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Antifungal Resistance Testing and Implications for Management

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Abstract

Purpose of Review Antifungal agents are the mainstay in the management of patients with invasive fungal disease. However, resistance to current antifungal agents can develop with clinical use, which may negatively impact clinical outcomes. We review the strengths and weaknesses of antifungal susceptibility testing and how the detection of resistance, either phenotypically or molecularly, correlates with clinical outcomes.

Recent Findings Phenotypic resistance is associated with worse outcomes, although this must be taken in context with other patient factors. Newer molecular assays have been developed that have shown promising results for the detection of resistance mechanisms, including azole resistance in *Aspergillus fumigatus* and echinocandin resistance in different *Candida* species. Further work is needed to improve the clinical utility of these assays for faster turn-around-time and direct use on specimens.

Summary Detection of antifungal resistance may provide useful information for the treatment of invasive fungal disease.

Keywords Azoles · Echinocandins · *Candida* · *Aspergillus* · Invasive fungal disease · Mycoses

Introduction

Resistance to antimicrobial agents is a growing clinical concern. This includes resistance to agents used in the treatment of infections caused by bacteria, viruses, parasites, and fungi. For invasive fungal disease (IFD), this is particularly concerning given the dearth of clinically available options, as well as the adverse effects/toxicities and drug-drug interactions that are often associated with available drugs used to treat these infections. Worldwide, resistance in non-*albicans Candida* species to fluconazole is a major concern given the widespread availability and use of this antifungal agent. This was highlighted in the World Health Organization global surveillance report on antimicrobial resistance, which documented fluconazole resistance at rates of 30% or higher in multiple countries and continents [1]. Recently, the problem of antifungal resistance has gained increased awareness with the emergence of *Candida auris* as a growing and clinically significant threat. Isolates of this emerging pathogen are often resistant to multiple antifungal agents [2], and pan-antifungal-resistant strains have also been discovered [3, 4]. Numerous articles have also documented the threat and spread of azole resistance in the ubiquitous mold *Aspergillus fumigatus* [5•, 6–8].

Clinical microbiology laboratories primarily test for antifungal resistance using in vitro phenotypic assays that measure the growth of a pathogen or the inhibition of its growth over a range of antifungal concentrations. However, such assays are time consuming and rely on the availability of a growing culture, which may not be available in many patients with IFD. New assays are becoming available that appear to enhance our ability to detect antifungal resistance in direct specimens, or at least predict the likelihood of resistance based on the infecting species combined with antifungal surveillance data (Fig. 1). In this review, we provide an overview of the various means by which antifungal susceptibility and resistance are determined, and discuss correlations with clinical outcomes and implications for patient management.

Topical Collection on Advances in *Diagnosis of Invasive Fungal Infections*

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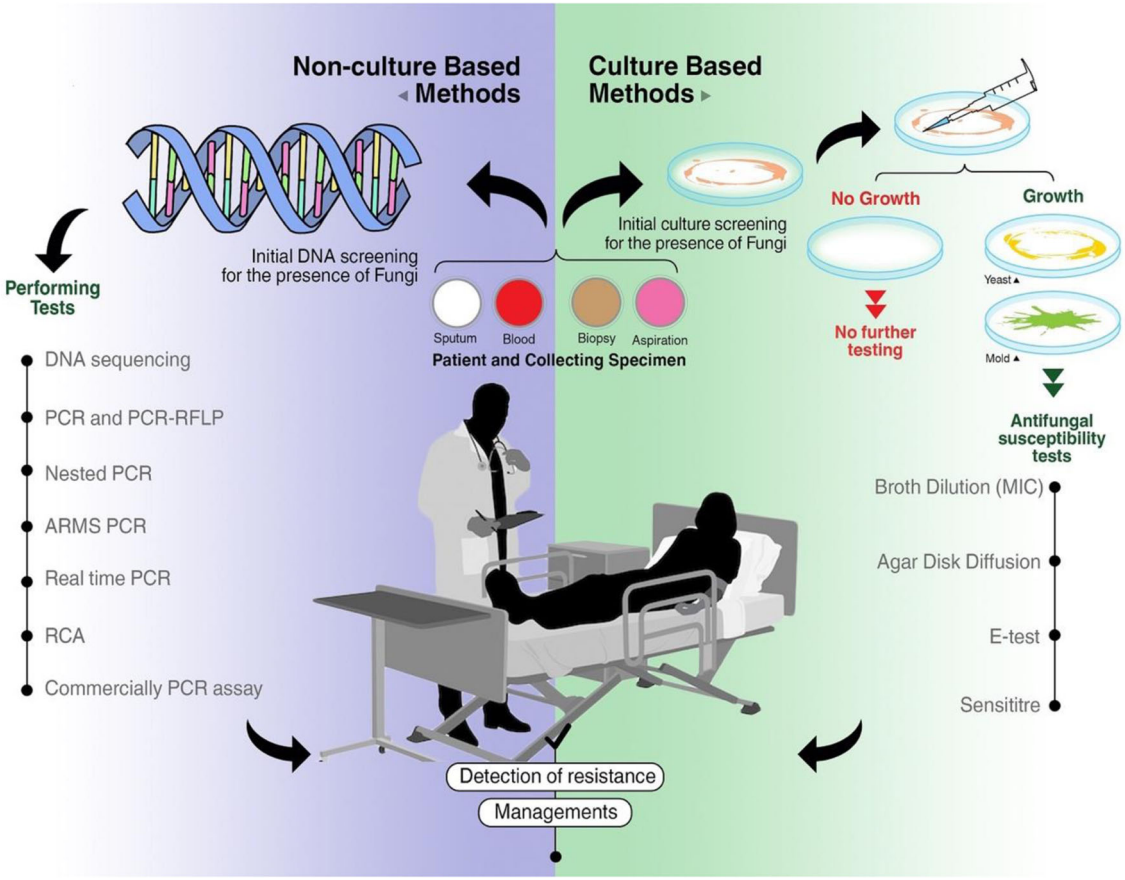

Fig. 1 Scheme describing culture-based (phenotypic) and non-culture-based (molecular) means for the detection of antifungal resistance. MIC, minimum inhibitory concentration; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ARMS, amplification refractory mutation system; RCA, rolling circle amplification

Phenotypic Methods of Susceptibility Testing and Resistance Detection

Phenotypic methods of antifungal susceptibility testing provide an estimate of the in vitro activity of an agent against the pathogen of interest. The readout of phenotypic methods is a minimum inhibitory concentration (MIC), which is the lowest concentration of the antifungal that inhibits the growth of the organism, and this value may then be used to classify fungi as susceptible or resistant to the antifungal agent being tested. For the echinocandins against filamentous fungi, the readout is different and is termed the minimum effective concentration (MEC), which is the lowest concentration that results in abnormal morphology (i.e., short, stubby hyphae, with abnormal branching). In order to define an isolate as susceptibility or resistance, a clinical breakpoint must be available. Below this threshold, MIC isolates are classified as susceptible and above which they are considered resistant. Clinical breakpoints are established by careful consideration of several components, including MIC distributions of specific antifungal agents against a specific species, pharmacokinetic/pharmacodynamic parameters, and correlations between MIC values and clinical outcomes [9–11]. Clinical breakpoints are subject to review and may change over time as new data become available [9].

The Clinical and Laboratory Standards Institute (CLSI) in the USA and the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) are two organizations that establish methods and standards for phenotypic antimicrobial susceptibility testing. For antifungal susceptibility testing, these are primarily broth microdilution methods [12–14], although standardized methods for broth macrodilution and disk diffusion are also available [14, 15]. Efforts have been made to harmonize broth microdilution methods between these two organizations, although some differences still exist, including the starting inoculum densities, glucose content, well types, and methods for endpoint reading [16]. Commercially available methods are also available for antifungal susceptibility testing. These include a colorimetric broth microdilution assay that uses the resazurin, a dye that is converted by metabolically active cells for endpoint detection (YeastOne Sensititre), gradient diffusion assays that use a plastic strip onto which a concentration gradient of a particular antifungal agent has been placed (e.g., Etest), and fully automated systems (e.g., Vitek 2).

Phenotypic susceptibility assays have several limitations that must be considered. In order for phenotypic antifungal susceptibility testing to be performed, an organism must be cultured from the patient and available for use. Thus, these assays cannot be used in culture-negative infections. Once a culture is available, additional time is required for phenotypic susceptibility assays to be completed, and this may range from 24 h (for *Candida* species and the Mucorales), 48 to 72 h for other yeast (*Cryptococcus* species) and many filamentous fungi, including *Aspergillus*, *Fusarium*, *Scedosporium* species, and black yeast-like fungi, up to 168 h for slower growing fungal species (e.g., *Histoplasma capsulatum*). Results of antifungal susceptibility testing should also be helpful in predicting clinical response to therapy. However, numerous factors may influence clinical response, and many may play a larger role in determining patient outcomes. These include, but may not be limited to, the host's immune response, the severity of the underlying infection and other co-morbidities, timely initiation of appropriate therapy, the concentration/overall exposure of the antifungal agent achieved at the site of infection, drug-drug interactions, which may in turn influence the concentration of the agent at the site of infection or result in adverse effects/drug toxicities that may limit the patient's ability to tolerate the therapeutic regimen, and patient adherence to the treatment regimens [10, 11, 17]. It should be remembered that the activity of the antifungal agent against the pathogen of interest is only one factor that may influence clinical outcomes. A low MIC value may not be predictive of a successful clinical outcome in an individual with profound and prolonged immunosuppression and multiple comorbidities, nor an elevated MIC predict treatment failure in an otherwise healthy individual when good exposures can be achieved at the site of infection. However, the chances of poor outcomes are increased when in vitro resistance is present along with other factors that may negatively influence response to therapy.

Currently, clinical breakpoints have been set for a limited number of antifungal agents against a limited number of species. This includes those for the echinocandins and some of the azoles against more common *Candida* species by both CLSI and EUCAST [18, 19], and currently some of the azoles against a limited number of *Aspergillus* species by EUCAST [19], although a voriconazole breakpoint against *A. fumigatus* is currently being considered by CLSI. Epidemiological cutoff values (ECOFFs or ECVs) are more frequently being used as guidance when clinical breakpoints are unavailable. These statistically derived MIC thresholds generally encompass approximately 95 to 97.5% of isolates within a wild-type MIC distribution for a given species and drug, and allow for discrimination between wild-type and non-wild-type isolates [20, 21, 22••]. CLSI has published ECVs for different antifungal species combinations [23], and these are also available for non-CLSI and non-EUCAST methods [24–26]. Although ECVs are commonly used by CLSI and EUCAST in setting clinical breakpoints, they should not be used in lieu of available breakpoints, which take into account other important characteristics in determining likelihood of response to therapy (i.e., pharmacokinetic/pharmacodynamic relationships, and results from clinical studies).

Phenotypic Antifungal Resistance and Clinical Correlations

Azoles and *Candida* Species In order for antifungal susceptibility testing and resistance detection to be useful, there must be a correlation with clinical outcomes. Most of the early clinical data linking antifungal susceptibility results to clinical outcomes are for fluconazole against *Candida* species in patients with oropharyngeal candidiasis [27]. However, some studies have reported correlations between in vitro fluconazole susceptibility profiles and clinical outcomes in patients with candidemia or other forms of invasive candidiasis. Baddley et al. reported a positive association between fluconazole resistance and worse survival in 84 non-neutropenic patients with candidemia from a single institution. In this study, lower mortality rates were observed in patients with bloodstream infections caused by fluconazole-susceptible isolates (6.7%) compared with those in whom fluconazole-resistant isolates were cultured (25%), although this difference was not statistically significant by multivariate analysis ($p = 0.08$) [28]. Similarly, Clancy et al. reported worse outcomes in patients with infections caused by fluconazole-resistant *Candida* isolates compared with fluconazole-susceptible isolates [29]. In this study, each of the 6 patients with infections caused fluconazole-resistant isolates (MIC > 64 μg/ml) failed therapy, while in contrast, 14 of the 21 patients from whom fluconazole-susceptible isolates were cultured responded to therapy with this azole. In both of these studies, the other important factor associated with response to fluconazole therapy was the overall exposure to this azole, which was measured as a dose/MIC ratio by Clancy et al., and an AUC/MIC ratio by Baddley et al., highlighting the importance of pharmacokinetic/pharmacodynamic relationships in determining clinical outcomes. Other studies have also reported associations between fluconazole doses, pharmacokinetic/pharmacodynamic parameters, and response to therapy in patients with invasive *Candida* infections [27, 30–34]. These further highlight the importance of both resistance detection and appropriate dosing to achieve adequate exposures and improve clinical outcomes with fluconazole therapy. However, positive correlations between fluconazole doses or exposures and susceptibility results with clinical responses have not been found in all studies [35–37]. Correlations between in vitro susceptibility and clinical outcomes have been observed for voriconazole against infections caused by certain *Candida* species (*C. albicans*,

C. parapsilosis, and *C. tropicalis*) but not against *C. glabrata* [38, 39]. Data are not currently available to determine if such associations exist between in vitro susceptibility and clinical outcomes for posaconazole and isavuconazole [22]. In addition, data are lacking regarding the correlation between azole susceptibility and clinical outcomes for other yeast species (e.g., *Geotrichum*, *Rhodotorula*, *Saccharomyces*, and *Trichosporon* species).

Azoles and *Cryptococcus* Species Mixed results have been observed in studies that have evaluated relationships between antifungal susceptibility and clinical outcomes in patients with cryptococcosis. In a prospective study conducted in France between 1997 and 2001 in which antifungal susceptibility testing was performed in HIV-positive and HIV-negative patients with first episode *C. neoformans* cryptococcosis, no relationship was observed between in vitro susceptibility results, which were performed by multiple methods, for fluconazole, amphotericin B, and flucytosine, and clinical outcomes 2 weeks after the initiation of antifungal therapy [40]. Similarly, in a study that evaluated the relationship between fluconazole MIC values, as determined by different methods (CLSI and EUCAST broth microdilution, Etest, and Sensititre YeastOne), against *C. neoformans* isolates from 16 patients who received this azole, either alone or in combination as part of induction or maintenance therapy, and clinical outcomes at 10 weeks of treatment, Vena et al. reported no correlation between fluconazole susceptibility and response to therapy [41]. The only variable associated with poor response to therapy was disseminated disease. Interestingly, the percentages of cryptococcal isolates with elevated fluconazole MICs (≥ 16 μg/ml) was quite variable between the different methods (CLSI 1.6%, EUCAST 16.1%, Etest 31.6%, Sensititre YeastOne 53.2%). In contrast to these studies showing a lack of correlation between fluconazole MICs against cryptococcal isolates and clinical outcomes, other studies have reported worse outcomes in patients with infections caused by fluconazole-resistant isolates. Lee et al. investigated the correlation between fluconazole susceptibility, as measured by CLSI broth microdilution, and outcomes in 46 patients with cryptococcal meningitis, and reported that fluconazole resistance (MIC > 8 μg/ml) was significantly higher in patients who failed therapy (11 of 25 [44%]) compared with those who responded (1 of 21 [4.8%], $p < 0.01$) [42]. Similarly, Aller et al. reported a significant correlation between fluconazole susceptibility as measured by CLSI broth microdilution and poor outcomes in 25 patients with cryptococcosis [43]. In this study, each of the five patients in which the fluconazole MIC was ≥ 16 μg/ml either died or failed fluconazole therapy compared with only 2 of 20 patients in which the MIC was < 16 μg/ml. Interestingly, the fluconazole MIC increased from 4 to > 16 μg/ml in 3 patients who relapsed while on maintenance therapy with this azole.

Echinocandins and *Candida* Species Studies have also evaluated the correlation between echinocandin MICs and response to therapy in patients with infections caused by *Candida* species. One early study that evaluated the caspofungin clinical trial database examined the clinical utility of in vitro susceptibility testing for this echinocandin since infections caused by isolates with caspofungin MICs of 1 or 2 μg/ml responded as well as those with lower MIC values (0.25 to 0.5 μg/ml) [44]. However, only 3 isolates with higher caspofungin MICs (≥ 4 μg/ml) were available from this dataset, limiting the capacity to draw any conclusions. In addition, numerous reports of clinical failures associated with *Candida* isolates with elevated echinocandin MICs have been documented in the literature [45–52]. Results from animal studies have also supported this link between in vitro resistance and reduced in vivo effectiveness [48, 53, 54]. Although in vitro susceptibility testing of the echinocandins is clinically useful, other factors should be considered when determining the likelihood of clinical resistance. Several single-center studies have reported that clinical failures were more likely to occur when in vitro resistance was detected in patients who have had previous echinocandin exposure [55–58]. Thus, echinocandin MICs may be best interpreted in the context of prior exposure to this class of agents. It should be noted that significant interlaboratory variability has been observed with caspofungin susceptibility testing against *Candida* species, especially *C. glabrata* and *C. krusei*, by both CLSI and EUCAST methodologies, which may lead to misclassification of isolates as resistant by some laboratories [59]. There is some suggestion that this may occur using the Sensititre YeastOne colorimetric assay [60]. Because of this, EUCAST recommends usage of anidulafungin and micafungin MIC results as surrogate markers for caspofungin susceptibility or resistance [61, 62], and this has also been shown to correlate well with predicting *fks* mutations per CLSI broth microdilution methods [63, 64].

Azoles and Aspergillus Species Data assessing correlations between antifungal susceptibility testing and clinical outcomes for molds, including common causes of IFD in humans, such as *Aspergillus* species, are lacking as few studies have been conducted. In addition, in vitro susceptibility testing against molds may vary from one region to the next due to the availability of such assays as well as differences in recommendations to perform such testing between different diagnosis and treatment guidelines [65, 66]. Currently, most of the studies assessing relationships between in vitro susceptibility of antifungal agents and clinical outcomes for mold infections are for the azoles against *Aspergillus fumigatus*, for which there has been interest due to reports of azole resistance in this species, including isolates obtained from patients without any previous azole exposure [7, 67]. It is known that mutations within the *CYP51A* gene can cause amino acid

substitutions within Cyp51a, the enzyme targeted by the azoles and which is responsible for the last step in ergosterol biosynthesis in *Aspergillus* species. The first reports of azole-resistant *A. fumigatus* were published in the 1990s from isolates collected in the USA and Sweden in patients administered itraconazole [68, 69]. Using a murine model of invasive aspergillosis, Denning et al. were also able to link in vivo failure with in vitro resistance to this azole [70], a finding that has been supported by other animal model studies that have found loss of azole effectiveness against *A. fumigatus* isolates demonstrating in vitro resistance [71]. It is now known that environmental exposure to azoles or azole-like compounds can also cause point mutations within *CYP51A* in addition to tandem repeats within the promoter regions of this gene (e.g., TR_{34}/L98H and TR_{46}/Y121F/T289A). Isolates harboring these mutations can cause invasive disease in patients without prior azole exposure [7, 67]. Recently, Heo et al. attempted to correlate azole in vitro susceptibility against *Aspergillus* species to crude mortality at 42 days in 107 hematologic malignancy patients and/or hematopoietic stem cell transplant recipients with invasive aspergillosis [72••]. In this single-center, retrospective analysis conducted between 1999 and 2015, an increase in the number of non-wild-type *A. fumigatus* isolates was observed over time, but there was no correlation between elevated azole MICs and mortality. Interestingly, no mutations within *CYP51A* were found in any of the phenotypically non-wild-type isolates. However, previous azole exposure, including fluconazole, was associated with azole resistance. In contrast, other case series have reported failures in patients with acute invasive or chronic pulmonary aspergillosis due to azole-resistant isolates [5, 7, 73, 74].

Molecular Assays for Resistance Detection

Much work is now being devoted to the development of molecular assays that can be used to detect antifungal resistance. These types of assays may allow for quicker turn-around-time as well as the use of direct specimens instead of cultures, which are often negative in patients with IFD. Table 1 provides examples of a few of the technologies that have been evaluated. Molecular diagnostic platforms are appropriate for the detection of antifungal resistance when the resistance mechanisms are both known and clinically validated [85]. For validation purposes, a genetic target for the detection of resistance should lead to an elevated MIC by standardized phenotypic susceptibility testing, result in dose-dependent resistance in animal models of infection, and be documented in patients with clinical failures.

The mechanism by which *Candida* species can develop resistance to the echinocandins is well understood and lends itself well to detection by molecular assays. The echinocandins non-competitively inhibit the (1,3)-β-D-glucan synthase enzyme, which leads to a weakening of the fungal cell wall [86]. Point mutations within highly conserved hot spot regions of the genes that encode this enzyme (i.e., *FKS* genes) lead to amino acid substitutions that decrease the sensitivity of (1,3)-β-D-glucan synthase to this class of antifungals by reducing enzyme velocity (Vmax) [87, 88]. The hot spot regions where mutations occur are highly conserved among different *Candida* species and have been found in isolates cultured from patients following echinocandin exposure [45–48, 89]. It is also known that the location of the mutation and resulting amino acid change can influence the degree which the phenotypic activity of the echinocandins is

Table 1 Examples of molecular assays that have been studied for the detection of antifungal resistance mechanisms

Molecular assay	Comments	References
PCR/PCR-restriction fragment length polymorphism	Targeted *CYP51A* and promoter region of this gene in *A. fumigatus*	[75]
Amplification refractory mutation system (ARMS)-PCR	Uses four primers in one reaction; external primers for detection of tandem repeats in *CYP51A* promoter; internal primers for detection of L98H point mutation in *A. fumigatus*	[76]
Pyrosequencing	Bioluminometric, nonelectrophoretic technique that monitors DNA synthesis; able to detect point mutations in *CYP51A* of *A. fumigatus* for azole resistance and *FKS* of *Candida* species for echinocandin resistance	[77, 78]
High-resolution melt curve (HRM) with fluorescent DNA binding dyes	Identification of point mutations in *CYP51A* and tandem repeats in promoter region of this gene in *A. fumigatus* associated with azole resistance; identification of *FKS* mutations in *C. glabrata* associated with echinocandin resistance	[79, 80]
Real-time multiplexed molecular beacon probes	Identify mutations in *FKS1* in *C. albicans* responsible for amino acid change at codon 645 for echinocandin resistance	[81]
Luminex-based multiplex microsphere assay	Detection of hot spot 1 mutations in *FKS1* and *FKS2* of *Candida glabrata* responsible for echinocandin resistance	[82]
Multiplex quantitative real-time PCR	Detection of *CDR1* expression in *C. glabrata* and azole resistance	[83]
Rolling circle amplification (RCA) and DNA sequencing	Identification of *ERG11* mutations in *C. albicans* associated with fluconazole resistance	[84]

decreased. Marked reductions in enzyme activity/velocity occur in *C. glabrata* isolates harboring a serine to proline amino acid change at codon 629 within *Fks*1p or codon 663 in *Fks*2p, or a phenylalanine to serine amino acid change at codon 659 in *Fks*2p [62, 87]. Patients infected with isolates harboring these mutations may also have worse outcomes (breakthrough or persistent infections while receiving an echinocandin) as reported in two retrospective studies [55, 56]. In *C. albicans*, similar amino acid substitutions for serine (at codons 641 and 645) account for a large number of resistant strains [90]. Various molecular assays have been published for the detection of mutations within hot spot regions of *FKS* genes associated with echinocandin resistance. These include those that have used classic PCR primer sets [91], real-time multiplexed molecular beacon probes, including those with melt-curve analysis [79, 81], and a microsphere-based assay that includes asymmetric PCR [82]. While these assays have shown promising results when used on cultures, further evaluation is needed using direct specimens in order to gauge their clinical utility.

Similarly, multiplexed real-time PCR assays have been used to detect point mutations within the *CYP51A* gene that are associated with azole resistance in *A. fumigatus*, including the detection of these changes in both cultured isolates and in direct specimens [92–95]. A comprehensive review of molecular assays for the detection of azole resistance has recently been published [96]. One assay that is commercially available for the detection of antifungal resistance using direct specimens is the AsperGenius (PathNostics), which uses multiplex real-time PCR to detect and identify *Aspergillus* species within bronchoalveolar lavage (BAL) fluid as well as point mutations within the *CYP51A* gene [97]. In a multi-center study that evaluated this assay using 201 BAL samples, azole treatment failure was reported in 6 of 8 patients in which *CYP51A* resistance mutations were found, compared with 12 of 45 patients without these genetic changes [98]. Mortality was also reported to be higher in patients with these mutations. While promising, the utility of this assay may be limited given the various reports of *A. fumigatus* that are phenotypically resistant to azoles but which harbor a wild-type *CYP51A* [6, 7, 72••, 74]. Other mechanisms of azole resistance in *A. fumigatus* have been described, including, but not limited to, overexpression of *CYP51B*, efflux pumps, gain-of-function mutations in transcription factors, mutations in sterol biosynthesis, and regulatory elements [99–103].

Secondary (i.e., acquired) azole resistance in *Candida* species can occur through many different mechanisms, including drug efflux, alterations or overexpression of the lanosterol 14α-demethylase enzyme target, and cellular response factors [104]. In addition, genomic plasticity is known to occur in *Candida* species, which may manifest as loss of heterozygosity, increases in chromosomal copy number, aneuploidy, or isochromosome formation, and these have been linked to altered expression of the *ERG11* gene, which encodes lanosterol 14α-demethylase, and efflux pumps [104, 105]. In *C. albicans*, several different mechanisms of resistance can be found in harboring high-level azole resistance. These factors have limited the ability to use molecular assays for the clinical detection of resistance [85].

Conclusions

The detection of antifungal resistance provides useful information to the clinician regarding the appropriateness of therapy for IFD. However, other factors, including both patient and drug characteristics, are also important factors that determine response to therapy. Currently, the detection of antifungal resistance is done primarily by phenotypic means. While standardized assays are available for phenotypic susceptibility testing, these are limited by delays in results and the inability to be performed in culture-negative infections. New molecular-based assays may be able to detect mechanisms associated with antifungal resistance. However, further work is needed to optimize their performance on direct specimens, as well as our understanding of the mechanism of antifungal resistance that may be targeted with these assays.

Compliance with Ethical Standards

Conflict of Interest Nathan Wiederhold reports grants from Astellas, grants from Cepheid, grants from Cidara, grants from bioMerieux, grants from F2G, grants from Viamet, personal fees from Mayne Pharmaceuticals, and personal fees from Gilead outside the submitted work. Hamid Badali declares no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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