 14-a-demethylase, whereas CYP51B is a redundant gene whose expression becomes important only in the absence of CYP51A.

Specific mutations in CYP51A may confer resistance to one, two or all triazoles, and various mutations in this gene have been described in clinical isolates and laboratory-generated mutants. Non-synonymous point mutations are reported more frequently in this gene, particularly in codons 54, 98, 138, 220, 431, 434 and 448. Among these mutations, isolates with changes in codons 98, 138, 431, 434 and 448 generally have the pan-azole resistance phenotype, i.e. resistance to all azoles.

Denning et al. [140] evaluated 25 sputum samples positive for Aspergillus from patients with chronic pulmonary aspergillosis and allergic bronchopulmonary aspergillosis and detected mutations in codon M220 in four (16%) samples. Three of the four patients with this marker experienced treatment failure when itraconazole and/or posaconazole were used. To date, more than 30 different point mutations in CYP51A conferring azole resistance in A. fumigatus have been identified.

Another type of azole resistance mechanism that occurs very frequently is a tandem repeat in a 34-bp sequence in the promoter region (TR34) of the CYP51A gene. This mechanism, combined with a leucine to histidine substitution at codon 98 (L98H), resulted in increased CYP51A expression.

This mutation is probably associated with the use of azole fungicides in agriculture and was first reported in Europe. The same resistance mechanism was subsequently reported in clinical and environmental azole-resistant A. fumigatus from all over the world including Africa, Americas, Asia, Australia and the Middle East. In contrast, more recently few studies from Japan, described azole resistance in clinical A. fumigatus isolates but interestingly none of them exhibited TR34/L98H resistance mechanism, instead several SNPs and novel mutations, F332K and P216L were reported. A single centre study from Japan reported 5.2% azole-resistant isolates harbouring only G54E/R/W and I266N mutation.

Recently, a novel mechanism of resistance involving the tandem repeat of a 46-bp sequence (TR46), together with the substitution of a few amino acids (Y121F/T289A), was associated with treatment failure in patients using voriconazole in the Netherlands. In addition to Belgium and the Netherlands, clinical and environmental isolates carrying the TR46/Y121F/T289A mutation have been identified in China, Colombia, Denmark, France, Germany, India, Spain, Tanzania and US. The TR46/L98H mutation induces pan-azole resistance, whereas the TR46/Y121F/T289A mutation induces high resistance to voriconazole.

Hodiamont et al. [159] evaluated a patient with chronic granulomatous disease and osteomyelitis due to A. fumigatus and found a mutation associated with the tandem repeat of a 53-bp sequence in the promoter region of CYP51A in this species. However, this mutation did not involve any amino acid substitution. It is of note that the patient received prophylaxis with itraconazole and that the isolate showed resistance to itraconazole and voriconazole and lower susceptibility to posaconazole. This same TR53 has recently been found in resistant environmental A. fumigatus isolates from Colombia.

In fact, not all isolates with increased MIC values have a mutation in the CYP51A gene, and not every mutation implies fungal resistance to antifungal agents.

### Increased production and inhibition of the target enzyme by the drug

The increased production of ergosterol due to the increase in the expression of the ERG11 gene (CYP51) constitutes a common azole-resistance mechanism in C. albicans and A. fumigatus. In C. albicans, two mechanisms are associated with ERG11 overexpression: (i) an increase in the expression of the transcription factor Ufc2, which is responsible for regulating the expression of most genes involved in ergosterol biosynthesis; and (ii) formation of an isochromosome with two copies of the left arm of chromosome 5, where the ERG11 gene is located, or full duplication of the chromosome.

The association between increased expression of ERG11 and azole resistance has also been reported in C. dubliniensis, C. glabrata, C. krusei, C. parapsilosis and C. tropicalis.

In A. fumigatus, the increased production of ergosterol may be associated with the transcriptional regulation of the CYP51A gene, and this regulation...
may be mediated by both transcription factors and tandem repeats in the promoter regions. SrbA, a transcriptional regulator belonging to the sterol regulatory element binding protein (SREBP) family, has important implications in triazole resistance in Aspergillus. The deletion of the SRBA gene in A. fumigatus induces hyper susceptibility to azoles and increases fungal susceptibility to fluconazole. This phenomenon may be associated with decreased expression of the CYP51A gene in the absence of SrbA because isolates with repressed CYP51A also exhibited increased susceptibility to this drug.

Recently, the complete genome sequences of two inbred isolates of A. fumigatus (one susceptible and one resistant) isolated from immunocompromised patients with chronic pulmonary aspergillosis were used to identify mutations that confer azole resistance. This study identified several non-synonymous mutations. However, only the mutation in the transcription factor associated with the CCAAT-sequence in a subunit of the HapE complex conferred resistance of this progeny to azoles by increasing the CYP51A gene expression levels. CCAAT-sequences are present in the promoters of a large number of eukaryotic genes. In general, they are formed by approximately 50- to 200-bp sequences upstream of the transcription initiation site and may occur in any orientation of the gene. These sequences are attached to different transcription factors, which can explain the diversity of functions associated with the modulation of the transcription levels in eukaryotic cells. Coincidentally, the CCAAT-sequence is present in the promoter of the CYP51A gene. Therefore, the hapE mutant can induce the increase in the expression of the CYP51A mRNA, demonstrating that transcription factors can regulate the expression of these genes. However, to date, these changes have not yet been described among resistant clinical isolates.

**Risk factors for the development of candidaemia by fluconazole-resistant isolates**

The first epidemiological studies that assessed the risk for developing bloodstream infection (BI) due to C. glabrata and C. krusei, which are intrinsic resistant to fluconazole, provided clinicians with information necessary for the early detection of patients at risk of developing IFIs due to fluconazole-resistant Candida isolates. In these cases, the independent variables associated with BI due to C. glabrata included the previous use of fluconazole or caspofungin, recent exposure to metronidazole, surgical procedures, the presence of central venous catheter (CVC) position, cancer, mechanical ventilation and senescence. For infections due to C. krusei, the independent risk factors included previous exposure to fluconazole or caspofungin, neutropenia, solid tumours, organ transplantation, splenectomy and use of antibiotics with antianaerobic coverage.

Over the past few years, an increase in the number of cases of candidaemia due to fluconazole-resistant isolates belonging to species primarily sensitive to this drug, including C. parapsilosis and C. tropicalis were noted. Several studies have explored the predisposing factors for the development of candidaemia due to fluconazole-resistant Candida species.

In this scenario, Garnacho-Monteiro et al. observed that neutropenia, chronic kidney disease and previous exposure to fluconazole were independent risk factors for the isolation of fluconazole-resistant Candida isolates. Recently, Cuervo et al. tested the susceptibility of more than 900 isolates to amphoterin B and itraconazole. The percentage of patients harbouring A. fumigatus isolates resistant to this drug class increased to 15% in 2007 and to 20% in 2009. In the Netherlands, azole resistance increased dramatically from 2.5% in 2000, to 4.9% in 2002, to 6.6% in 2004, to 10% in 2009. By 2013 in certain centres resistance rates of up to 15% are found in high risk haematology patients. This trend represents a yearly increase of 6%, and in this case, the patients were infected by resistant isolates but without prior exposure to antifungal therapy. Therefore, although higher rates of resistance in A. fumigatus were described in the Netherlands and the UK (Manchester),
it is essential to recognise that the mode of resistance development had distinct determinants in these countries.

In Manchester, where the National Aspergillosis Centre is located, the hypothesis of resistance is attributed to the use of long-term antifungal therapy in patients with chronic forms of pulmonary aspergillosis, more specifically, aspergilloma and chronic cavitary aspergillosis. In the latter case, some researchers suggested the occurrence of microevolution of the fungus in the lung tissue because both sensitive and resistant isolates with identical or closely related genotypes were reported to infect the same patient.177,178

In the Netherlands, the occurrence of triazole resistance in Aspergillus is closely associated with so called environmental mutations. It is believed that the use of azoles as fungal pesticides has led to the emergence of mutations in the CYP51A gene.46,57 From approximately 30 azole fungicides commonly used in agriculture, seven showed cross-resistance with azoles in clinical use.79,179 Two resistance mechanisms associated with point mutations in CYP51A (TR34/L98H and TR46/Y121F/T289A) have been reported in clinical and environmental isolates, and the latter were recovered from patients not exposed to antifungal agents.151–153

An increasing number of cases involving azole-resistant environmental isolates of A. fumigatus have been documented in Asia and Europe.78,79,83,180 It is suggested that most of the clinical cases of resistance reported between 2009 and 2011 from Dutch patients were due to the dissemination of resistant environmental isolates.181 Between 2012 and 2015, other countries, including Austria, Belgium, Denmark, France, Germany, Ireland, Poland, Spain, Sweden and the UK, also reported the identification of clinical isolates of A. fumigatus harbouring the TR34/L98H mutation.12,65,141,152,181–183

The wide geographical distribution of the TR34/L98H mutation has raised questions about the origin of azole-resistant Aspergillus isolates, including (i) the geographical migration of resistant conidia carrying this mutation, (ii) the independent local development of this mutation and subsequent selection of the TR34/L98H mutation in unrelated isolates and (iii) the previous two hypotheses occurring simultaneously.144 To clarify the origin and dissemination of this genotype, Camps et al. [181], using molecular markers, evaluated the genetic relationship of 142 European isolates recovered between 1998 and 2007. The authors noted that the isolates with the TR34/L98H resistance mechanism showed less genetic variation compared with wild-type susceptible isolates or with those with other resistance mechanisms. In addition, the crossing of these isolates indicated that the TR34/L98H isolates could cross with azole susceptible isolates of different genetic origins, suggesting that TR34/L98H isolates could complete their natural sexual cycles. This finding suggests the occurrence of a common ancestry for the TR34/L98H mechanism.181

Chowdhary et al. [79] evaluated nine loci in azole-resistant and susceptible isolates from India and observed the occurrence of a wide genetic diversity among environmental and clinical isolates susceptible to azoles, and these isolates were highly polymorphic. The azole-resistant isolates harbouring the TR34/L98H mutation shared the same genotype, indicating clonal propagation. However, this genotype was not detected in other isolates from other countries, such as China, France, Germany and the Netherlands. Therefore, the authors suggested that the Indian genotype was an adaptive recombinant progeny derived from the crossing between an azole-resistant isolate (that migrated into India) and an azole-sensitive isolate (that was native to India); this crossing underwent mutation and quickly dispersed to various parts of India.28

Of note, 61% of the global agricultural fungicide market is concentrated in Western Europe and Asia, suggesting that the prevalence of the TR34/L98H mutation in the CYP51A gene is directly associated with geographical parameters and the indiscriminate use of these antifungal fungicides.184

With regard to the emerging TR46/Y121F/T289A mutation, to date, clinical isolates carrying this mutation have been detected in China, Belgium, Denmark, France, Germany, the Netherlands, Spain and the US, whereas environmental isolates with this mechanism have been reported in Belgium, Colombia, France, Germany, India, the Netherlands and Tanzania.12,64,150–152,157,158,184

At this point, an increased concern about the rapid spread of isolates harbouring these two mutations should be underscored because these cases have been reported in hospitals and homes throughout Europe.12,73 Furthermore, the exposure of A. fumigatus to azole fungicides can induce the emergence of novel resistance mechanisms over time, thereby compromising the clinical use of azoles in the treatment of diseases associated with Aspergillus.

Rates of echinocandin resistance in Candida spp. and Aspergillus spp. worldwide

Echinocandins represent the most recent antifungal class, consisting of three agents – micafungin,
anidulafungin and caspofungin – that act by inhibiting cell wall synthesis. Caspofungin was the first echinocandin approved by the Food and Drug Administration (FDA) in 2001 in the US, followed by miconazol and anidulafungin, which were approved in 2005 and 2006, respectively, and the three drugs are available only in parenteral preparations. Since their introduction, echinocandins have been widely used for the treatment and empirical therapy of fungal infections due to *Candida* and *Aspergillus*.188–190 Although echinocandin resistance is still considered unusual, cases of resistance by using this therapeutic class have become increasingly frequent.42,191 Recently, Cleveland *et al.* [192] conducted a surveillance study in two US cities between 2008 and 2013 involving invasive candidiasis in a prospective cohort. In this series, increases in cases of echinocandin resistance were observed in Atlanta (from 1.2% to 2.9%, a 147% increase) and Baltimore (2% to 3%, a 77% increase). In addition the authors reported that 44% of the multidrug-resistant isolates that exhibited an echinocandin- and fluconazole resistance phenotype were recovered from patients without prior exposure to echinocandins, suggesting the occurrence of horizontal transmission of this resistant phenotype.

Although this resistance mechanism remains uncommon in isolates of *C. albicans*, *C. krusei* and *C. tropicalis*, echinocandin resistance has become increasingly common among isolates of *C. glabrata* and other species such as *C. lusitaniae* and *C. kefyr*.42,193

Recently, Alexander *et al.* [194] reported that in the US, the resistance rate of *C. glabrata* isolates increased from 4.9% to 12.3% between 2001 and 2010. In Europe, the same trend is observed, although on a smaller scale. Accordingly, a study reported that the resistance rate of *C. glabrata* isolates increased from 0% to 3.1% between 2004 and 2013 in Denmark.195 It is important to mention that the factors associated with the increased development of resistance in *C. glabrata* isolates remain unknown.195

In Latin America, a single case report has demonstrated clinical and microbiological echinocandin resistance in *Candida*. Bizerra *et al.* [196], using sequencing methodologies for the study of FKS genes and quantification of glucan synthesis, reported the occurrence of a mutation associated with this resistance phenotype in *C. glabrata* isolated from a single patient.

To date, little is known about echinocandin resistance in *Aspergillus*, particularly because susceptibility tests are not performed routinely and present technical difficulties and problems associated with reproducibility and the clinical-in vitro testing correlation.197,198

Because the therapeutic outcome of treatment of aspergillosis with echinocandins is poorer than with polyenes and triazoles, the use of echinocandins in these patients is limited and restricted to cases involving empirical or combination therapies.189,198,199 Consequently, few cases of secondary resistance of *Aspergillus* to this therapeutic class have been reported. However, non-*Aspergillus fumigatus* species may exhibit primary resistance, as is the case in *A. lentulus*.200,201

**Molecular mechanisms of echinocandin resistance in *Candida* spp. and *Aspergillus* spp.**

Echinocandins present predominantly fungicidal activity against *Candida* spp. and fungistatic activity against *Aspergillus* spp.202–204 Experimental data have shown that the primary mechanism of action of echinocandins is associated with inhibition of the synthesis of the fungal cell wall via inhibition of β-1,3-glucan synthase (FKs1/AFks1 for *Aspergillus fumigatus*), which is responsible for the synthesis of β-1,3-glucans.205 In fungi belonging to the phylum *Ascomycota*, the paralogous genes FKS1, FKS2 and FKS3 are responsible for the synthesis of β-1,3-glucan synthase. In most species, the FKS1 gene is more active, whereas FKS2 is expressed in adverse conditions and only during the sexual cycle and sporulation.205 The FKS3 gene is involved in the formation of the cell walls of spores.206 In some species, including *C. glabrata*, FKS1 and FKS2 are functionally redundant.207 In *Aspergillus* spp., only FKS1 has been described.208 The echinocandin resistance mechanisms reported in *Candida* spp. and *Aspergillus* spp. will be discussed below.

**Mutations in the FKS genes**

The main resistance mechanism described for echinocandins involves the occurrence of mutations in the FKS1 gene, resulting in conformational changes in the enzyme encoded by this gene (FKs1), decreased affinity between echinocandins and Fks1, and the consequent resistance to these compounds.209–212 These mutations occur in two specific, highly conserved regions of the FKS1 gene known as hot spot 1 (HS1), corresponding to positions 641-649 in the amino acid sequence of the Fks1 protein, and hot spot 2 (HS2), spanning positions 1345-1365 of the amino acid sequence.211,213

In *C. albicans*, among the mutations already described, the replacement of serine with proline,
phenylalanine or tyrosine at position 645 has been described as the most frequent event. In fact, Balashov et al. [209] found that, among 85 caspofungin-resistant isolates of *C. albicans*, 93% had a mutation in the serine residue at position 645.

Mutations in FKS1 have been described not only in *C. albicans* but also in *C. krusei*, *C. parapsilosis* and *C. tropicalis* and, more recently, in *C. kefyr*. Several studies have demonstrated that these drugs exhibit inhibitory activity against *C. parapsilosis* infections. However, their antifungal activity is only fungicidal and not fungistatic, which may explain the higher incidence of persistent candidaemia with *C. parapsilosis* in patients treated with echinocandins.

Despite the advances made in the characterisation of echinocandin resistance mechanisms in *Candida*, to date, little is known about the molecular basis of *Aspergillus* resistance to these drugs. Moreover, few clinical isolates associated with treatment failure have been investigated. Arendrup et al. [230] evaluated the echinocandin resistance mechanism of an isolate of *A. fumigatus* isolated from a patient with invasive pulmonary aspergillosis who experienced treatment failure with caspofungin; this isolate did not harbour mutations in the *AFKS1* gene; therefore, the β-1,3-glucan synthase was completely sensitive in vitro. However, the *AFKS1* gene was overexpressed, indicating that treatment with caspofungin did not decrease the expression of this gene. Similarly, Arendrup et al. [231] studied the amplified DNA of *A. fumigatus* from liver and lung tissue samples from a patient with aspergillosis subjected to long-term therapy with caspofungin, but did not find any mutations in the sequence of the hot spot region of *AFKS1*.

Because the reports of echinocandin resistance in clinical isolates are rare, several researchers have sought in vitro models for the induction of resistance in the laboratory with the aim of better characterising the resistance mechanisms associated with these drugs. Gardiner et al. [232] inserted a site-directed mutation in *AfKS1* consisting of a substitution of serine with tyrosine at codon 678 (S678Y). This mutation decreased the susceptibility to caspofungin 16-fold, with a minimum effective concentration (MEC) of 4 μg/mL. This same study used mini-array analysis of 220 target genes to evaluate the expression of relevant genes that could contribute to the decrease in the susceptibility to caspofungin in mutant isolates. Their results allowed the identification of (i) increased expression levels of 28 genes encoding structural components of the cell wall (*AGS2*, *CWPI*, *MC136*), (ii) enzymes responsible for cell wall biosynthesis (*Kre1*, *Mci35*) and (iii) signal transducers (*MAPK*, *MC1106*). The expression levels of genes encoding proteins involved in transportation, such as *MDR1* and *MDR4*, were also increased in this mutant.

Rocha et al. [233] generated a mutant by the substitution of serine with proline at codon 678 (S678P) in *AfKS1*, and this change was sufficient to confer resistance to three echinocandins, demonstrating that the modification in Fks1 is a conserved mechanism in echinocandin resistance among pathogenic fungi.

Of note are the differences in the in vitro susceptibility profile to echinocandins between different species of *Aspergillus*. For example, *A. niger* exhibited higher MECs to echinocandins compared with other species, suggesting the occurrence of differences in the cell wall composition in these fungal species. In contrast, *A. lentulus* was less susceptible to caspofungin, although it was apparently susceptible to micafungin and anidulafungin. The analysis of the *FKS1* region of this fungal species indicated no polymorphism in the hot spot region of this gene. One hypothesis to explain this phenomenon is the excessive production of glucan by this species, which could limit the effective concentrations of antifungal agents.

**Increase in chitin synthesis**

The increase in chitin synthesis has been described as an important resistance mechanism against echinocandins. Walker et al. [235] have shown that the exposure of *C. albicans* to low levels of echinocandins induces the expression of genes encoding chitin synthases, increases the concentration of chitin in the cell wall and consequently decreases the activity of
Risk factors for the development of candidaemia with echinocandin-resistant isolates

Although still rare, cases of echinocandin resistance in clinical isolates of *Candida* have been increasingly reported, in particular among *C. glabrata* isolates.193

Using case-control studies, several authors have highlighted that prior exposure to echinocandins results in a higher risk of developing candidaemia due to the lower susceptibility of these isolates to this class of antifungal agents.215,219,240 For infections due to *C. glabrata*, in addition to the previous exposure to antifungal agents, exposure to total parenteral nutrition (TPN) has also been reported to be a predisposing factor.241–243 Recent studies have indicated the development of echinocandin resistance after short periods of exposure to these antifungal agents. In fact, Ruggero and Topal identified a *C. albicans* isolate with a FKS1 mutation after 14 days of prophylactic therapy with micafungin.244

Resistance to amphotericin B

Amphotericin B is an antifungal agent belonging to the class of polyenes which are natural antifungal compounds produced by species of the genus *Streptomyces*. Since the 1950s until the discovery of azoles, standard therapy for IFIs involved the use of amphotericin B. Because of its broad spectrum of activity against yeasts, filamentous and dimorphic fungi and its fungicidal activity, amphotericin B based drugs are still considered the gold standard for the treatment of most fungal infections, particularly in cases of severe IFI, when a quick response is required.89,200,245–247

The classical mechanism of action of amphotericin B involves the irreversible binding to ergosterol present in the plasma membranes of fungal cells, resulting in the formation of pores in the cell membrane, with loss of K+ and Na+ ions, which leads to impairment of the cellular osmotic balance and consequent cell death.89,248,249 Furthermore, recent findings have shown that exposure to amphotericin B induces the production and accumulation of reactive oxygen species, which eventually causes cell death.250,251

Although amphotericin B has a broad spectrum of activity, this antifungal drug is highly toxic to the host. Nephrotoxicity is the major side-effect, which has limited its clinical use.188,252–256 To decrease drug toxicity, lipid formulations have been developed which are currently first choice of treatment of IFIs.257–261

Secondary resistance to amphotericin B is rare among isolates of *Candida* spp., but some cases of resistance have been reported.135,262–267 Several studies have shown resistance of *C. lusitaniae* to this drug. Although these studies reported that *C. lusitaniae* isolates have low rates of resistance *in vitro*, this species seems to develop secondary resistance during treatment with amphotericin B, and several cases of therapeutic failure are on record.267–274

Similar to *Candida*, secondary resistance to amphotericin B has not been observed in *Aspergillus*, although some patients experienced treatment failure when using this drug.262,263,275 However, primary resistance to amphotericin B has been reported among isolates of *A. flavus*, *A. lentulus*, *A. terreus* and *A. ustus*.48,53,276–280

Previous studies have also shown that isolates of *A. flavus* exhibit higher MIC values to amphotericin B compared with isolates belonging to the section *Fumigati*.65,199,276,277,279,281

Hadrich et al. [277] evaluated the *in vitro* susceptibility to amphotericin B of 37 *A. flavus* isolates recovered from 14 patients with haematological malignancies, of which 13 were treated with amphotericin B deoxycholate. Of the total isolates evaluated, 84% (*N* = 31) exhibited resistance to this drug *in vitro*. Among the patients infected with resistant isolates, nine were treated with amphotericin B, and 88% (*N* = 7) died.277 Similar results were observed by Lass-Flörl et al. [281], who compared the *in vitro* susceptibility to amphotericin B in strains of *A. flavus* with clinical outcome of patients undergoing bone marrow transplantation. In this study, 12 *A. flavus sensu lato* isolates were evaluated. Only patients infected with susceptible isolates (MIC to AMB <2 μg/mL, *N* = 4) survived, whereas those infected with resistant isolates (CIM to AMB ≥2 μg/mL, *N* = 8) died.281

Alastruey-Izquierdo et al. [65] evaluated 280 clinical isolates of *Aspergillus* from 29 Spanish hospitals and reported a rate of resistance to amphotericin B of 10.8%. In this series, four of 27 (14.8%) *A. flavus* isolates and seven of 26 (27%) *A. terreus* isolates were resistant to this drug. In Brazil, Gonçalves et al. [54]...
observed a high rate of resistance (49%) to amphotericin B in a series consisting of 77 clinical isolates belonging to the section Flavi. Likewise studies from India show high amphotericin B MICs in 31 out of 37 isolates (84%) [277]. However, the genetic mechanisms responsible for acquired resistance to amphotericin B have not been elucidated. Some studies have indicated that mutations in the genes involved in the biosynthetic pathway of ergosterol (ERG), including ERG2, ERG3, ERG5, ERG6 and ERG11, may lead to decreased concentration of ergosterol and the formation of intermediate sterols in the fungal cell membrane.\textsuperscript{286} These quantitative and qualitative changes in plasma membrane sterols decrease the affinity of amphotericin B for its target sites and constitute the major mechanisms of resistance of Candida isolates to this drug.

Although most of the data related to resistance mechanisms for amphotericin B have been observed in trials with yeast, Chamilos and Kontoyiannis thoroughly reviewed these mechanisms in Aspergillus. These authors reported that these mechanisms can be attributed to two phenomena: (i) a decreased concentration of membrane ergosterol via mutations in the biosynthetic pathway of ergosterol and/or prior exposure to triazoles, and (ii) increased production of reducing enzymes (e.g. catalase), which confer resistance to oxidative stress and can also act as oxidising agents.\textsuperscript{287}

Although A. terreus is the best studied species, its intrinsic mechanism of resistance to amphotericin B is not fully elucidated but has been attributed to the high expression levels of catalase, an enzyme that causes damage to the fungal cell membrane via generation of reactive oxygen species.\textsuperscript{288} A recent study showed that application of pro-oxidants significantly affects amphotericin B efficacy by rendering resistant isolates more susceptible to this drug which was also confirmed in vivo in a Galleria model.\textsuperscript{289}

Conclusions

Over the past few years, the significant increase in the use of antifungal agents for the treatment of candidiasis and invasive aspergillosis has resulted in the emergence of resistant clinical isolates, particularly against triazoles and echinocandins. In addition, a recent emergence of fluconazole resistance among isolates that were primarily sensitive to this drug, including C. parapsilosis and C. tropicalis has been noted. For echinocandins, despite the low resistance rates, the occurrence of isolates with lower susceptibility to this drug has been increasingly reported, particularly among C. glabrata isolates.

Therefore, we highlight the need to conduct antifungal resistance surveillance studies using clinical isolates of Candida and Aspergillus in different geographical regions and monitoring of the infection rates in distinct population groups with the aims of early detection of resistance to these drugs and implementation of efficient policies for infection control and treatment.

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Potential conflicts of interest

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Molecular mechanisms of antifungal resistance


We are interested in understanding the mechanisms of antifungal drug resistance and their implications for clinical practice. The R467K amino acid substitution in the Erg11 protein of the fungal pathogen Aspergillus fumigatus is known to confer resistance to azole antifungal drugs. We used site-directed mutagenesis to examine the structure-function relationship of the Erg11 protein and its role in azole drug resistance.

Our results suggest that R467K substitution alters the catalytic domain relative to a bilayer and that fine-tuning of sterol 14α-demethylase might orient the catalytic domain relative to a bilayer. Membrane spanning cytochrome P450 suggests constraints that might have evolved from lungs of patients with chronic fungal disease. The structure-function relationship of the Erg11 protein and its role in azole drug resistance is related to different azole target alterations in azole-resistant isolates.

Amino acid substitutions in the Erg11 protein can lead to changes in the fold and function of the protein. We have identified missense mutations that contribute to resistance toazole antifungal agents. The structure-function relationship of the Erg11 protein and its role in azole drug resistance is related to different azole target alterations in azole-resistant isolates.

New evidence indicates that the R467K substitution in the Erg11 protein is a major contributor to azole drug resistance. Our results also suggest that the R467K substitution may alter the fold of the Erg11 protein, which could have implications for the design of new azole drugs.


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