Responding to the emergence of antifungal drug resistance: perspectives from the bench and the bedside

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The incidence of serious fungal infections is increasing rapidly, and yet the rate of new drugs becoming available to treat them is slow. The limited therapeutic armamentarium is a challenge for clinicians, because the available drugs are often toxic, expensive, difficult to administer, ineffective or a combination of all four. Given this setting, the emergence of resistance is especially concerning, and a review of the topic is timely. Here we discuss antifungal drug resistance in Candida spp. and Aspergillus spp. with reference to the most commonly used first-line antifungal agents – azoles and echinocandins. We review the resistance mechanisms of the leading pathogens, how resistance can be identified in the diagnostic lab and the clinical implications of resistance once detected.

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The global incidence of fungal disease has increased dramatically in recent years. Current (likely under-) estimates suggest that there are approximately 300 million life-threatening fungal infections annually, resulting in 1.6 million deaths [1]. Health impacts worldwide include high morbidity, an overall mortality of 30–80% and a multibillion dollar annual economic burden. The limitations of current therapies – including one or more of toxicity, poor bioavailability and relative inefficacy – are now further amplified by the emergence and escalation of drug resistance.

In considering the impact of drug resistance in the clinical setting, it is important to note that resistance does not always equate with clinical failure. Many factors contribute to clinical outcome. In particular, host factors such as an impaired immune system, other comorbidities, site of invasive fungal disease; therapeutic factors such as dosage regimen, compliance and drug toxicity; and ancillary factors such as source control all impact clinical outcomes. Nevertheless, there is evidence that rapid initiation of appropriate antifungal therapy reduces mortality in invasive candidiasis in the critically ill and in invasive aspergillosis in at-risk hematology patients [2]. Detection and characterization of drug resistance in vitro can assist clinicians to select the best antifungal regimen. A review of how this can be achieved is timely.

This review focuses on Candida and Aspergillus. The yeast Candida is a normal part of human skin flora, but can cause serious invasive infections. Aspergillus, a mold, is associated with serious invasive infections, and allergic conditions. Azole resistance in Candida and Aspergillus and echinocandin resistance in Candida are among the most challenging problems in clinical mycology. We will discuss the drivers of antifungal resistance and the clinical value of characterizing resistance. We will discuss different laboratory methods for detection of resistance and whether rapid detection can improve outcomes in outbreaks or for individual patients. In addition, we will discuss strategies for management of resistant fungal infections.

Epidemiology of azole resistance
Antifungal resistance, whether intrinsic (primary) or acquired (secondary), can result in worse clinical outcomes or clinical failure. Pressure exerted by the use of antifungals may select for infection with intrinsically resistant strains,
or strains may acquire resistance during prolonged therapy. Furthermore, environmental organisms with intrinsic or acquired resistance may infect antifungal naive patients. See Figure 1.

**Epidemiology ofazole resistance in Candida**

In *Candida*, susceptibility to azoles varies by species. A global surveillance study showed 92% of all cases of candidemia were caused by: *Candida albicans* (65.3%), *Candida glabrata* (11.3%), *Candida tropicalis* (7.2%), *Candida parapsilosis* (6%) and *Pichia kudriavzevii* (formerly and more commonly known as *Candida krusei*) (2.4%) [3]. These species display important differences in susceptibility. In the surveillance period 2005–2007, 98 and 98.5% of *C. albicans* were susceptible to fluconazole and voriconazole, respectively. For *C. glabrata*, these figures were 68 and 83%, *C. tropicalis* 91 and 90% and *C. parapsilosis* 93 and 97%. Only 9% of *C. krusei* were susceptible to fluconazole, while 83% were susceptible to voriconazole. The sixth commonest species, *Meyerozyma guilliermondii* (previously *Candida guilliermondii*), was only responsible for 0.7% of candidemia cases, but 74% of those showed reduced susceptibility to fluconazole and 91% displayed reduced voriconazole susceptibility [3].

Although it remains an uncommon pathogen, particular concern has been raised about *Candida auris* as a cause of nosocomial outbreaks since its emergence in multiple countries in 2009 [4]. *Candida auris* is resistant to multiple antifungal agents; only 7 and 46% of isolates are susceptible to fluconazole and voriconazole, respectively.

The frequency of different *Candida* species in clinical isolates (and therefore the anticipated susceptibility patterns) varies with geographic location and patient characteristics. For example, *C. glabrata* is over-represented in North America, occurring in 21% of patients [3]. Similarly, *C. krusei*, is over-represented in Europe (3.4%) and North America (3.1%). *Meyerozyma guilliermondii* ranks in the top five causes in Latin America, being responsible for 2.2% of cases. Finally, *C. tropicalis* is notably more common in Asia Pacific (11.7%) and Latin America (13.2%) than the global average.

Variation in species distribution with patient age was noted in the SENTRY surveillance study [5]. Candidemia due to *C. albicans* and *C. parapsilosis* decreased with advancing age, whereas that due to *C. glabrata* increased. The influence of underlying diseases was demonstrated in an Australian study of hematology and oncology patients. While *C. albicans* was the leading cause of candidemia in both patient groups (causing 51 and 33% of episodes in oncology and hematology patients, respectively), there were differences in the proportion of non-*albicans* species [6]. For example, *C. glabrata* was the second commonest in oncology patients (causing 19.3% of episodes) and fourth in hematology patients (causing 12.3% of episodes). *Candida krusei* caused <1% of episodes in oncology patients, but 16.6% in hematology patients. This study also highlighted that prior antifungal therapy reduced the proportion of infections caused by *C. albicans* and increased the proportions due to *C. glabrata*, *C. parapsilosis* or *C. krusei*. Forty-four percent of patients receiving more than 6 days of fluconazole therapy prior to the appearance of candidemia
were infected with *C. krusei*, compared with 11% of those who had received less than 6 days of therapy. Furthermore, the odds of having a fluconazole-resistant isolate were significantly increased by prior azole therapy.

**Epidemiology of azole resistance in Aspergillus**

Azoles are the first-line treatment for most infections caused by *Aspergillus* species [7]. Globally, *Aspergillus fumigatus* is the most frequent cause of invasive aspergillosis, causing over 90% of such infections [8], followed by *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus terreus*. Distribution varies by geographic location, for example, *A. flavus* is isolated at least as frequently as *A. fumigatus* in subtropical and tropical regions [9,10].

Intrinsic resistance to azoles varies by *Aspergillus* species. They are all intrinsically resistant to fluconazole and ketoconazole, because of a naturally occurring amino acid substitution in 14-α sterol demethylase A, encoded by *CYP51A* [11]. However, they are generally considered intrinsically susceptible to other azoles. *Aspergillus fumigatus* is actually a species complex containing up to 63 members, many of which can only be distinguished by molecular methods [12]. *Aspergillus fumigatus sensu stricto* is the commonest species in the complex, accounting for approximately 95% of all isolates [13]. Although it is intrinsically susceptible to all *Aspergillus*-active azoles, some of its sibling species, including *Aspergillus lentulus*, *Aspergillus thermomutatus* and *Aspergillus udagawae*, have caused disease refractory to azole therapy [13]. Furthermore, within the *A. niger* species complex, itraconazole resistance has been observed in 25–50% of clinical isolates, with some evidence of cross-resistance with voriconazole and posaconazole [14–16]. Both cases highlight the importance of molecular epidemiology.

**Mechanisms of azole resistance**

Azoles inhibit the enzyme lanosterol 14α-demethylase, preventing the synthesis of ergosterol, a fungal cell-membrane component. In *Candida* and *Aspergillus*, the major causes of azole resistance are changes in this target enzyme (reduced drug–target affinity, upregulated production or both) or upregulation of efflux pumps [17].

**Mechanisms of azole resistance in Candida**

Two mechanisms are responsible for most azole resistance in *Candida* species. These are reduced affinity for the target enzyme, lanosterol 14α-demethylase, and overexpression of efflux pumps.

Mutations in lanosterol 14α-demethylase, encoded by the gene *ERG11*, were first described in 1997 [18]. Many point mutations have been identified subsequently and have been reviewed in detail [19–21]. *ERG11* mutations are responsible for intrinsic fluconazole resistance in *C. krusei* [22] and have been reported in *C. albicans*, *C. parapsilosis* and *C. tropicalis* [21]. In addition, upregulation of lanosterol 14α-demethylase production can overcome drug concentrations achievable within fungal cells and has caused resistance in *C. albicans* and *C. tropicalis* [21].

Two families of efflux pump, the ATP-binding cassette pumps (encoded by *CDR* genes) and major facilitator superfamilies (encoded by *MDR* genes) [23] are both involved in antifungal resistance. Increased expression of *CDR* genes occurs in *C. albicans*, *C. glabrata* and *C. krusei* and is generally associated within a broader spectrum of antifungal resistance [19]. Increased expression of *MDR* genes has only been described in *C. albicans* and *C. parapsilosis* [21].

Azoles both prevent the formation of ergosterol and cause intracellular accumulation of toxic 14α-methyl-3,6-diol. Resistance in *Candida* can result from bypassing these metabolic pathways. Isolates with an inactive *ERG3* gene do not form 14α-methyl-3,6-diol from 14α-methylfucosterol [24]. The accumulated 14α-methylfucosterol provides a functional substitute for ergosterol in the cell membrane, thus negating the impact of the azole. This mechanism occurs in *C. albicans* and *C. tropicalis* [21]. A related mechanism, whereby exogenous sterols are taken up by the cell, has been described in *C. glabrata* [21]. See Figure 2.

**Mechanisms of azole resistance in Aspergillus**

The problem of acquired azole resistance in *A. fumigatus* has been well-described. Resistance acquired following prolonged therapy has been recognized since the late 1980s. Contributory mechanisms include increased efflux pump activity and decreased target enzyme affinity [25].

However, there has been growing interest in acquired resistance in environmental isolates of *A. fumigatus* over the past decade, which may have emerged through exposure to agricultural azoles. Such isolates have been associated with infection in azole naive patients [26,27]. In a global surveillance study, over 50% of azole-resistant clinical isolates shared common resistance mechanisms, with point mutations in *CYP51A* and variable tandem repeat insertions in the promoter region (TR34/L98H or TR46/Y121F/T289A). These mechanisms confer multi-azole resistance,
and have been frequently identified in environmental samples [28]. See Figure 3. The mechanism of intrinsic azole resistance in the cryptic species described above is yet to be clarified.

Echinocandin resistance

Echinocandins inhibit the membrane-bound enzyme β-(1,3)-D-glucan synthase and prevent formation of β-(1,3)-D-glucan, a vital cell-wall component. The enzyme complex is comprised of three functional (Fks1, Fks2 and Fks3) subunits and one regulatory (Rho1) subunit [29]. The specific targets for echinocandins are the Fks1 and Fks2 subunits [17], and resistance arises from conformational changes in these target sites [30]. Upregulation of efflux pumps has not been implicated in echinocandin resistance [31].

Echinocandin resistance in Candida

Candida develops resistance to echinocandins through point mutations in the FKS1 or FKS2 genes, although the exact biochemical mechanism is yet to be described [20]. Candida parapsilosis and C. guillermondii naturally contain mutations in FKS1, which are responsible for their reduced susceptibility to echinocandins [32,33], although the clinical impact of this mutation has not been established. Of concern, one study reported echinocandin resistance in 7% of C. auris isolates [4]. Acquired mutations in FKS1 arising after prolonged drug exposure, and associated with breakthrough infections, have been observed in C. albicans, C. tropicalis, C. krusei, and C. glabrata [34]. Acquired resistance mutations in FKS2 have, thus far, only been observed in C. glabrata [35].

Echinocandin resistance in Aspergillus

None of the pathogenic Aspergillus species are intrinsically resistant to echinocandins. Indeed, the only noted variation between the species complexes is that A. niger is hypersusceptible to caspofungin [56]. Development of
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**Review**

Azoles

Echinocandins

Aspergillus spp.

Cell wall

Membrane

Efflux pump

Fks

Rho1

1 2 3

Cyp-51A

Figure 3. **Overview of the major resistance mechanisms in Aspergillus spp.** (A) Shows normal binding of azoles (red) to 14-α-sterol demethylase (Cyp-51A); and normal binding of echinocandins (peach) to β-(1,3)-D-glucan synthase (Fks, including the three functional subunits Fks1, Fks2, and Fks3, and the regulatory subunit Rho1). (B) Shows reduced azole binding due to conformational changes in 14-α-sterol demethylase. (C) Shows reduced azole efficacy due to increased production of 14-α-sterol demethylase (+/- conformational changes). (D) Shows reduced azole efficacy due to increased efflux pump activity. (E) Shows conformational changes in the Fks1 subunit of β-(1,3)-D-glucan synthase leading to reduced echinocandin binding.

echinocandin resistance in Aspergillus, by similar mechanisms to those described for Candida, is possible. There is laboratory evidence that mutations in FKS can reduce susceptibility in Aspergillus, but so far only a handful of clinical cases have been reported [37].

**Laboratory methods to detect drug resistance in Candida & Aspergillus spp.**

*In vitro* antifungal susceptibility (AFST) testing aims to detect drug resistance or the probability that antifungal treatment will fail [38]. Clinical microbiology laboratories rely on AFST to guide therapeutic choice and to develop an understanding of local resistance patterns [39]. AFST measures the ability of a specific organism to grow in the presence of a particular drug *in vitro*, generating a minimum inhibitory concentration (MIC) [40,41]. As the echinocandin activity against molds is fungistatic, end points are not clear-cut and the minimum effective concentration (MEC) is used instead. The MEC is the lowest concentration of a drug resulting in growth of small, rounded, compact hyphal balls compared with the filamentous hyphal growth seen in control wells [42].

**Reference antifungal susceptibility testing methods**

The Clinical and Laboratory Standards Institute (CLSI) [43,44] and the European Committee on Antibiotic Susceptibility Testing (EUCAST) [45,46] have developed standardized reference methods for the AFST of yeasts and filamentous molds. Both employ variations on broth microdilution methods, where the growth of isolates is observed in concentrations of an antifungal agent, prepared by serial dilution with growth medium. There are some technical differences between CLSI and EUCAST methods, but both reliably discriminate between susceptible ‘wild-type’ strains and resistant strains [38,47–48].

Test standardization has enabled the generation of clinical break points (CBPs) and/or epidemiologic cut-off values (ECVs) for the triazoles, echinocandins (and amphotericin B) against the main *Candida* spp. [42,44–45] and *Aspergillus* spp. [43,46]. The CBP is the concentration of drug which categorizes an organism as suscepti-
ble, susceptible dose-dependent/intermediate or resistant. CBPs are based on a combination of MIC values, pharmacokinetic/pharmacodynamic (PK/PD) parameters and clinical outcome data, in other words, an MIC value predictive of whether a microorganism will respond in vivo [41]. In contrast, the ECV is the upper limit of the wild-type population and usually includes 90–95% of a given population for a specific agent [42], in other words, an MIC separating a population into those with and without resistance based on their phenotype. ECVs can be generated against any species provided there are MIC data from enough isolates (typically ≥ 100) [41], but they do not reliably predict clinical response. The CLSI and EUCAST methods produce MICs which can differ by as much as three dilutions, so results cannot be used interchangeably [41,47]. However, the two methods generally agree on susceptibility categorization when testing azoles and echinocandins against Candida spp. [49,50] and azoles against Aspergillus species [51].

**Commercial antifungal susceptibility assays**

Because the ‘gold standard’ reference techniques for AFST are labor intensive and complex [52], many clinical laboratories use commercial tests. These are convenient, but are not fully standardized against the reference tests for all drug–fungus combinations and can result in the misclassification of susceptibility results [53].

The Sensititre YeastOne® ( Trek Diagnostics Systems, OH, USA) is a commercial broth microdilution assay, based on CLSI methodology, which determines the MIC (or MEC) of nine antifungal drugs. It has been approved by the US FDA for use in clinical care with Candida spp. [58]. For Candida spp., several multicenter evaluations of YeastOne have demonstrated excellent reproducibility and results comparable with both reference methods [48–49,54]. Species-specific YeastOne ECVs of echinocandins have been defined for eight common Candida species. These correctly classified 88.9–93.8% of 81 well-characterized FKS mutant strains of five Candida spp., demonstrating the potential of the YeastOne test to help monitor the emergence of echinocandin resistance in routine diagnostic laboratories [55]. There are fewer studies of YeastOne among mold isolates, however high levels of agreement with reference methodologies have been observed for amphotericin B and the azoles with Aspergillus spp. [56–58], including detection of azole resistance in all A. fumigatus isolates (n = 10) with a CYP51A mutant genotype [59]. Data are limited for echinocandins, although a recent study demonstrated detection of A. fumigatus echinocandin-resistant isolates after 24 h incubation using YeastOne [60].

The Vittek 2® system (bioMérieux, Marcy d’Etoile, France) is a fully automated identification and AFST system, which provides results in 14–18 h [54]. Seven antifungal agents are included, although only fluconazole, caspofungin and voriconazole are approved by the FDA [48]. Results are accurate and reproducible when compared with CLSI/EUCAST reference methods [49,54,61–62]. Of note, the range of reported MIC values using the Vittek 2 system is limited, making interpretation of intermediate results difficult [26]. For caspofungin, the lowest concentration tested (0.25 μg/ml) is above the CLSI susceptible break point for C. glabrata (≤0.12 μg/ml) [63] and therefore misclassifies C. glabrata isolates with known mechanisms of resistance (i.e., FKS hotspot mutations) as susceptible [64]. Additionally, this method resulted in higher MICs than the CLSI reference method, which could lead to inappropriate therapy recommendations [65]. The Vittek 2 system is not suitable for antifungal susceptibility testing of molds.

Another frequently used commercial method for AFST is the agar-based Etest (bioMérieux, Marcy d’Etoile, France). Agreement between the Etest and both reference methods has been reported to be >90% [54]. However, in one study, 13% of reference method-susceptible Candida isolates (n = 497) were misclassified as intermediate or resistant by Etest [66]. The majority of those misclassified were C. krusei (73.1%) and C. glabrata (33.1%). Etest-specific ECVs were recently published for Candida spp. to amphotericin B and the echinocandins. Using these ECVs, 92% of the Candida FKS mutants were classified as non-wild-type (non-WT) for anidulafungin. However, the performance was inferior for caspofungin (75%) and micafungin (84%). These results suggest andulafungin Etest ECVs may serve as a useful screen for echinocandin resistance in Candida spp. [67].

Etest-specific ECVs for the four most common Aspergillus species complexes (i.e., A. fumigatus, A. flavus, A. niger and A. terreus) have only been determined for amphotericin B and no CBPs are available. Preliminary data evaluating an isavuconazole Etest strip revealed that it showed promise in separating wild-type (n = 40) from CYP51A mutant (n = 39) A. fumigatus strains [68]. To date, the FDA has not approved any Etest strip for clinical use in mold infections.

Four-well azole supplemented screening plates (Balis Laboratorium VOF, Boven Leeuwen, The Netherlands) containing itraconazole (4 mg/l), posaconazole (0.5 mg/l) and voriconazole (1 mg/l) are a convenient and
inexpensive, rapid screening method for azole-resistance in *A. fumigatus* [28]. They cannot provide an exact MIC value, but identify potentially resistant isolates for further investigation.

**Molecular methods to detect azole resistance in Candida spp.**

AFST of non-*glabrata* Candida species against the azoles remains a core function of the clinical laboratory, as interpretative CBPs predict outcomes [52,69]. However, molecular assays have the advantage that they can determine the underlying genetic basis of azole resistance [70]. These molecular methods are not standardized and individual mutations do not necessarily correlate with MICs – since multiple mechanisms can lead to reduced azole susceptibility [52]. Molecular methods to detect azole resistance must be multiplexed and capable of determining the presence and upregulation of housekeeping genes.

Point mutations in the three ‘regional hotspots’ of the *ERG11* gene (amino acid positions 105–165, 266–287 and 405–488) and the *ERG3* gene (T330A and A351V) can reliably and easily be detected with high-throughput DNA sequence analysis, allele-specific real-time molecular probes, high resolution melt curve analysis and DNA microarray technology, as reported by Perlin [70,71]. These techniques are robust and can be used with amplified DNA from primary specimens, but they are technically demanding. Whether such mutations in the *ERG11* gene confer phenotypic resistance to the azoles requires additional experiments to demonstrate causal effect – not all amino acid substitutions have been validated [72,73].

Most recently, next generation sequencing (NGS) has been used to detect alterations in genes associated with azole resistance in *Candida* spp. (*ERG11, ERG3, TAC1* and *CgPDR1*) [4,74–75]. NGS has the ability to detect novel mutations implicated in phenotypic resistance of clinical isolates that may otherwise be missed by targeted DNA sequence analysis [75]. Although the practicability and cost (~$60 USD sample) of NGS mean that it is currently beyond the scope of most routine clinical laboratories [76], rapid technological advances will reduce these barriers and make routine application of NGS more likely.

**Molecular methods to detect echinocandin resistance in Candida spp.**

AFST is unable to reliably distinguish WT isolates from echinocandin-resistant (FKS mutant) isolates, particularly for caspofungin, due to substantial interlaboratory variability in MIC results [77]. *FKS* genotype analysis has been deemed a better predictor of resistance than AFST alone because: an *FKS* mutation is an independent risk factor for therapeutic failure; the number of *Candida* strains exhibiting resistance *in vitro* is low; and there is a limited spectrum of mutations conferring resistance [52,70,78,79]. Echinocandin resistance can be assessed by real-time PCR, DNA sequencing or NGS, [70,74,75,80–82], with the latter being more suited (and potentially more cost effective) to accommodate the larger number of mutations in multiple *FKS* genes seen with *C. glabrata*.

In one recent study, ECVs established by CLSI were used to select isolates for screening for *FKS* mutations by PCR and DNA sequencing. Among the *C. glabrata* isolates selected, 73% (11/15) harbored mutations in *FKS1* (S663P, S629P or F625S) and/or *FKS2* (F659S/Y) genes. No known *FKS* mutations were detected in the remaining four, highlighting that some isolates may be categorized as non-WT due to elevated MICs in the absence of known resistance mechanisms [74].

*FKS* mutations have been detected using NGS in clinical isolates of *C. glabrata* with high MICs to the echinocandins [75,82]. Biswas *et al.* [82] recently used NGS to retrospectively study three strain pairs of *C. glabrata* from three patients where antifungal resistance developed during treatment. Two of three isolate pairs developed a >60-fold increase in the MICs to all echinocandins and NGS detected mutations in either the *FKS1* (S629P) or *FKS2* (S663P) genes of the resistant isolates [82].

**Molecular methods to detect azole resistance in Aspergillus spp.**

The application of molecular testing for azole resistance in *Aspergillus* spp. has been more straightforward than in *Candida* spp. as there are fewer resistance mechanisms. Resistance-associated mutations have been detected in *Aspergillus* spp. by real-time PCR, with molecular beacon probes, sequencing, high resolution melt curve analysis and most recently NGS [70,83,84]. Although a method for detection of *CYP51A* gene mutations has not been standardized, the most commonly used is a simple PCR amplification of the entire coding and promoter regions, followed by Sanger sequencing [85]. Ahmad *et al.* [86] developed a simple PCR-restriction fragment length polymorphism assay for simultaneous detection of *TR34* and *TR40* repeats (and likely others), as well as the L98H substitution. NGS, however, has the ability to determine the genome-wide basis of azole resistance. Hagiwara *et al.* [83] used NGS to identify nonsynonymous mutations in *A. fumigatus* strains sequentially isolated from each of two patients, which
would not have been detected by traditional methods such as PCR-restriction fragment length polymorphism or microsatellite genotyping.

Although routine AFST is recommended for all clinically significant isolates of *Aspergillus* spp., to monitor for resistance [87], this is dependent on fungal culture and invasive aspergillosis is frequently diagnosed by culture-independent methods. Thus it is possible that azole resistance is underdiagnosed [85]. To overcome this problem, non-culture-based molecular assays that identify *Aspergillus* spp. and detect azole resistance directly from clinical samples have been developed. These methods combine high sensitivity with high specificity to ensure amplification of the small amounts of *Aspergillus* DNA present in biological samples. Most formats are PCR-based with subsequent DNA sequence analysis to detect the mutations [85]. Two commercially available multiplex real-time PCR assays are available; AsperGenius® (PathoNostics BV, Maastricht, The Netherlands) and MycoGENIE (Ademtech, Pessac, France). AsperGenius detects DNA from *A. fumigatus* and *A. terreus*. It uses melt curve analysis, rather than DNA sequencing, to identify TR34/L98H and Y121F/T289A CYP51A substitutions. It has performed well on bronchoalveolar lavage fluid (including culture-negative fluid) and blood samples [88,89]. The MycoGENIE detects only *A. fumigatus* DNA and specific TR34 and L98H mutations in CYP51A. Its sensitivity and specificity were 92.9 and 90.1%, respectively, using respiratory samples (n = 88) and 100 and 84.6%, respectively with serum samples [90].

Management of drug-resistant *Candida* infections
The intrinsic susceptibility patterns of various *Candida* species have been discussed above. Perspectives on the treatment of invasive fungal diseases caused by *C. glabrata*, and selected species which are less susceptible to antifungal agents (including the more recently-described *C. auris*), are outlined below.

*Candida glabrata*

The rising incidence of invasive *C. glabrata* infections should prompt a revisit of the antifungal treatment algorithms used in hospitals. This is especially pertinent given reported resistance rates to azoles of 11–13% [91] and to echinocandins of >13% [30]. Knowledge of local susceptibility patterns is essential for clinical decision-making.

Echinocandins are first-line therapy for *C. glabrata* infections and are recommended for empiric therapy of invasive candidiasis in many clinical settings [92]. This is appropriate unless there is a strong suspicion of echinocandin resistance in high-risk individuals, for example, in patients with previous echinocandin exposure, a previous episode of invasive candidiasis, or known azole resistance [93]. Concerningly, co-resistance to the azoles and echinocandins has been reported [94]. In such cases, treatment should be initiated with a different class of antifungal such as a polyene [92].

*Candida auris*

*Candida auris* is increasingly recognized worldwide as a cause of nosocomial candidemia and other forms of invasive candidiasis, with a high mortality [4,95–97]. It is often misidentified on conventional tests and is typically resistant to fluconazole and variably susceptible to the other azoles, amphotericin B and the echinocandins. Lockhart et al. in a US CDC-based survey reported that of 54 isolates, 93% were fluconazole-resistant, 35% were amphotericin B-resistant and 7% were echinocandin-resistant [4]. Of note, 41 and 4% of isolates were resistant to 2 and 3 drug classes, respectively. These data were derived using CBPs established for related *Candida* species as species-specific CBPs for *C. auris* have not yet been endorsed.

At present, first-line antifungal therapy for *C. auris* candidiasis remains an echinocandin [93]. Following AFST, treatment should be tailored to a drug against which this isolate is susceptible. Some clinicians favor combining antifungal drugs despite limited evidence favoring combination therapy. Fahkim et al. demonstrated *in vitro* synergy between miconafungin and voriconazole against multidrug-resistant *C. auris* although combinations of caspofungin and fluconazole or voriconazole were indifferent [98]. This is consistent with findings in mice with invasive candidiasis, where clinically relevant doses of micafungin were more effective than fluconazole and amphotericin B [99].

The recommended duration of antifungal therapy is similar to that for other *Candida* spp. Where feasible, indwelling devices such as central venous catheters should be removed [93]. Critically, as *C. auris* can persist in the environment and colonize patients and clinical staff, aggressive infection control measures are recommended. These include daily and terminal cleaning of patient rooms, elimination of the organism from colonized body sites using...
chlorhexidine body washes, from the environment using disinfectants and from hands of personnel by compliance with hand hygiene guidelines [100].

**Management of drug-resistant A. fumigatus infections**

The Infectious Diseases Society of America (IDSA) in 2016 updated guidelines for management of invasive, chronic and allergic aspergillosis [7]. Azoles remain the preferred first-line therapy, for both treatment and prevention [7]. However, experimental evidence to guide treatment of azole-resistant *A. fumigatus* infections is scarce and recommendations have not been included in the management guidelines.

The choice of empiric antifungal therapy will be guided by the prevalence of azole resistance in the country, region or preferably, in the treating institution. Where an elevated MIC to an azole is demonstrated by a validated test method (discussed above), treatment with one or more alternative antifungal agents is recommended. As the number of effective drugs remains limited, regimens to extend azole effectiveness and overcome decreased susceptibility by optimizing drug exposure have also been explored [101].

**Alternatives to azole monotherapy**

Infections caused by *A. fumigatus* confirmed to be voriconazole-resistant, expert consensus recommends discontinuation of voriconazole as monotherapy and either substitution of a different class of antifungal agent in its place or combining the azole with an echinocandin [102]. Despite the absence of large systematic studies correlating *in vitro* azole resistance with clinical outcome, observations of higher mortality in patients infected with azole-resistant strains compared with those affected by azole-susceptible *A. fumigatus* (88 vs 30–50%) are concerning [105]. Alternative regimens such as voriconazole plus an echinocandin or in combination with liposomal amphotericin B (L-AMB) have been used without clinical efficacy studies to support either [102]. Echinocandin monotherapy is another option but success rates in invasive aspergillosis have been only in the vicinity of 50% or less [106]. As a single agent, *in vitro* studies indicate comparable efficacy of L-AMB against invasive aspergillosis due to azole-susceptible and azole-resistant *Aspergillus*, consistent with the absence of cross-resistance between these drug classes [107]. Hence, L-AMB monotherapy remains an important therapeutic option in the setting of azole resistance.

Where the laboratory can perform AFST, this is recommended. Although differential azole susceptibility may occur, frequently isolates are resistant to more than one azole including isavuconazole. Hence substitution with a different azole should be undertaken with caution.

### High (>10%) prevalence of azole resistance

Many experts consider a prevalence of azole resistance >10% to be ‘high’ and would recommend against primary treatment with voriconazole. Either a combination of voriconazole plus an echinocandin or L-AMB have been recommended as empiric therapy [102] prior to obtaining susceptibility results; when these are known, therapy can be tailored accordingly.

Where infection due to azole-resistant *Aspergillus* is confirmed, antifungal therapy should be continued using either of the above options or with an agent to which the isolate is susceptible. If the isolate is shown to be susceptible to voriconazole, voriconazole monotherapy can be instituted [102]. Where voriconazole cannot be used or is not tolerated by the patient, then posaconazole or isavuconazole may be considered. In all instances, therapeutic drug monitoring is desirable to ensure that azole serum levels are adequate.

In the absence of a cultured isolate, the choice of antifungal relies on clinical judgement and local epidemiology. One approach may be to employ initial therapy with 2–4 weeks of L-AMB followed by step down oral therapy with an azole.

### Intermediate (5–10%) prevalence of azole resistance

Opinions differ regarding preferred empiric treatment in settings with intermediate rates of resistance. Unnecessary fear of voriconazole (or other azole) resistance may lead to use of another agent with lower efficacy. Voriconazole, a combination of voriconazole and echinocandin, or L-AMB monotherapy could be considered for initial use.
No head-to-head comparative clinical studies between L-AMB and voriconazole have been performed. However, observational reports have shown that the efficacy of voriconazole in invasive aspergillosis is about 15–20% higher than that of all amphotericin B formulations and echinocandins (reviewed in [102]).

**CNS aspergillosis**

International guidelines recommend the use of voriconazole as first-line therapy for CNS aspergillosis [7] despite limited clinical data comparing it with other antifungal treatments. Schwarz et al. [108] showed that the use of voriconazole led to more favorable clinical outcomes compared with the polyenes. Unlike the other mold active azoles itraconazole and posaconazole, which cannot be detected in appreciable amounts in the cerebrospinal fluid (CSF), voriconazole has favorable CNS PK properties, including penetration into brain tissue [109].

If the use of voriconazole is precluded due to drug resistance, alternatives are limited, as neither itraconazole, posaconazole, nor lipid formulations of amphotericin B penetrate well into the CSF (although concentrations in the brain are often adequate to achieve a clinical response). Based on PK data in animals, experts prefer L-AMB over other amphotericin B formulations [102,110].

Clinical outcomes in patients with CNS aspergillosis due to azole-resistant strains have been poor, despite combination antifungal therapy [105]. Many experts recommend combining amphotericin B with a second agent, specifically, 5-flucytosine [7,102]. The agent 5-flucytosine achieves good CSF drug levels. Echinocandins achieve only very low CSF levels and are not recommended in CNS aspergillosis.

**Increasing exposure to azoles**

Depending on specific gene mutations corresponding to a particular resistant phenotype, loss of activity of an azole may be complete or partial. For isolates in which MICs have increased only by a few twofold dilutions, escalating the drug dose may translate to a clinical response. Such an approach must take into account PK/PD parameters and binding of the drug to the target site. Seyedmousavi et al. reviewed the use of azole monotherapy for azole-resistant aspergillosis [101]; based on in vitro susceptibility, PK/PD studies, and experimental models of infection, they concluded that there may be a case for voriconazole dose escalation. Where the MIC is 2 mg/l (intermediate susceptibility), plasma drug levels should exceed 1.03 mg/l; where the MIC is 4 mg/l (resistant), a plasma level of \(>2.65\) mg/l is recommended. In the latter case, the likelihood of drug toxicity may be increased, and intravenous administration is required. For isolates with an MIC of \(>4\) mg/l, very high levels of drug are required (plasma level \(>5.3\) mg/l). At present, there appears to be no role for posaconazole when the MIC of the isolate is outside the WT, although data using the intravenous formulation have not yet been published.

**Newer antifungal agents for Candida & Aspergillus**

Several newer antifungals are in development for use against Candida. T-2307 (Toyoma Chemical Company) is active in vitro against azole- and echinocandin-resistant Candida including C. glabrata, and has good in vivo efficacy in animal models (reviewed in [111]). CD101 (biafungin, Cidara Therapeutics, CA, USA), an echinocandin under development, is a long-acting compound with good anti-Candida activity [111]; against 16 C. auris isolates, the MIC\(_{90}\) was 0.25 mg/l [112]. CD101 is also effective against Candida species in biofilms [113].

The new oral glucan synthase inhibitor SCY-078 (Scynexis, Inc., NJ, USA) has potent activity against Candida species, which includes those in biofilms and isolates with known FKS1 and FKS2 mutations. MIC values against C. auris range from 0.5 to 2 mg/l, with an MIC\(_{90}\) of 1 mg/l [114].

The drug F901318, developed by F2G Ltd (Manchester, UK) (http://www.f2g.com/), is a promising antifungal agent, now entering Phase III clinical studies. It selectively inhibits the fungal dihydro-orotate dehydrogenase enzyme resulting in perturbed pyrimidine synthesis. In vitro, F901318 is highly active against azole-resistant Aspergillus isolates (MICs against A. fumigatus 0.06–0.12 mg/l) including against isolates with CYP51A mutations known to confer azole resistance (summarized in [115]).

Other promising anti-Aspergillus agents include the arylamidine T-2307 (Toyoma Chemical Company, Tokyo, Japan) which selectively targets fungal mitochondria, E121010/APX001 (Amplyx Pharmaceuticals, CA, USA) which inhibits glycosylphosphatidylinositol-dependent anchoring of the fungal cell wall, and ASP2397 (Astellas Pharma, Tokyo, Japan) which interferes with fungal siderophore transport [115].
Conclusion & future perspective

Here we have discussed current knowledge of, and controversies regarding, antifungal resistance, with a focus on Candida and Aspergillus. Coordinated surveillance of fungal pathogens including antifungal resistance remains inadequate. Since many routine diagnostic laboratories, especially in lower and middle-income settings, lack the capacity to perform even routine species identification, it is important that clinicians everywhere have access to local epidemiology and susceptibility patterns from reference laboratories to guide treatment choices.

At the same time, development of diagnostic capacity for fungal infections is a priority. A recent review outlined a ‘roadmap’, calling for immediate access to point of care testing for endemic fungal infections, followed by staged development of microscopy, culture and ultimately molecular techniques for low and middle-income countries  

Executive summary

- The incidence of fungal infections is increasing, but the availability of new agents to treat them is not.
- Although results of antifungal susceptibility testing do not correlate precisely with treatment response in vivo, they can be used to guide treatment choices.
- This review focuses on azole and echinocandin resistance in Candida spp. and Aspergillus spp.

Azole resistance in Candida spp. & Aspergillus spp.
- Intrinsic resistance patterns vary by species. The likelihood of a particular species causing disease in a given patient is affected by geographic location and patient characteristics.
- The principle mechanisms by which Candida spp. acquire resistance to azoles are structural alterations to lanosterol 14α-demethylase resulting from mutations in its encoding gene ERG11, upregulated production of lanosterol 14α-demethylase and upregulation of ATP-binding cassette or major facilitator’s superfamily efflux pumps.
- Cryptic Aspergillus species within complexes such as Aspergillus fumigatus and Aspergillus niger, which can usually only be identified by molecular testing, are frequently azole-resistant. This complicates treatment recommendations, and reinforces the importance of performing antifungal susceptibility testing.
- Most azole resistance in Aspergillus arises from mutations in the CYP51A gene. The alarming emergence of TR34/L98H and TR46/Y121F/T289A as the dominant global mutation has raised the possibility of environmental/agricultural selection pressure.

Echinocandin resistance in Candida spp. & Aspergillus spp.
- Intrinsic reduced susceptibility to echinocandins is uncommon and of uncertain significance in Candida spp. other than Candida auris. Most species can acquire resistance through mutations in the FKS1 and FKS2 genes, leading to alterations in subunits of the target enzyme β-(1,3)-D-glucan synthase, and refractory infections. Clinically relevant echinocandin resistance in Aspergillus is rare.

Laboratory methods to detect drug resistance in Candida & Aspergillus spp.
- Clinical and Laboratory Standards Institute and European Committee on Antibiotic Susceptibility Testing reference broth dilution methods for both Candida and Aspergillus spp. are discussed, including some of the commercial kits that have been developed from these methods.
- Molecular methods, including next generation sequencing, are also discussed. They are useful for describing resistance mechanisms at the epidemiological level, and as diagnostic tests in the routine laboratory.
- In particular, the value of identifying FKS mutations in Candida is highlighted as an area where molecular methods may better predict echinocandin treatment failure than standard antifungal susceptibility testing.

Management of drug resistant Candida & Aspergillus spp. infections
- Treatment strategies are discussed with particular reference to Candida glabrata and C. auris. Strategies covered include combination, substitution, dose-escalation and new antifungal agents, and which indicators to consider when selecting empiric therapy.
- Azoles are first line therapy for Aspergillus infections. Empiric management of Aspergillus spp. infections are discussed in light of known low, high or intermediate rates of azole resistance.
- CNS infections with Aspergillus are especially challenging to manage, even in the absence of azole resistance. Echinocandins are not recommended, and treatment relies on combination therapy with amphotericin and flucytosine.
- New mold active agents are also discussed.

Conclusion & future perspective
- Enhanced global surveillance efforts are required to better understand the prevalence and drivers of resistance in fungal pathogens, and to ensure that they are identified early.
- Lab capacity, especially in low and middle income settings where disease burden is highest, needs development. Point-of-care testing for mycoses should be made available rapidly, while molecular testing is likely to be key for both diagnostics and epidemiological surveillance in coming years.
- New drugs are required to improve therapeutic options, especially in light of emerging antifungal resistance.
and we endorse that roadmap. NGS is a new technology that can be used to identify emerging threats; its value has been illustrated in identifying and tracking azole resistance in *A. fumigatus*. As molecular technologies continue to evolve and their widespread use becomes more financially viable, molecular data can be integrated into clinical management guidelines.

Finally, we note the importance of new drug development. Increasing azole and echinocandin resistance heightens the urgency of the need for a larger antifungal armamentarium to improve the availability, efficacy and tolerability of therapeutic options for these serious but neglected diseases.

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