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Review

New approaches for antifungal susceptibility testing

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ABSTRACT

Background: Invasive fungal diseases, including those caused by (multi)drug-resistant *Candida* and *Aspergillus* species, still represent global public health concerns. Information about the antifungal susceptibility testing (AFST) of fungal isolates must be quickly produced to help clinicians in administrating appropriate antifungal therapies. Unfortunately, reference AFST methods, albeit accurate, are labour-intensive and take several hours before patients' results can be available to the treating clinicians.
Aims and sources: This review is a blend of evidence obtained from PubMed literature searches, clinical laboratory experience and the author's opinions that is aimed to summarize recent significant advances and ongoing challenges in the AFST area.
Content: Particular attention is given to the new approaches based on genetic or phenotypic recognition of antifungal resistance that are destined to enhance the clinical usefulness of AFST in the near future. Following short-term exposures of fungal cells to antifungal drugs, new antifungal susceptibility end-points have been established, and novel diagnostic assay platforms have been proposed for the genotyping assessment of fungal isolates with resistance-associated mutations. Overall, new approaches provide a rapid, reliable means of identifying those fungal isolates with phenotypically detectable acquired resistance mechanisms, independently from the clinical susceptibility categorization of the isolates as obtained in a classical AFST way.
Implications: Despite holding promise as a surrogate diagnostic method to better direct antifungal therapy, the AFST approaches described in this review need to be evaluated in multicentre laboratory studies to enable their standardization and refinement. **M. Sanguinetti, Clin Microbiol Infect 2017;▪:1**

Introduction

Antifungal susceptibility testing (AFST) of pathogenic fungal organisms remains an indispensable though challenging tool in the battle against fungal diseases [1], despite multiple factors beyond the microbiological resistance that affect the *in vivo* outcome in opportunistic invasive mycoses. Measuring fungal growth in the presence of different antifungal drug concentrations allows the determination of the MIC, a value that helps to predict the likelihood of efficacy of the antifungal therapy [2,3]. However, one of the limitations when using reference (i.e. CLSI or EUCAST) AFST methods [4–7] or the commercial adaptations (e.g. Etest (AB Biodisk, Solna, Sweden) or Sensititre YeastOne (SYO, Thermo Fisher Scientific, MA, USA)) of microdilution or disc diffusion MIC assays, is that these methodologies have a time-consuming set up and/or intrinsically slow turnaround time—this is also true for antibacterial agents [8]. Again, there are important issues that cannot be ignored. First, the interlaboratory variability in caspofungin MICs has limited the *in vitro* testing of this antifungal drug using both the reference methods. Second, the general subjectivity in reading MIC end-points may lead to unreliable and non-reproducible results, especially when the visual MIC determination is adopted. Third, the scarce correlation between the results of reference AFST and therapeutic outcomes has precluded CLSI or EUCAST from establishing clinical breakpoints for some antifungal agents and fungal species. Fourth, CLSI and EUCAST clinical breakpoints often differ, making the interpretation of antifungal MICs difficult in the clinic setting.

A key function of the reference AFST would be to detect fungal resistance, i.e. to determine which antifungal agents are likely to be clinically inactive [2]. To this end, the epidemiological cut-off value,

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defined as 'the upper end of the wild-type (WT) or beginning of the non-wild-type (NWT) MIC distribution', is useful to indicate whether or not a given fungal isolate 'is devoid of phenotypically detectable acquired resistance mechanisms or not' [9]. This indication is irrespective of the clinical susceptibility categorization as susceptible, intermediate (or susceptible-dose-dependent) or resistant for that isolate. As in the case of echinocandin or azole antifungal agents, clinical breakpoint MICs and epidemiological cut-offs may not agree [10,11].

As a response to the uncertainties about the reference AFST methods, new technologies, particularly those that are nucleic-acid-based, have emerged as promising tools for the detection of antifungal resistance. Although not yet a reality, with future advances in diagnosis of fungal infections and resistance [12], the potential of rapid (<4 h to result) diagnostic approaches for antifungal resistance is plausible to ensure that a patient quickly receives appropriate antifungals. The prophecy that 'very soon we shall not need breakpoints for phenotypic susceptibility testing as these will be replaced by genetic methods' [13] is not so far from coming true. However, it is required that newer-generation methods (e.g. whole genome sequencing) are capable of predicting not only resistance but also susceptibility, as well as quantifying the level of resistance [14]. It should be noted that nucleic acid sequence-based amplification techniques have the ability to detect mRNA transcripts of the target gene, so offering a more confident prediction of resistance phenotypes. In contrast, phenotype-centred (or semi-molecular) approaches combining a culture step with molecular analysis (i.e. by real-time PCR or matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS)) could be very promising alternatives to the classical phenotypic AFST [15,16].

In this review, we will briefly describe new technologies that could be considered really as competitors to the current methods for AFST. Furthermore, we will discuss questions that still remain to be addressed regarding these technologies, which undermine their suitability for routine use in the clinical microbiology laboratory.

Nucleic acid-based diagnostic assay platforms for detection of echinocandin resistance

Clinical resistance to echinocandins has been associated with a number of amino acid substitutions caused by single nucleotide polymorphisms in specific hot spot (HS) regions of the genes *FKS1* (all *Candida* species) and *FKS2* (*Candida glabrata*), which encode the drug target β-1,3-D-glucan synthase. These mutations reduce by 30-fold to several thousand-fold the echinocandin inhibition of the target enzyme and arise in *C. glabrata* and *Candida albicans* isolates recovered almost exclusively from patients with previous echinocandin exposure [17]. The most dominant mutation in all *Candida* species seems to involve codon S645, which is equivalent to S629 in *C. glabrata* (reviewed in ref. [10]), altering the amino acid sequence in the HS1 region. However, the degree of MIC elevation, i.e. the level of resistance, depends on the location as well as the single amino acid substitutions [3], with mutations at S629 resulting in higher echinocandin MIC values than mutations at R631 or D632 in *C. glabrata* [18]. It was also noted that, for the functionally equivalent mutations S629P and S663P, the mutation in the *FKS2* gene (S663P) was associated with higher echinocandin MIC values than the mutation in the *FKS1* gene (S629P) [18]. Nevertheless, the *FKS2* S663P alteration was seen to rapidly disappear after the discontinuation of echinocandin treatment in a patient with persistent, clonal *C. glabrata* fungaemia [19].

Since the majority of mutations that affect *C. glabrata* susceptibility to echinocandins would be in the *FKS1* HS1 and *FKS2* HS1 [18,20,21] it is reasonable that these regions were chosen as the primary targets of molecular assays for resistance detection [22–24]. Until recently, DNA sequencing has been the only available method for the identification of mutations in *FKS1* and *FKS2*, but it is impractical for most clinical microbiology laboratories because of its high costs and laboriousness. Alternatively, multiple PCR assays where each oligonucleotide probe is specific for a defined genotype could be employed, but the complex interactions occurring between oligonucleotides would make the optimization of assay conditions quite difficult. By contrast, the Luminex MagPix technology using xMAP microspheres has been developed as a platform for the establishment of multiplex assays, which permits high-throughput analysis of up to one hundred different target molecules in a single test.

In 2014, Pham et al. [22] employed the microsphere-based technology to create a Luminex probe-based assay for the rapid identification of single nucleotide polymorphisms that are known to confer resistance to one or more echinocandins in *C. glabrata*. The targets for the *FKS* probes were generated using asymmetrical PCRs—where single-stranded PCR products allow the probes to anneal; hence, the discriminatory affinity of *FKS* probes for their targets was determined by using a panel of reference isolates with known *FKS* mutations. The MagPix assay was first validated with a set of 102 isolates obtaining 100% of results concordant with the isolates' DNA sequencing profiles; after that, the assay was used for high-throughput screening of 1032 *C. glabrata* surveillance isolates, enabling the detection of 16 new isolates with mutations, the presence of which was confirmed by DNA sequencing of the corresponding region [22]. Of the isolates displaying an irregular *FKS2* HS1 probe signal pattern, three had the F659del mutation—not included in the original assay but known to confer echinocandin resistance. The remaining two isolates had the S663P mutation in addition to a silent mutation at G1986A. As a rapid and highly versatile format—*FKS1* HS1 and *FKS2* HS1 profiles of up to 95 isolates can be determined in as little as 5 h—the multiplex *FKS* MagPix might be used in place of DNA sequencing, particularly for those laboratories that are already equipped with the Luminex technology [22].

In the meanwhile, Dudiuk et al. [23] developed a set of classical PCRs able to detect ten of the most frequent mutations associated with clinical echinocandin resistance in *C. glabrata* within 4 h. Using a blind collection of 50 *C. glabrata* strains, including 16 *FKS1* and/or *FKS2* mutants, the assay allowed that 98% (49/50) of the strains were correctly identified as echinocandin susceptible or resistant compared with the echinocandin AFST results. The molecular diagnostic results from the PCR assay were 98% concordant with those obtained from DNA sequencing. The one false result regarded an *FKS2* mutant, in which Fks2p carried the aforementioned deletion at the 659 residue (F659del). In this case, the deletion could not be detected because a few nucleotides were deleted and the nucleotide sequence where the primer was aligned did not change [23].

Very recently, Zhao et al. [24] proposed a novel diagnostic assay platform for rapid *FKS* genotyping of *C. glabrata* isolates, to identify echinocandin resistance-associated mutations without the assay design and set-up complications of previously described assays [22,23]. Using asymmetrical PCR in conjunction with molecular beacon probe-based melting curve analysis, a dual assay for *FKS1* and *FKS2* was developed to accurately discriminate WT from mutated *FKS* genes within 3 h. In this assay, signature melting profiles and corresponding T_m values were generated, through direct colony PCRs from reference strains, for eight *FKS1* HS1 and seven *FKS2* HS1 genotypes. Hence, the *FKS* genotype of the testing isolate could be easily identified by comparing the T_m value with those of the reference strains representing all the mutations

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included in the dual assay. In the subsequent proof-of-concept clinical validation study, using a blinded panel of 188 *C. glabrata* isolates, both *FKS1* HS1 and *FKS2* HS1 assays showed 100% accuracy for WT/NWT genotype discrimination compared with DNA sequencing results [24].

Collectively, these studies show that the molecular testing can be ideal for detecting echinocandin resistance in *Candida* species, whereas this is not the same for azole resistance because of the complexity of underlying resistance mechanisms in these yeasts [2].

MALDI-TOF MS-based assays for detection of echinocandin or azole resistance

MALDI-TOF MS has been adopted as a rapid and robust tool for the accurate identification of microorganisms with medical importance [25,26], including yeasts and filamentous fungi [27,28]. Recently, MALDI-TOF MS profiling of microbial isolates has taken exciting new trajectories beyond microbe identification, offering opportunities for detection of antimicrobial susceptibility/resistance in fungal organisms [29]. So, as with bacteria [30], promising approaches have been developed for the detection of antifungal resistance in *Candida* and *Aspergillus* species [29]. Similar to MALDI-TOF MS-based species identification, these approaches may considerably decrease the time to result associated with the current AFST assays, so facilitating the implementation of targeted antifungal therapy.

The initial demonstration of the potential of MALDI-TOF MS for testing antifungal susceptibility of pathogenic fungi came from a proof-of-concept study by Marinach et al. [31] who monitored the changes of the proteome of *C. albicans* cells exposed to different concentrations of fluconazole. Using a rigorous statistical approach, the authors assessed the similarity of the mass spectra at each of the different intermediate concentrations with each of the mass spectra at two extreme concentrations (fluconazole 128 mg/L and fluconazole 0 mg/L), i.e. the 'extreme' spectra, to provide a classification of 'nearer to the 128 mg/L' or 'nearer to the 0 mg/L' spectrum. Hence, the minimal profile change concentration (MPCC) value was proposed as a new AFST end-point, that was defined as the lowest concentration of drug (i.e. fluconazole) at which a mass spectrum profile change is detected. Surprisingly, MPCCs did not differ by more than one dilution step from the MICs determined using the CLSI reference method, so that all the *C. albicans* isolates tested except one were correctly assigned to the category (resistant or susceptible) to which they were assigned by CLSI [31].

Taking advantage of these observations, we described in 2012 [32] the development of a MALDI-TOF MS-based assay for testing the susceptibility of fungal species to caspofungin. In this assay, we applied the composite correlation index (CCI) analysis to calculate a correlation matrix based on the mass spectra acquired after the fungal cells were exposed to different drug concentrations for 15 h. Following matching of the 'intermediate' mass spectra with each of the 'extreme' mass spectra, MPCC was assessed as the CCI value at which a spectrum is more similar to the spectrum observed at the maximal caspofungin (32 mg/L) concentration (maximum CCI) than the spectrum observed at the null caspofungin (0 mg/L) concentration (null CCI). Using a panel of WT and *FKS1*/*FKS2* mutant isolates of *Candida* (34 isolates) and *Aspergillus* (ten isolates) species, we showed that the MPCC values were fully concordant with the CLSI MIC or the minimum effective concentration values for 100% of the isolates tested [32].

Although the MALDI-TOF MS-based assay may eliminate subjective visual end-point determination, it provides slight time savings compared with the CLSI reference method (15 h versus 24 h). Therefore, in 2013 [33] we simplified our MALDI-TOF MS-based assay to allow discrimination between susceptible and resistant isolates of *C. albicans* after 3 h of incubation of yeast cells in the presence of three caspofungin levels only: no drug (null concentration), intermediate ('breakpoint' concentration) and maximum (maximal concentration). The decision about susceptibility/resistance was achieved by determining the caspofungin concentration at which the CCI values obtained by matching the 'breakpoint' spectrum with the 'maximal' spectrum were, respectively, higher (for susceptible isolates) or lower (for resistant isolates) than the CCI values obtained when the 'breakpoint' spectrum was matched with the spectrum at null concentration. Analysis of WT and *FKS1* mutant *C. albicans* isolates revealed that 100% (51/51) and 90.9% (10/11) of the isolates tested were correctly classified as caspofungin-susceptible and caspofungin-resistant, respectively, in accordance with the *FKS1* genotype. The categorical agreement was 98.4%, with only one major error for an isolate harbouring a D648Y genotype—this mutation is known to confer a lower level of echinocandin resistance [33].

Although these data suggest that the MALDI-TOF MS-based assay has the potential to be introduced into clinical microbiology laboratories, the reproducibility and robustness of the assay still have to be definitively proven. Unsurprisingly, in a study performed similarly to that described originally by us [32] but modified to facilitate the discrimination of susceptible and resistant isolates of *Candida* species (35 *C. albicans*, 35 *C. glabrata* and 37 *C. tropicalis*) to triazoles [34], the overall essential agreement between the MALDI-TOF MS and the CLSI AFST methods ranged from 54% to 97%, with the best essential agreement observed for *C. glabrata* isolates. The reproducibility of the MALDI-TOF MS-based assay varied between 54% and 83%, and it was higher for *C. glabrata* isolates than for *C. albicans* or *C. tropicalis* isolates. However, as very major and major errors with MALDI-TOF MS-based assay were noticed, applying a 5% tolerance for CCI ratio evaluation did result in error percentages decreased by up to 33.3% [34].

Future view and implications

The selection and spread of multi(drug)-resistant microorganisms over the last decades has become a major and global public health concern [35]. Two growing threats originating from the fungal kingdom, i.e. *Aspergillus fumigatus* and *Candida auris* [36,37], re-emphasize the need for reproducible, medically relevant AFST [2,3]. As the most important outcome of any AFST is the fast and reliable prediction of resistance in pathogenic fungi, laboratory scientists are anxious to have antifungal assays run concomitantly with fungal identification. Rapid and parallel detection of genetic (e.g. *FKS* mutations) or phenotypic (e.g. MALDI-TOF MS profiling) recognition of antifungal resistance should become part of the routine identification protocols for clinical isolates in the mycology laboratory. Therefore, assessment of *FKS* mutations in *C. glabrata* by the aforementioned assay platforms should require some optimization [22,24], whereas work is in progress in our laboratory to extend MALDI-TOF MS-based AFST to *C. glabrata*.

To the best of our knowledge, no studies have yet been published that evaluate the performance and utility of new AFST approaches in a clinical context, i.e. as the primary method of diagnosis. Hence, although these approaches offer much promise, their place in the clinical diagnostic laboratory is not yet validated. Future studies will help to enhance the ability of AFST for detecting drug resistance, which may provide useful adjuncts to optimize the efficacy of antifungal therapy, to improve the treatment outcome of the patients, and to preserve the antifungal susceptibility of fungal isolates.

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Transparency declaration

MS and BP declare that there are no conflicts of interest in connection with this article.

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