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Antifungal drug resistance: do molecular methods provide a way forward?

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Abstract

Purpose of review—Antifungal drug resistance is a confounding factor that negatively impacts clinical outcome for patients with serious mycoses. Early detection of fungi in blood or other specimens with a rapid assessment of drug susceptibility could improve the survival of patients with invasive disease by accelerating the initiation of appropriate antifungal treatment. Recent years have seen the growth of molecular technology that is ideally suited for fungal identification and assessment of drug resistance mechanisms.

Recent findings—Elucidation of the genetic mechanisms responsible for triazole and echinocandin resistance in prominent *Candida* spp. and *Aspergillus* spp. provides an opportunity to develop molecular diagnostic platforms suitable for rapid detection of primary and secondary drug resistance. Several highly dynamic and robust amplification/detection methodologies are now available that can provide simultaneous species identification and high fidelity discrimination of resistance alleles.

Summary—Molecular diagnostic platforms are ideal for rapid detection of fungal pathogens, and they provide an opportunity to develop in parallel molecular assays that can evaluate antifungal drug resistance.

Keywords

fungal infections; antifungal drug resistance; molecular diagnostics

Introduction

Opportunistic fungal infections are widespread in immunosuppressed individuals and are a growing concern for the management of such patients. In the past two decades, the frequency of invasive fungal infections has risen and there is a striking increase in mortality due to invasive mycoses [1]. Numerous yeasts and moulds contribute to clinical disease with *Candida* spp. and *Aspergillus* spp. representing the most common life-threatening infections. Candidemia is the 4th leading cause of bloodstream infections and carries a 35-55% mortality [2]. The most common infecting species is *C. albicans*, which, when combined with *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* cause ~99% of all human cases [3]. As these organisms show a range of susceptibilities to existing antifungal drugs,

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distinguishing them is important for the selection of antifungal therapy. The incidence of invasive mould infections has also risen in the past decade, especially those caused by *Aspergillus* spp. [4,5]. Despite highly active antifungal drugs, mortality remains high at 50-70% [6,7]. *Aspergillus fumigatus* is the dominant species causing invasive mould disease, although other *Aspergillus* spp. are also important [5,8]. Effective therapeutic management of patients requires early diagnosis of *Aspergillus* infections, which remains a significant challenge. In recent decades, there has also been an increase in rare mould infections which are often resistant to antifungal drugs [9].

Need for early diagnosis

Early diagnosis of invasive fungal infections remains a major problem since signs and symptoms are nonspecific, blood cultures are commonly negative, and patients are often unable to undergo invasive diagnostic procedures [10,11]. For candidiasis, diagnosis is usually based on the isolation of *Candida* species from blood cultures or tissue biopsy specimens. However, in the early stages of infection, the sensitivity of blood cultures for diagnosis of systemic candidiasis is low, and may require 2-5 days for growth. Clinical outcomes are improved when treatment is started early [12,13], but empiric antifungal therapy has unwanted consequences as it increases selective pressure towards drug resistant species [3,14]. For invasive mould infections, the diagnosis is often based on clinical and non-specific radiographic findings, which has led to broad use of prophylaxis, empiric and pre-emptive therapeutic approaches [15-17]. Definitive diagnosis requires demonstration of hyphae in blood, respiratory specimens or tissue. Yet, an evaluation of morphological features, reproductive structures and biochemical properties may take days to weeks. Too often, deep-seated fungal infections are diagnosed at autopsy.

The resistance problem

The extensive use of triazole drugs has raised concern about resistant infections. It results from both primary resistance, which is observed as a shift toward colonization with inherently less susceptible organisms, and secondary resistance, which involves the emergence of cell-specific resistance mechanisms in normally susceptible strains. Triazole resistance is the most significant problem because these antifungal agents are commonly used to treat *Candida* spp., which represent the most abundant fungal mycoses [3,18,19]. Triazole drugs (fluconazole, voriconazole, itraconazole and posaconazole) interfere with ergosterol biosynthesis by blocking a key enzyme, lanosterol 14- α demethylase (Erg11p). The widespread application of triazole antifungal drugs promotes colonization with less susceptible species like *C. glabrata* or *C. krusei* but also helps select for resistant sub-populations of normally susceptible organisms like *C. albicans* [3,20]. In a recent study involving more than 140,000 *Candida* spp. isolates collected over an 8.5 year period overall resistance to fluconazole and voriconazole was 6.2% and 3.1%, respectively [19]. The level of resistance for all *Candida* spp. has remained relatively constant over a decade [19,20]. Triazole resistance in *A. fumigatus* is increasingly being recognized [21]. In the Netherlands, triazole resistance among isolates occurred with an annual prevalence of 1.7% to 6% over a 14 year period [21,22], and a similar trend has emerged in the United Kingdom [23]. The new echinocandin drugs (caspofungin, micafungin and anidulafungin) are expanding in use because they are effective against a wide range of *Candida* spp., including azole resistant species [24-27]. They inhibit β -1,3-D-glucan synthase, which disrupts the structure of the growing cell wall in susceptible yeast cells [25,28]. Global surveillance studies indicate that there has been no significant epidemiologic shift in the susceptibility of *Candida* spp. isolates to the echinocandin drugs suggesting that resistance is not a pervasive problem [29]. Clinical failures remain uncommon, although reports of echinocandin resistance with *Candida* spp. are more prevalent with expanding drug use [30-36].

Overall, the level of resistance to antifungal agents is relatively low, but antifungal resistance remains a serious clinical management challenge for individual high risk patients and those with persistent mycoses.

Drug resistance testing

In the United States, standardized antifungal testing utilizes either broth microdilution or disc diffusion assays performed in accordance with guidelines of the Clinical Laboratory Standards Institute (CLSI) CLSI M27-A3 standard [37] and EUCAST Definitive Document E.DEF 7.1. Drug threshold levels for *in vitro* growth inhibition yield a minimum inhibitory concentration (MIC). The CLSI has established antifungal MIC breakpoints for azole resistance and echinocandin susceptibility based on *in vitro* susceptibility data obtained from epidemiological surveillance, *in vivo* outcomes and pharmacokinetic/pharmacodynamic studies [38]. Overall, the MIC of an infecting organism is a presumptive measure for predicting clinical outcome. Defining this *in vitro* measure of susceptibility is important for management of high risk patients. Yet, susceptibility testing is not routinely performed. Antifungal susceptibility testing requires 48-72 h following identification, which often comes too late to influence a timely decision on patient management. More rapid tests that can be used in parallel with primary identification are urgently needed.

Molecular diagnostics

Molecular techniques provide a faster and more accurate assessment of both primary and secondary resistance than classical methodologies. Nucleic acid-based diagnostics are the fastest growing component of many clinical laboratories. These applications are gradually replacing or complementing culture-based, biochemical, and immunological assays for the detection of a wide range of microorganisms. The detection of ribosomal genes, 18S or 28S [39,40], or intervening non-coding regions, ITS1 and ITS2 [41], facilitate accurate detection of genera and species. These targets are present in hundreds to thousands of copies per genome enabling detection of low microbial burdens (<10 colony forming units) in a primary specimen [42]. Polymerase Chain Reaction (PCR) and Nucleic Acid Sequence Based Amplification (NASBA) based amplification-detection platforms [43-48] have the potential to diagnose fungal infections at an early stage that can influence patient management. Real-time PCR using self-reporting fluorescent probes allows the kinetics of the amplification process to be observed and analyzed yielding higher quality diagnostic information. To date, nucleic acid assays have been focused on the detection and identification of microbial pathogens. Newer probing technologies providing allelic discrimination enable parallel determinations of drug resistance [49].

Assessing primary resistance

Antifungal resistance is dominated by primary resistance to triazole drugs due to inherently less susceptible fungal species. Knowing species-specific information provides a workable inference of drug susceptibility that guides treatment choices. For example, rapidly identifying organisms that show a propensity for reduced triazole susceptibility like *C. glabrata* and *C. krusei* [20,50] or polyene resistance like *A. terreus* [51] influences primary therapy. The faster the information can be provided, the greater the likelihood of impacting clinical care. In the case of *Candida* infections, initiating appropriate therapy at an early stage (<12 hours) following the first culture positive blood sample significantly improves outcome [13]. Molecular diagnostic assays are ideal for rapid species determination. They can be used with primary specimens because of their sensitivity, which eliminates the need for overnight growth. In principle, a blood or tissue specimen can be analyzed in a matter of hours and species-specific information generated by high fidelity real-time probing of amplicons generated by PCR or NASBA.

Assessing secondary triazole resistance in *Candida*

Molecular probing technology is ideal to determine specific triazole resistance mechanisms that result from genetic changes in drug target site genes, chromosomal aneuploidy or genetic elements controlling expression of drug efflux transporters (Fig. 1). Real-time PCR platforms that reliably distinguish single nucleotide changes (allele discrimination) are highly dynamic and suitable for multiplexed assays [52,53]. When used in conjunction with genus and/or species-specific probes, this approach can rapidly identify a specific fungal pathogen and determine whether a drug resistant determinant is present. The mechanisms of secondary resistance are complex, but their underlying genetic basis is ideal for molecular probing technology. Several major mechanisms of triazole resistance have been elucidated in recent years for prominent yeasts and moulds. Firstly, mutations in the gene encoding the drug target Erg11 (yeasts) and Cyp51A (moulds) alter the apparent drug-binding domain. These mutations are well characterized and associated with resistance phenotypes [54,55]. They are easily assessed with high throughput DNA sequencing [56], allele-specific real-time molecular probes [57], LightCycler(TM) melt curve analysis [58] or DNA microarray technology [59]. These techniques are robust, although they are technically demanding. Secondly, prominent triazole resistance arises from overexpression of the sterol pathway genes and up regulation of two principal families of efflux pumps, the ATP binding cassette (ABC) (*Cdr1*, *Cdr2*) and the major facilitator superfamily (MFS) (*Mdr1*) [60]. The level of transcript for these genes provides a measure of relative resistance. In recent years, quantitative RT-PCR has replaced semi-quantitative Northern blot analysis to assess transcript levels [57,61,62]. Each gene linked to resistance is assessed relative to a constitutive, highly expressed normalization control gene. Expression profiling of genes requires cell cultures grown in the presence/absence of drug, unlike specific mutation detection, which can be used with amplified DNA from primary specimens. In addition, threshold levels of expression need to be assessed and associated with the resistance phenotype, which requires assay standardization and evaluation of large numbers of isolates. Miniarrays have been constructed with numerous targets and internal controls [59]; this multiplex approach facilitates simultaneous expression profiling of many genes. Overall, expression profiling can be subjective when relating transcript levels to resistance phenotypes. This subjectivity can be effectively eliminated by evaluating gain of function mutations in transcription factors that promote expression of specific drug resistance genes (Fig. 1). Up-regulation of *Cdr1/Cdr2* and *Mdr1* leading to triazole resistance arises in *C. albicans* from mutations in Zn(2)-Cys(6) transcription factors *Tac1* and *Mrr1*, respectively [63-66]. High level azole resistance results from hyperactive alleles following loss of heterozygosity at the *Tac1* locus on chromosome 5, which includes *Erg11* [66]. In *C. glabrata*, CgPdr1p is a related transcription factor that regulates drug efflux pumps *CgCdr1*, *CgCdr2*, and *CgSnq2* [67-69]. Finally, the zinc cluster transcription factor Upc2p positively regulates expression of *Erg11* and other ergosterol biosynthesis genes upon exposure to azole antifungals [70,71]. These gain of function mutations can be directly targeted without cell culture by microarray analysis, high throughput sequencing and multiplexed real-time PCR. Yet, clinical application will require a more complete validation between mutations and relative azole resistance.

Triazole resistance in *Aspergillus*

The variety of genetic mechanisms makes molecular detection of triazole resistance in *Candida* species complex. In contrast, triazole resistance in *A. fumigatus* is more restricted and predominantly involves mutations in *Cyp51A* (equivalent to *Erg11*). Drug pumps play an unclear role. Limited mutations in *Cyp51A* confer resistance to one or more triazole agents (voriconazole, itraconazole or posaconazole), which is ideally suited for molecular detection [72,73]. The narrow range of resistance-associated mutations facilitated the application of real-time PCR with molecular beacon probes [72], pyrosequencing [74] and

high resolution melt curve analysis [75] for mutation profiling. Allele-specific molecular beacons could profile eight separate alleles in a single multiplexed PCR reaction [72]. The reaction was ideal for high-throughput applications and was designed either to identify specific alleles by assigning separate fluorophores to each mutation or overall resistance by assigning a single fluorophore to all resistance alleles and a separate fluorophore to the wild type susceptible allele. In this format, high-throughput evaluation of isolates facilitated a designation as susceptible or resistant. Recently, a more comprehensive two-tiered molecular diagnostic assay for triazole drug resistance was described to detect resistance-associated mutations. The first tier consisted of a molecular beacon probe panel that broadly distinguished triazole-susceptible from triazole-resistant *A. fumigatus*. As specific mutations confer resistance to different triazole drugs (Fig. 2), the second tier panel provided possible therapeutic options depending on the mutation detected [73].

Detecting echinocandin resistance

Resistance to echinocandin drugs remains relatively low but breakthrough infections involving high MIC strains are rising due to more extensive use of echinocandins. When they occur, mechanism-specific resistance has been reported [76-78]. Resistant isolates show cross-resistance across the entire class of echinocandin drugs. It is now well recognized that high MIC isolates of *Candida* spp. from patients failing therapy often contain amino acid substitutions in Fks subunits (Fks1p and/or Fks2p), which comprise the 1,3- β -D-glucan synthase target [79]. There is an emerging perspective that the *FKS* genotype is a better predictive indicator of resistance than MIC alone [76]. The limited spectrum of mutations conferring resistance is ideal for detection by molecular diagnostic tools. Real-time PCR with allele-specific molecular beacons have been used to detect prominent mutations associated with echinocandin resistance [80]. Echinocandin resistance in *C. albicans* can easily be assessed by real-time PCR or high throughput sequencing. Microarray technology may be more suited to accommodate the larger number of mutations in multiple *FKS* genes seen with *C. glabrata* [77].

Conclusion

The overall prevalence of antifungal resistance is relatively low, but it remains a serious clinical management challenge for individual high risk patients and those with persistent mycoses. Molecular diagnostics have the potential to transform the modern clinical microbiology laboratory by providing rapid identification of infecting organisms while profiling the presence of inherently resistant species or acquired genetic mechanisms that alter susceptibility to antifungal drugs. These assays cover a range of platforms including real-time PCR or NASBA with high fidelity self-reporting probes, microarrays, high throughput sequencing and hybridization melt profiles. These assays are well suited to molecular diagnostic laboratories and are useful for both patient management and epidemiological surveillance. The key to the successful application of this technology for antifungal resistance is a strong association between genetic mechanisms, in vitro reduced susceptibility, and clinical outcome. The multifactorial nature of triazole resistance in *Candida* spp makes detection complex, especially differential expression of drug efflux pumps. But the recent elucidation of gain of function mutations in transcription factors helps pave the way for specific targeting. The limited spectrum of resistance mutations identified for triazole resistance in *A. fumigatus* and echinocandin resistance *Candida* is ideally suited for the development of robust diagnostic assays. Long standing issues of standardization and nucleic acid extraction are slowly resolving as improved techniques for cell lysis coupled with automated nucleic acid extraction, along with new commercial kits for nucleic acid amplification/detection, are emerging.

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Genetic Target			
	<i>Candida</i>	<i>Aspergillus</i>	
Triazoles			
• Target site mutations	<i>C. albicans</i>	<i>Erg11</i>	<i>Cyp51A</i>
• Target site Upregulation	<i>C. albicans</i>	<i>Erg11</i>	<i>Cyp51A</i>
•Drug Efflux Transporters			
ABC	<i>C. albicans</i>	<i>Cdr1,Cdr2</i>	<i>Mdr1, Mdr4</i>
	<i>C. glabrata</i>	<i>Cdr1, Cdr2, Snq2</i>	
MFS	<i>C. albicans</i>	<i>Mdr1</i>	<i>Mdr3</i>
•Transcription factors			
ABC	<i>C. albicans</i>	<i>Tac1</i>	
	<i>C. glabrata</i>	<i>Pdr1</i>	
MFS	<i>C. albicans</i>	<i>Mrr1</i>	
ERG	<i>C. albicans</i>	<i>UpC2</i>	
Chromosomal aneuploidy	<i>C. albicans</i>	Chromosome 5	
Echinocandins			
Target site mutations	<i>C. albicans</i>	<i>Fks1</i>	<i>Fks1</i>
	<i>C. glabrata</i>	<i>Fks1, Fks2</i>	

Fig. 1.
Summary of genetic mechanisms leading to triazole and echinocandin resistance in *Candida* spp. and *Aspergillus* spp.

Genotype	G54X	G54W	L98X	TR/L98X	G138X	G138C
Drug						
Itraconazole	R	R	S	R	R	R
Voriconazole	S	S	S	R	R	R
Posaconazole	S	R	S	S	S	S
Ravuconazole	S	S	S	R	R	R

Fig. 2.

Association between *Cyp51A* mutations and triazole resistance in *Aspergillus fumigatus*. The expected susceptibility associated with specific alleles is designated as either resistant (R) or susceptible (S) for agents itraconazole, voriconazole, posaconazole or ravuconazole. Modified with permission from Garcia-Effron et al. (73).