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Paving the Way to Overcome Antifungal Drug Resistance: Current Practices and Novel Developments for Rapid and Reliable Antifungal Susceptibility Testing

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The past year has established the link between the COVID-19 pandemic and the global spread of severe fungal infections; thus, underscoring the critical need for rapid and realizable fungal disease diagnostics. While in recent years, health authorities, such as the Centers for Disease Control and Prevention, have reported the alarming emergence and spread of drug-resistant pathogenic fungi and warned against the devastating consequences, progress in the diagnosis and treatment of fungal infections is limited. Early diagnosis and patient-tailored therapy are established to be key in reducing morbidity and mortality associated with fungal (and cofungal) infections. As such, antifungal susceptibility testing (AFST) is crucial in revealing susceptibility or resistance of these pathogens and initiating correct antifungal therapy. Today, gold standard AFST methods require several days for completion, and thus this much delayed time for answer limits their clinical application. This review focuses on the advancements made in developing novel AFST techniques and discusses their implications in the context of the practiced clinical workflow. The aim of this work is to highlight the advantages and drawbacks of currently available methods and identify the main gaps hindering their progress toward clinical application.

1. Introduction

Invasive fungal infections (IFI) are a rising cause of morbidity and mortality among humans with underlying medical conditions, causing more than 1.4 million deaths annually worldwide.[1,2] As only four antifungal classes (azoles, echinocandins, polyenes, and pyrimidine analogs) are currently available for treating IFI, rising resistance to these drugs is a cause of major concern.[1,3] For example, the globally emerging multidrug-resistant species *Candida auris* is now recognized by the Centers for Disease Control and Prevention (CDC) as an urgent threat.[4] Thus, its proper treatment is therefore crucial for both individual therapeutic outcomes and preventing its spread.[5] The increasing and undirected use of antifungals in medicine and agriculture is associated with the rising numbers of acquired resistance in fungi.[3] The readers are referred to the following excellent reviews on fungal pathogens[6,7] and antifungal resistance[3] for further reading. The latter, in combination with the challenges associated with developing novel antifungals, emphasizes the need for rapid disease detection and adequate antifungal therapy protocols.[3,8] Implementing such measures is part of a proper antimicrobial stewardship aimed at reducing the excessive usage of antimicrobial therapies in clinical settings by encouraging physicians to prescribe appropriate antimicrobials only when truly required.[9]

Antimicrobial susceptibility testing (AST), specifically antifungal susceptibility testing (AFST), is employed to reveal the susceptibility or resistance of fungal pathogens to clinically relevant antifungals.[10,11] In these tests, pathogenic fungi are exposed to various concentrations of a panel of antifungals to determine the minimum inhibitory concentration (MIC) values, which are typically defined as the lowest drug concentration inhibiting the pathogen's growth.[12] MIC values allow physicians to predict the success of antifungal treatments.[13] As such, rapid AFST is an essential tool to improve antifungal therapy by choosing the correct and most effective antifungal drug in a timely manner. Early therapy initiation is also crucial for enhancing the therapeutic outcome of patients with invasive fungal infections, such as life-threatening bloodstream infections.[14] Clinical AFST is conducted according to standardized methods and protocols, published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or the Clinical and Laboratory Standards Institute (CLSI).[12,15] Yet, as these gold-standard methods are laborious and typically require at least 24 h (and in many cases, several days) for completion,[12,16] significant research efforts are now being directed toward the development of more expedited phenotypic and molecular AFST techniques.[17,18]

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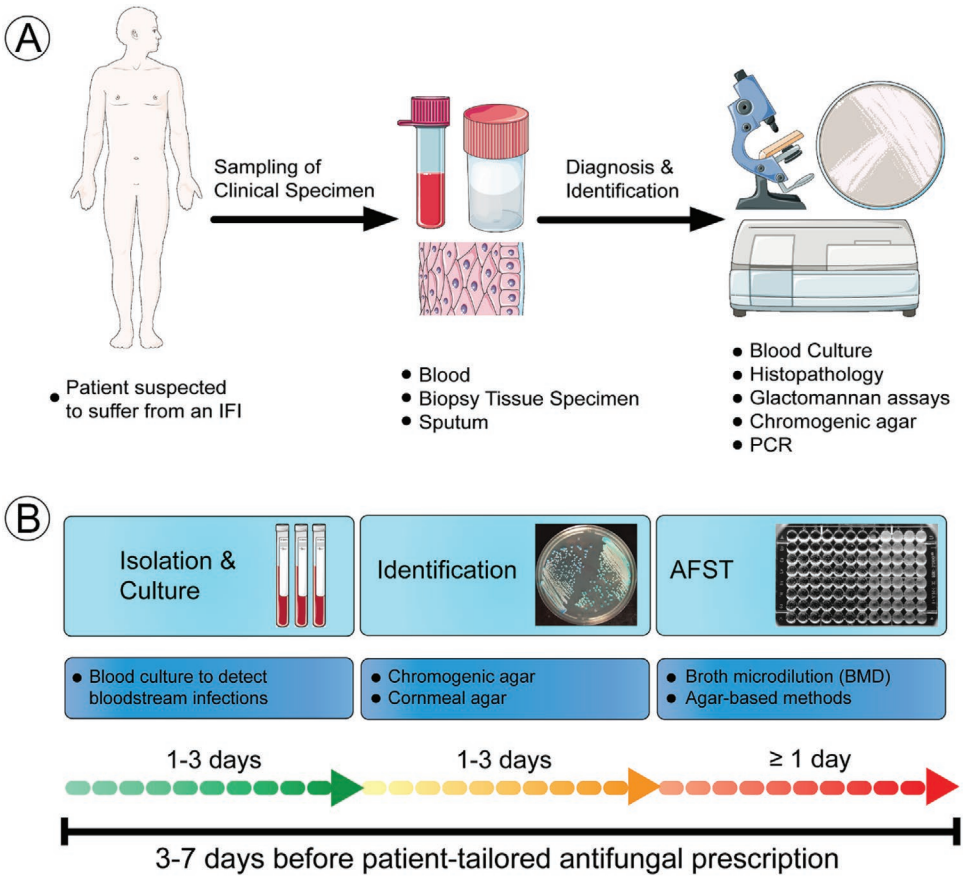

Figure 1. A) General workflow for IFI diagnosis. First, clinical specimens, such as blood or tissue, are sampled from a patient suspected to suffer from an IFI. Methods such as blood culture and histopathology, among others, are used for fungal diagnosis. Subsequently, procedures such as selective chromogenic agar or PCR are employed to discriminate fungal species. B) Workflow for suspected candidemia includes fungal pathogen isolation, identification, and AFST. First, blood cultures reveal the presence of bloodstream pathogens in a patient within 1–3 days. Subsequently, the employment of agar-based methods such as chromogenic agar or cornmeal agar enables fungal species identification within 1–3 days. Eventually, AFST and MIC determination requires at least another day but allows a patient-tailored antifungal prescription to improve antifungal treatment and reduce the spread of antifungal resistance. Images are adapted with permission under the terms of the Creative Commons CC BY license from Servier Medical Art (smart.servier.com). Chromogenic agar plate and BMD plate are adapted with permission.[34,35] Copyright 2006, Oxford University Press (chromogenic agar plate) and 2003, American Society for Microbiology (BMD plate) respectively.

2. Fungal Disease Diagnosis Precedes AFST

The diagnosis of IFIs predates AFST, and **Figure 1**A presents a generalized workflow. First, the fungal pathogen is isolated from a patient sample (i.e., blood, tissue biopsy specimens, etc.), and subsequently, various techniques are applied for disease diagnosis and species identification to confirm the type of infection (e.g., fungal, bacterial, or viral).[14] For instance, blood culture detects fungal bloodstream infections,[19] while histopathology reveals fungi in tissue samples.[20] Furthermore, β-glucan and galactomannan assays detect fungal cell wall antigens in body fluids such as serum.[14] Species identification can be performed on chromogenic agar,[21] and recently, molecular approaches like polymerase chain reaction (PCR) detecting species-specific DNA sequences have become of increasing importance.[22] For a comprehensive overview of IFI diagnosis techniques, the readers are referred to the recent review articles of Sanguinetti et al.[22] and Ruhnke et al.[19] Unfortunately, due to the emergence of antifungal resistance in fungi, proper therapy cannot rely on IFI diagnosis alone, and subsequent AFST is inevitable.[3,23]

Conventional AFST methods, as practiced in clinical laboratories, include the gold standard broth microdilution (BMD) and the commercial agar-based Etest (bioMérieux, France). While these techniques are simple and well established, they are slow and require multiple preparation steps. The latter stems from the prerequisite for high cell densities; for example, for yeasts, $0.5–2.5 \times 10^5$ CFU mL^{-1} and $1–5 \times 10^6$ CFU mL^{-1} are required for BMD and Etest assays, respectively.[12,24] Thus, culturing remains an essential prerequisite before standard AFST.

Figure 1B schematically illustrates a typical workflow of a sample derived from a patient suspected of suffering from candidemia (a bloodstream infection with *Candida*), one of the most common types of life-threatening invasive fungal diseases.[2] First, blood samples are taken from the patient

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and then added to designated bottles containing blood culture medium to improve fungal pathogen recovery.[25,26] These bottles are thereafter placed in blood culture systems, such as the automated BD BACTEC (Becton Dickinson, USA) or the BacT/ALERT (bioMérieux, France).[25] In the case of candidemia, these systems typically achieve detectable fungal growth within 1–3 days[14] (termed a "positive blood culture result"). Subsequently, the second step of species identification, using conventional agar-based techniques, requires another 1–3 days.[14,27] For instance, chromogenic agar enables differentiation of *Candida* species, relying on species-specific enzyme-based cleavage of chromogenic compounds, by naked eye observation of differently colored colonies.[21] Another approach employs cornmeal agar, a chlamydospore-inducing medium. It allows for morphological identification of *Candida albicans* (*C. albicans*), the most frequently found pathogenic yeast in humans,[6] by microscopically investigating its chlamydospore-formation.[28]

The subsequent and final diagnostic step of AFST takes at least another day when traditional broth and agar-based techniques are employed.[12,24] In these methods, the determination of MIC values for a specific pathogen drug is achieved by visual detection of fungal growth on agar plates or in a liquid medium in the presence of various antifungal concentrations.[12,29,30] Thus, the whole process (Figure 1B) of pathogen isolation, identification, and AFST, which precedes appropriate antifungal prescription, ideally takes between 3 and 7 days, assuming it is not even further delayed (e.g., by slow-growing strains or logistic influences such as transportation or laboratory opening hours).[31,32]

Accelerating AFST will, of course, shorten the time for a patient-tailored antifungal therapy, and more importantly, the introduction of new AFST methods may profoundly impact the lengthy preceding preparation steps. For example, genotypic techniques detecting antimicrobial resistance genes can be directly performed from sputum, swabs, and blood culture specimens alongside pathogen identification.[33] Analyzing single microbial cells by time-lapse microscopy reduces the required number of cells,[11] potentially avoiding the time-consuming prerequisite of obtaining high cell densities as required in conventional approaches. Thus, we believe that AFST techniques that are readily integrable with pathogen isolation, detection/identification could be a game-changer and potentially practiced in point-of-care settings.

3. The Ideal AFST Method

If we could design the perfect AFST method, this approach would be sensitive, accurate, and reliable in terms of the MIC value determination while also being easy to use and cost-effective. Furthermore, this method would allow parallelization and multiplexing to analyze multiple antimicrobials or pathogens simultaneously with a broad microorganism spectrum, minimal sample pre-processing steps, and integrability. Other factors, such as the preferential phenotypic or genotypic resistance determination and the test setting (e.g., centralized laboratories vs. point of care), should also be considered. Most importantly, this approach should yield MIC values and allow "resistant" and "susceptible" determinations as quickly as possible to improve therapy outcomes and reduce the spread of antifungal resistance.

3.1. Phenotypic versus Genotypic AFST

In general, susceptibility testing methods can be divided into phenotypic and genotypic approaches. Phenotypic methods monitor the growth and other physiological changes (e.g., size and shape) of cells in the presence of different antimicrobials at varying concentrations.[11] As such, they reveal specific phenotypic susceptibility profiles and provide a comprehensive assessment of what antimicrobials agents can be used to treat infections caused by pathogenic microorganisms.[36] However, these phenotypic methods are time-consuming as, in most cases, they are culture-based. By contrast, genotypic techniques, representing a nonculture methodology, rely on detecting established resistance-conferring genes and mutations on the DNA level.[11] While offering rapidity, genotypic approaches only reveal the presence of resistance factors;[37] they do not allow susceptibility determination, and as such, they are correctly referred to as genotypic antimicrobial resistance (AMR) detection methods.[38] Nevertheless, genotypic AMR detection is fast and preferred if seeking specific resistance-conferring genes.[11,33]

In this review, we mainly focus on phenotypic methods that enable MIC determination and a more definitive prediction of how fungal pathogens will behave in the presence of clinically relevant antifungal drug concentrations. Although recent research efforts have increasingly focused on novel methods for phenotypic AFST, in an attempt to improve the current situation, traditional methods that have not changed since the first days of AST remain the gold standard in the context of clinical laboratory practice.

4. Standard Methods and Their Commercial Adaptations

AFST was first described in the 1970s and included broth and agar-based methods, such as BMD, disk diffusion, and agar screening.[39,40] Today, nearly half a century later, these techniques, validated by the EUCAST and the CLSI, are still widely used in clinical laboratories, and several commercial adaptations are available. **Table 1** summarizes these AFST techniques and briefly describes both their concept and typical assay time. That table also provides a short comparison of the main advantages and disadvantages of these methods, as discussed in the following few paragraphs.

4.1. Standard Methods—Frequently Used But Not Changed for Decades

BMD is the current gold standard for AFST of yeasts and molds. Twofold serial dilutions of antifungals are made in a liquid medium (broth) and inoculated with a predefined and standardized cell number, as shown in **Figure 2**A. Growth is determined visually or spectrophotometrically in a 96-well plate,

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Table 1. Summary of standard AFST methods and their commercial adaptations.

Name	Measuring principle	Assay time	Advantages	Disadvantages	Ref.
		Standard methods			
Broth microdilution (BMD)	Visual or spectrophotometric measurement of turbidity in a liquid medium	24 h for *Candida* species, 46–50 h for *Aspergillus* species and up to 72 h for *Cryptococci*	Standardized by EUCAST and CLSI Available for yeasts and molds Cheap when prepared in the laboratory	Laborious and lengthy Subjective when read visually	[12,15,16,18,41]
Disk diffusion	Zone of inhibition around antifungal disks on agar plates	24 h for *Candida* and *Aspergillus* species	Standardized by CLSI Available for yeasts and molds Easy to perform and cheap	Lengthy No MIC values	[18,30,42]
Agar screening	Visual detection of growth in a liquid medium	48 h	Standardized by EUCAST Cheap, easy to perform, and read	Lengthy No MIC values Only screening of *Aspergillus* species	[43–45]
		Commercial adaptations			
Vitek2 (bioMérieux, France)	Turbidity in a liquid medium	Usually 12–18 h	Automated Objective results Accelerates AFST compared to BMD	High investment costs Only available for yeast	[18,46,47]
Sensititre YeastOne (Thermo Fisher Scientific, USA)	Colorimetric detection in a liquid medium	24 h	Easy to perform Less subjective than BMD	Lengthy Not validated for molds	[18,48–50]
Etest (bioMérieux, France)	Zone of inhibition around a strip with an antifungal gradient	24–48 h for yeast (up to 72 h for *Cryptococcus* species) and 16–72 h for molds	Easy to use Available for yeast and molds An agar-based method that provides MICs	Lengthy Sometimes subjective	[18,24,51–53]

and the MIC is defined as the lowest growth-inhibiting drug concentration.[12,15,16,41] Both EUCAST and CLSI standardized this reference method for routine AFST of yeasts and molds. While BMD is a relatively cheap method, it is laborious and lengthy. For example, for *Candida* and *Aspergillus* species, the MIC is determined only after 24 or 48 h, according to EUCAST protocols.[12,16] Furthermore, these methods require high cell numbers for both yeasts and molds, typically $\geq 10^5$ CFU mL^{-1} according to EUCAST.[12,16]

Disk diffusion is a widely used agar-based AFST method standardized by CLSI for yeast and filamentous fungi.[30,42] Paper disks with a specific antifungal concentration are placed on an agar plate with a standardized inoculum. Antimicrobial activity is determined by measuring the inhibition zone diameter of the growth-free area around the disk (see Figure 2B), which correlates to the antifungal's diffusion rate through the agar and the fungus susceptibility to that drug.[18] While disk diffusion is simple and cheap, its main disadvantage is its inability to produce MIC values, impairing the interpretation of results for emerging fungal pathogens and novel antifungal agents.[18] Furthermore, this agar-based approach takes a long time of 24 h for *Candida* and *Aspergillus* species and requires a large inoculum size of $\geq 10^6$ CFU mL^{-1}.[30,42]

Azole agar screening, relevant only for *Aspergillus* species, was recently established by EUCAST and is based on the commercially available VIP check assay (Mediaproducts BV, Netherlands).[43–45] A 4-well agar plate contains 4 μg mL^{-1} itraconazole, 2 μg mL^{-1} voriconazole, 0.5 μg mL^{-1} posaconazole, and a drug-free well. *Aspergillus* growth is determined visually after 48 h (see Figure 2C) and used to screen for azole-resistant isolates.[43,45] While this method is easy to perform and cheap, it is time-consuming, requires a high number of cells (0.5 McFarland, $\geq 10^6$ CFU mL^{-1})[16,43], and importantly does not allow for MIC values determination.

4.2. Commercial AFST Approaches—Adaptations of Standard Methods

The BMD method has several commercially available adaptations that simplify the assay procedure and MIC readout. For example, the Sensititre YeastOne (ThermoFisher) assay is performed in 96-well plates containing a growth medium with serial dilutions of the antifungal agent and the Alamar Blue redox indicator.[18] When entering the cells, the latter undergoes a color change from blue to red when reduced by enzymes of metabolically active fungi (reducing resazurin to resorufin; see Figure 2D).[50,57] Accordingly, the MIC is defined as the lowest antifungal concentration where no color change to red occurs, and the medium remains blue.[50] The endpoints are read after

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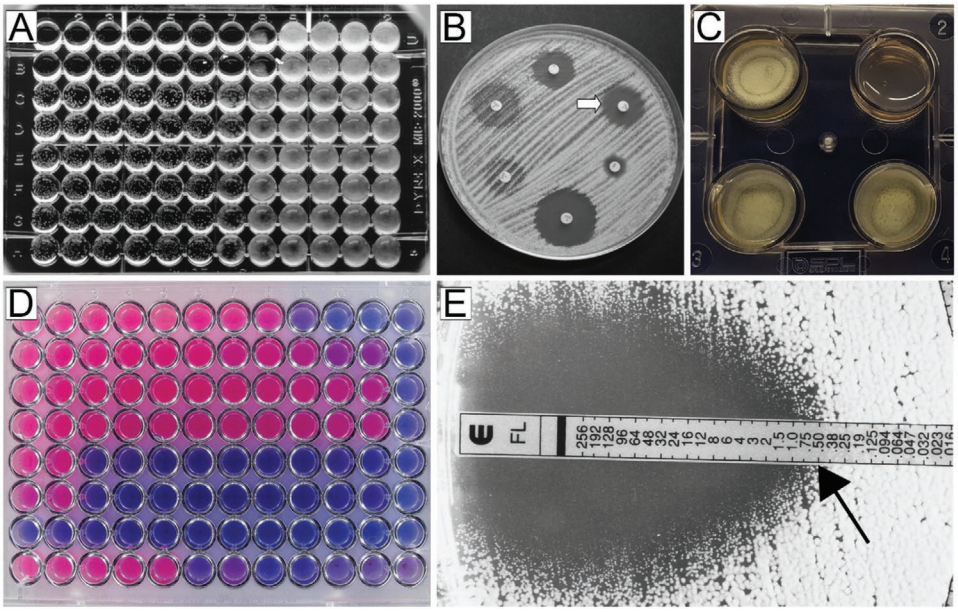

Figure 2. Standard methods and commercial adaptations for routine AFST. A) Visual detection of fungal growth in a liquid medium with twofold antifungal dilutions in the BMD method. Adapted with permission.[35] Copyright 2003, American Society for Microbiology. B) Characteristic disk diffusion assay with zones of inhibition (as indicated by the arrow) around paper disks on an agar plate. Adapted with permission.[54] Copyright 2007, American Society for Microbiology. C) Growth of *Aspergillus sp.* in a 4-well agar plate during the azole agar screening method. Reproduced with permission.[43] Copyright 2018, European Society of Clinical Microbiology and Infectious Diseases. D) The Sensititre YeatOne assay uses a colorimetric redox indicator to indicate fungal growth. Adapted with permission under the terms of the Creative Commons CC BY license.[55] Copyright 2011, the Authors. Published by PLoS ONE. E) Etest strip inhibiting the growth of *Candida* sp. on an agar plate. The MIC is read where the ellipse-shaped zone of inhibition and the strip intersect, as indicated by the arrow, and determined to be 0.5 μg mL^{-1}. Adapted with permission.[56] Copyright 1998, American Society for Microbiology.

24 h and are found to be in good agreement with the reference MICs.[50] Although the Sensititre YeastOne assay is only validated for yeast species, some studies demonstrated its potential for AFST of molds.[49]

An automated commercially available BMD adaptation for susceptibility testing of yeast is the Vitek2 (bioMérieux). This system uses AFST cards with 64 wells containing dried medium with antifungal agents at varying concentrations. The machine introduces the cells into the wells, and their growth is monitored by turbidity measurements. MIC values are typically determined after 12–18 h for *Candida* species and comply well with MIC values determined by BMD.[46,47] The Vitek2 system is mostly used in centralized clinical laboratories where high throughput measurements are crucial. It should be noted that this system is not established for molds and suitable Vitek2 AFST cards are not available.

Another commercially available AFST assay is the agar-based Etest assay (bioMérieux, France), which is an adaptation of the disk diffusion test, where plastic strips containing an antifungal gradient and concentration scale are placed on an agar plate with a standardized inoculum size and incubated.[51,53] The MIC value is determined based on the intersection of the formed ellipse-shaped zone of inhibition and the drug concentration on the strip, as shown by the arrow in Figure 2E.[51,53,56] The Etest is inexpensive, easy to perform, and the MIC values comply well with the BMD gold standard[52]; yet, the time to readout is lengthy (24–48 h for yeasts and 16–72 h for molds).[24]

While these methods have simplified the AFST assay and, in some cases, allowed its automation, the required size of the initial inoculum size remains high and comparable to that of standard methods. For AFST of yeast, the Etest and the Sensititre YeastOne use an inoculum size of ≥10^6 CFU mL^{-1}, whereas the Vitek2 employs a 2.0 McFarland equivalent (≈10^7 CFU mL^{-1}).[24,48,58,59]

5. New Tools for Phenotypic AFST

The time to readout remains the main bottleneck of current state-of-the-art phenotypic AFST methods. Therefore, a significant research effort is directed toward expediting phenotypic AFST, and new techniques, relying on mass spectrometry, flow cytometry, calorimetry, fluorescence microscopy, and optical on-chip assays, are emerging.[60–64] **Table 2** summarizes these new AFST approaches and briefly describes their concept and typical assay time. The table also provides a short comparison of these methods' main advantages and disadvantages, as discussed in the next paragraphs.

5.1. Molecular Analysis by Mass Spectrometry

Mass spectrometry (MS) is already used in many clinical laboratories for the identification of bacteria and fungi, and, as such, it accelerates treatment decisions by classifying pathogens into high or low resistance prevalence categories, as shown in **Figure 3**A. In recent years, MS-based methods are emerging for AST of microorganisms.[66,67,73] For example, MS was used to detect the presence of resistance-conferring enzymes, such as β-lactamases, revealing their activity by monitoring antibiotic

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Table 2. Overview of new AFST methods.

Name	Measuring principle	Assay time	Advantages	Disadvantages	Ref.
Mass spectrometry	Shifts within mass spectral profiles	15 h incubation for minimal profile changing concentrations (MPPC) 3 h for differentiation between susceptible and resistant isolates	Rapid differentiation of susceptible and resistant isolates Different approaches possible (e.g., whole-cell profiles, antibiotic degradation analysis)	High investment costs for instrumentation Arduous workflow	[11,38,65–67]
Flow cytometry	Fluorescence signal to differentiate dead and viable cells	1–9 h exposure of fungal cells to antifungals	Rapid AFST Demonstrated for yeast and molds	Labor-intensive workflow and required technical expertise	[18,63,68–70]
Calorimetry	Heat flow related to fungal metabolism	24 h for *Candida* species and 48 for *Aspergillus* species	Available for yeast and molds	Does not accelerate AFST compared to reference methods	[64,71,72]
Fluorescence microscopy	Fluorescence microscopy analyzes microcolony area on porous aluminum oxide	3.5–7 h for various *Candida* species and 14 h for *Aspergillus* species	Rapid AFST	Arduous workflow including culturing, staining, and microscopy	[61]
On-chip optical assays	Intensity changes of light reflected from a silicon diffraction grating	10–12 h for *Aspergillus niger*	Rapid compared to reference BMD Label-free and real-time monitoring	Only demonstrated for *Aspergillus niger*	[60]

degradation, as depicted in Figure 3B.[66] In this case, cells suspected of encoding for antimicrobial degrading enzymes are incubated with antimicrobials, and subsequent MS reveals their degradation and allowed researchers to determine if resistance-conferring enzymes were present or not.[66] Although this concept requires only a few hours of incubation time (up to 3 h), it only provides information about specific resistance mechanisms.[11,65] A more holistic approach involves analyzing the entire MS profiles or the proteomic profiles of microorganisms after their exposure to varying concentrations of antimicrobial agents, as shown in Figure 3C, and is mainly applied for AFST of fungi. For example, in a study from 2009, *C. albicans* cells were exposed to different fluconazole concentrations, and the shifts in the proteome were examined after 15 h of incubation by MS.[62] Minimal profile changing concentrations (MPCC) were defined as the lowest antifungal concentration, leading to a shift in the mass spectrum compared to a no-drug control.[62] de Carolis et al. have extended this methodology to other *Candida* and *Aspergillus* species and demonstrated a complete agreement of MPCCs with CLSI MICs after 15 h of incubation.[74] Differentiation of resistant and susceptible isolates without determining MPCC values was even achieved within 3 h of incubation.[75] The main drawbacks of MS for AFST include the high costs of the instrument and, so far, insufficient validation.[11,65] Also, it should be noted that fungal cell incubation in the presence of various antifungal agents before MS-based AFST requires high cell densities (typically 10^6 cells mL^{-1} and above).[62,74,75]

5.2. Flow Cytometry

In flow cytometry assays, the fungal cells are cultured in the presence of different antifungal concentrations following staining with fluorescence dyes such as propidium iodide, ethidium bromide, or acridine orange.[63,69,70,76] These nucleic-acid selective dyes penetrate damaged membranes (propidium iodide, ethidium bromide)[63] or indicate alterations in the DNA's secondary structure during cell death by switching fluorescence from green to red (acridine orange).[77] Therefore, changes in the fluorescence are used to distinguish between dead and viable cells by flow cytometry, as depicted in **Figure 4**A-i. Characteristic flow cytometry results, where acridine orange is used to stain *Candida glabrata* (*C. glabrata*) after exposure of 4 h to various concentrations of the antifungal caspofungin, are presented in Figure 4A-ii. In this work, the MIC is referred to as the minimum fluorescence-enhancing concentration (MFEC), defined as the minimum drug concentration yielding an increased fluorescence signal in a predefined number of cells.[69] The determined MFEC values were found to agree well with BMD and Etest reference methods for most fungi–drug combinations.[69]

Flow cytometry allows for rapid AFST of various *Candida* and *Aspergillus* species; the exposure time of fungal cells to the antifungal agents varies between species and protocols but typically lies in the range of 1–9 h.[63,68–70,78] Yet, it should be noted that also flow cytometry requires high cell numbers (commonly around 10^6 CFU mL^{-1}).[63] Furthermore, the labor-intensive workflow, the necessary technical expertise, and its limitations for resource-limited settings are perceived as disadvantages of this technique.[18]

5.3. Calorimetry

In calorimetric AFST techniques, such as isothermal microcalorimetry (IMC), vials contain growth medium at varying antifungal concentrations and cells at defined inoculum size (typically around 10^4–10^5 CFU mL^{-1}).[64,71,72] Changes in the differential heat flow between the sample vial and a reference vial, ascribed to the fungus metabolism, are measured over time at a constant temperature; see Figure 4B-i for a schematic layout

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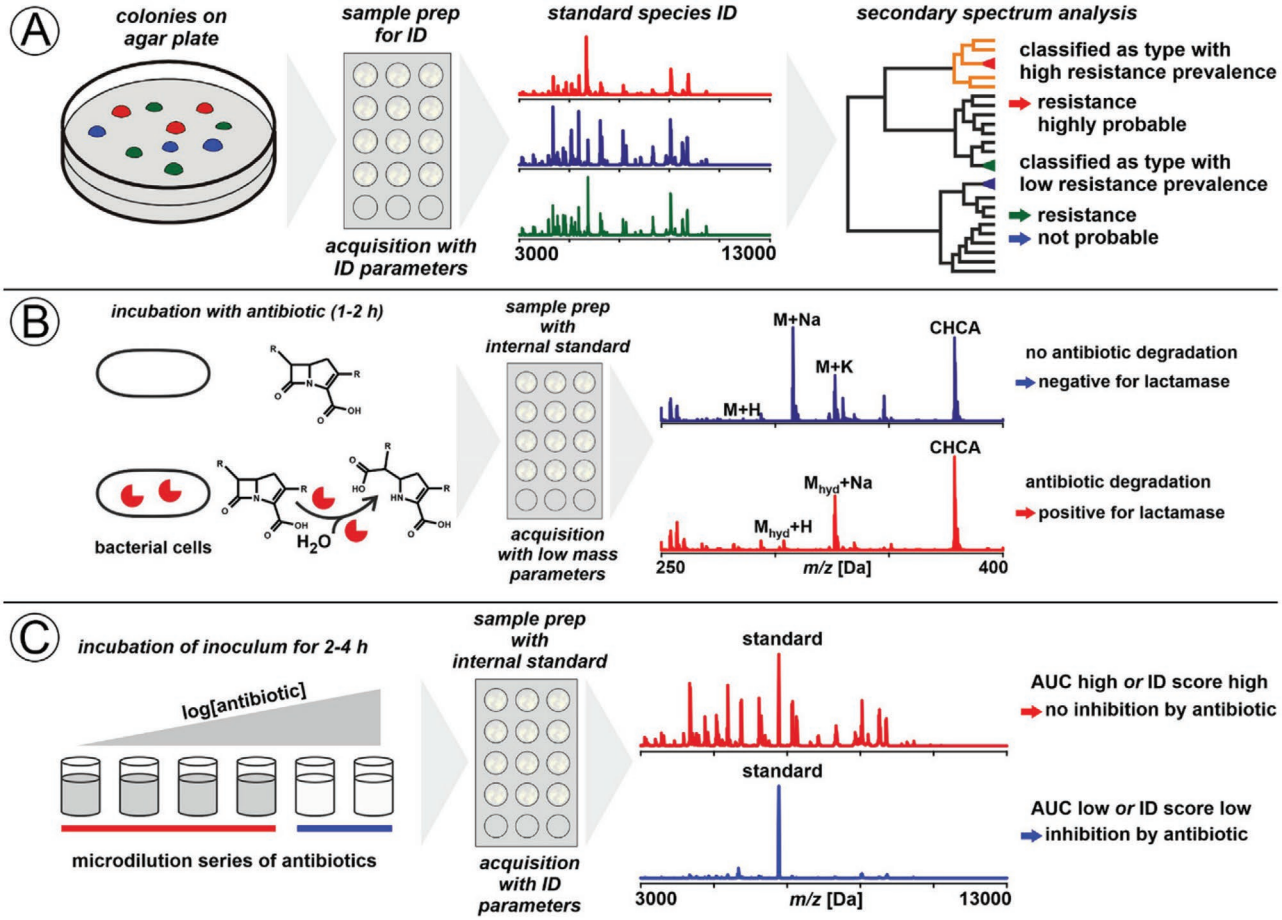

Figure 3. Mass spectrometry-based concepts to advance AST of bacteria and fungi. A) Species identification by MS gives a hint about drug resistance in the microbial pathogen. B) MS-based analysis of enzymatic antimicrobial degradation reveals the presence of resistance-conferring enzymes. C) Incubation of microbial pathogens in the presence of varying antimicrobial concentrations and analysis of entire mass spectral profiles or proteomic profiles. Reproduced with permission under the terms of the Creative Commons CC BY license.[66] Copyright 2019, the Authors. Published by Frontiers Media S.A.

of a typical microcalorimeter. The produced heat is correlated to microbial growth and enables MIC or minimum heat inhibitory concentration (MHIC) value determination as depicted in Figure 4B-ii.[64,71] Here, the MHIC was defined as the lowest drug concentration that reduces by 50% the total heat produced (after a designated time) compared to a drug-free fungus control.[71] IMC has been successfully applied for molds, planktonic yeast, and yeast biofilms; yet, it does not expedite AFST compared to reference methods. For example, MHIC values were determined after 24 h for *Candida* species and after 48 h for *Aspergilli* and biofilms.[64,71] However, IMC is recognized as a potential method for high-throughput AFST and testing novel antifungal drugs.[18]

5.4. Fluorescence Microscopy to Analyzes Microcolonies on Porous Aluminum Oxide

Fluorescence microscopy has been applied for AFST of yeast and mold species by monitoring microcolonies on porous aluminum oxide (PAO).[61,80] This ceramic material retains cells on its surface, whereas nutrients and antimicrobials contained in agar can diffuse through the porous structure (pore sizes 20–200 nm; 60 µm thick) when a PAO strip is placed on an agar plate, see **Figure 5**A. Therefore, environmental conditions can be rapidly altered by transferring PAO strips between agar plates of different compositions. Importantly, the PAO substrate enables effective imaging of microcolonies by using microscopy techniques such as fluorescence and scanning electron microscopy (SEM).[81]

The employment of PAO substrates for AFST was first demonstrated for *Candida* species: In this assay, sterile PAO strips are placed on agar plates with a specific antifungal concentration (see Figure 5A representing a PAO strip on a sheep's blood agar plate), and subsequently, *Candida* cells are seeded onto the PAO strip (at a density of 2×10^3 to 2×10^4 CFU mm^{-2}), as presented in Figure 5B and incubated. The microcolony area at varying antifungal concentrations is monitored by fluorescence microscopy following staining with Fun-1/Calcofluor White, as depicted in Figure 5C. These dyes stain nucleic acids (Fun-1) and the fungal cell wall (Calcofluor White), respectively.[82] Analyzing changes in the microcolony area allows for MIC determination after 3.5–7 h for *Candida* species, and the results agree well with standard BMD testing and Etest.[61] In another work, Ingham et al. extended this approach to AFST of *Aspergillus* species enabling

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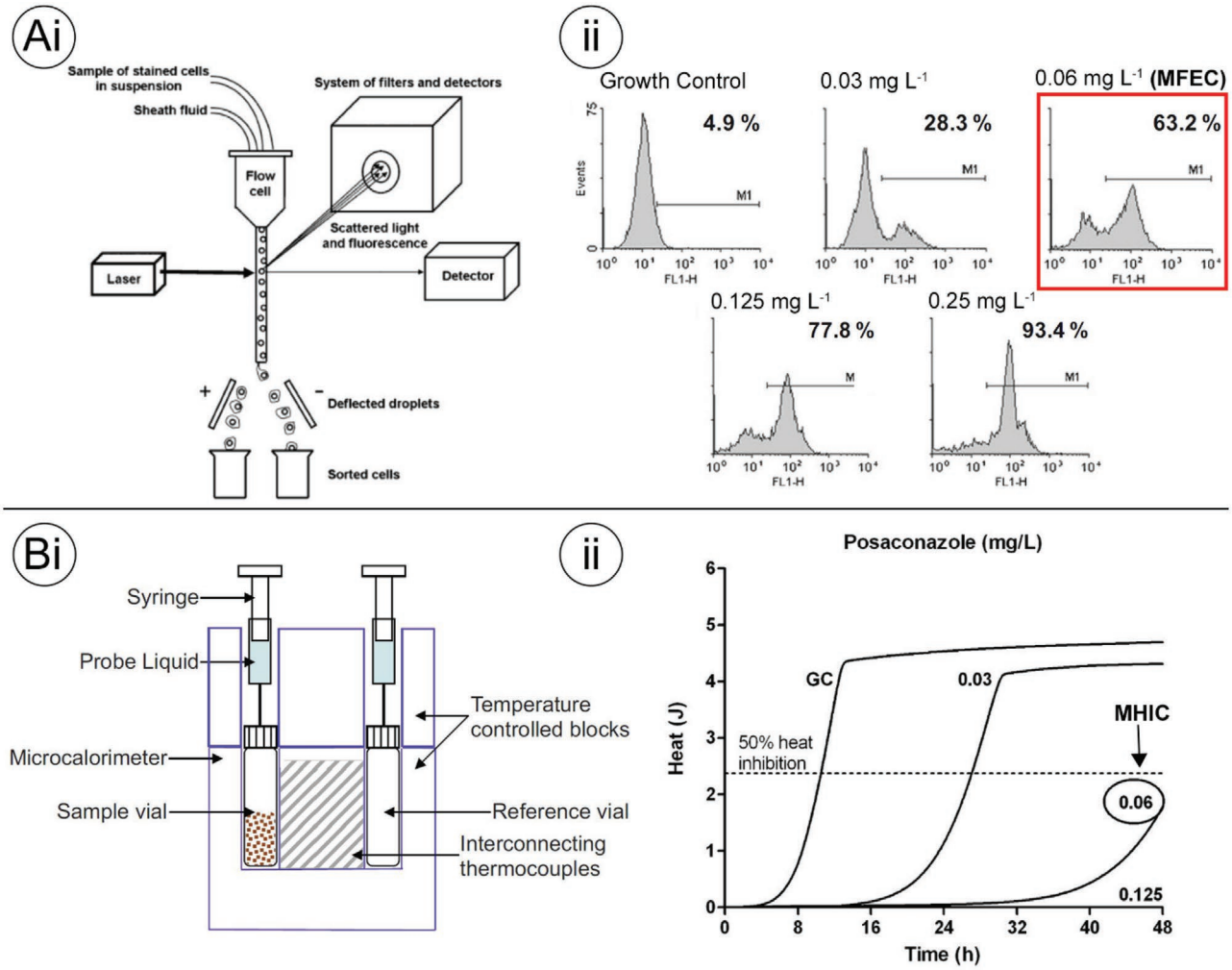

Figure 4. Flow cytometry and calorimetry for phenotypic AFST. A-i) Fungal cells are introduced into a flow cytometer which acquires light scattering and fluorescence emission of individual fungal cells using a system of laser beams, filters, and detectors after exposure to varying antifungal concentrations and staining with a fluorescence dye. A-ii) Flow cytometry histograms depict the number of *C. glabrata* cells versus fluorescence intensity caused by acridine orange upon entering the cells after exposure to different caspofungin concentrations. The MFEC is determined to be 0.06 mg L^{-1}, as indicated by the red frame. At this concentration, the number of cells exhibiting elevated fluorescence correlated to cell damage is 63.2%; this value is above the specific cutoff value of 50%. Adapted with permission.[63,69] Copyright 2006, Blackwell Verlag GmbH (A-i) and Copyright 2011, Springer Nature (A-ii), respectively. B-i) A schematic of an IMC device consisting of a sample vial and a reference vial within a temperature-controlled block. Thermocouples interconnecting both ampoules measure the differential heat flow between sample and reference. B-ii) Characteristic IMC heat flow curves of *Aspergillus fumigatus* (*A. fumigatus*) at varying posaconazole concentrations. The MHIC is determined to be 0.06 mg L^{-1} (indicated by the arrow) as at this concentration, a 50% inhibition of heat flow compared to a growth control without antifungal is visible after 48 h. Adapted with permission.[71,79] Copyright 2009, American Society of Civil Engineers (B-i) and Copyright 2012, European Society of Clinical Infectious Diseases (B-ii), respectively.

MIC determination for echinocandin antifungal agents within 14 h.[80] Although these PAO assays are a rapid AFST approach, and MICs mostly agree with standard AFST methods,[61,80] the workflow is complex as it requires fluorescence labeling, microscopy, and image analysis. Moreover, this method does not directly track changes in the cells' physiology but only relies on obtaining a fluorescence signal of stained cells.

5.5. On-Chip AST on Photonic Silicon Arrays

A recent approach for rapid AST employs optical sensors based on photonic silicon arrays for label-free monitoring of bacterial and fungal behavior during exposure to antimicrobials in real time.[60,83–85] These sensors are based on diffraction gratings, consisting of periodic micropatterned silicon architectures of micrometer-dimensions, which are used as the optical transducer element and preferential surface for microbial colonization as presented in **Figure 6**A. These sensors were first demonstrated to optically track the growth of *Escherichia coli* (*E. coli*) and determine MIC values within 2–3 h (compared to 8 h with state-of-the-art automated methods) by monitoring bacterial growth patterns in the presence of varying concentrations of clinically relevant antibiotics.[83]

These on-chip optical assays were further extended for AFST of *Aspergillus niger* (*A. niger*) by tuning the chip architecture

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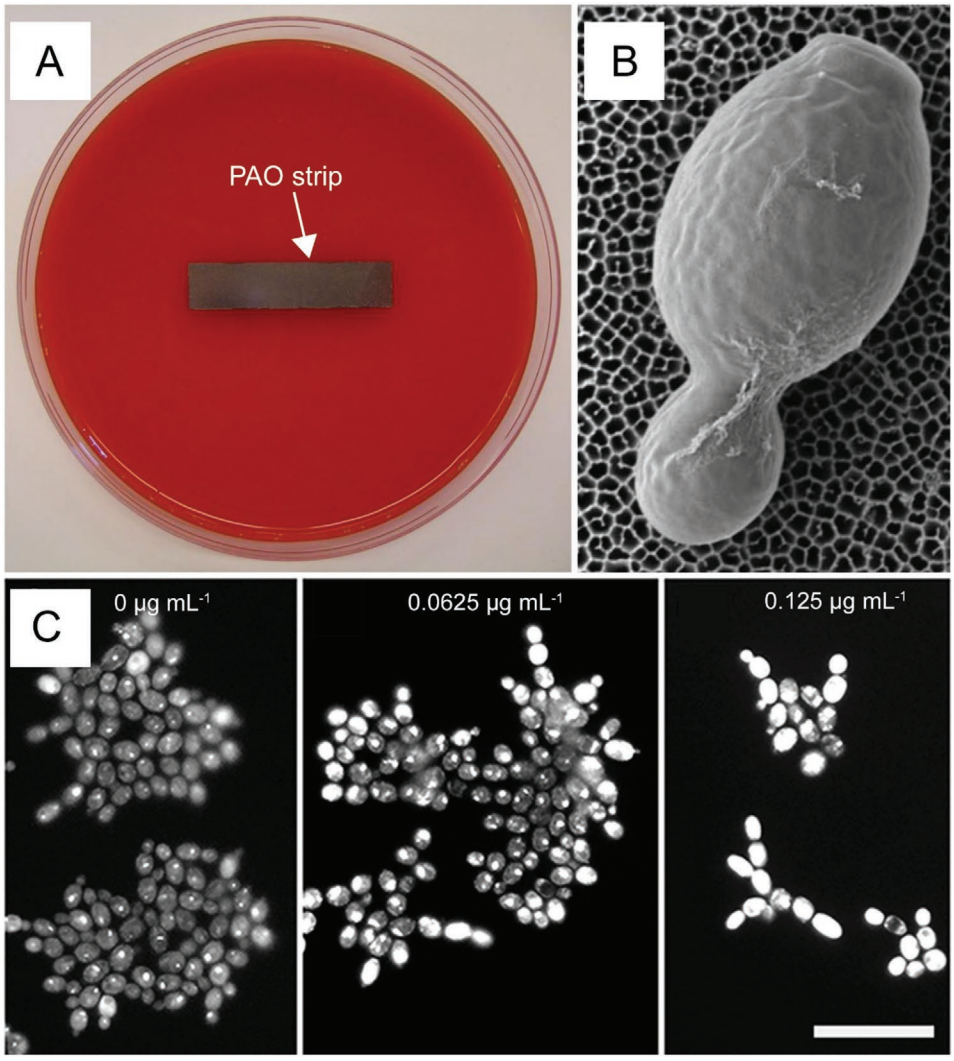

Figure 5. Fluorescence microscopy-based PAO assays for AFST. A) PAO strips of 36 × 8 mm are placed on an agar plate allowing nutrients and antimicrobials to pass through the highly porous structure while B) *Candida* cells seeded on the porous material are retained on its surface. C) Microcolonies of *Candida tropicalis* on PAO are analyzed by fluorescence microscopy after exposure to increasing concentrations of voriconazole and fluorescence staining. The white scale bar indicates 20 µm. Adapted with permission.[81] Copyright 2007, National Academy of Sciences, USA. (A) and adapted with permission under the terms of the Creative Commons CC BY license.[61] Copyright 2012, the Authors. Published by PLoS ONE (B,C).

to arrays of microwells with a width of ≈3 µm and a depth of ≈4 µm, allowing entrapment of individual *A. niger* conidia within the wells (see inset of Figure 6A-iv). The experimental setup is simple and includes a flow cell with temperature-controlled microfluidic channels in which the photonic chips are fixed individually and illuminated by a conventional white light source, as depicted in Figure 6B. The chip reflectance spectrum shows characteristic interference fringes as light is partially reflected from the silicon microstructure's top and bottom interfaces, as illustrated in Figure 6C. Applying frequency analysis to the collected spectrum results in a single peak (Figure 6E), where the peak amplitude corresponds to the intensity of the reflected light, and the peak position corresponds to the value $2nL$ (n is the refractive index of the medium which fills the microstructure and L represent the depth of the microstructure/wells). The AFST assay is performed in two steps: first, fungal conidia are introduced onto the photonic chip and given 15 min to settle within the microstructure. Subsequently, clinically relevant antifungals (e.g., amphotericin B and voriconazole) at varying concentrations are introduced into the channels (see Figure 6D), and fungal growth is monitored by analyzing changes in intensity as a function of time.[60] At drug levels at the MIC and above, growth is inhibited, as illustrated in Figure 6D-i, and no intensity reduction occurs, as depicted in Figure 6E-i. In contrast, at subinhibitory concentrations, the fungal hyphae formation on top of the photonic silicon chip, as shown in Figure 6D-ii, causes an intensity decrease in the reflected light, as illustrated in Figure 6E-ii. Accordingly, the MIC is defined as the lowest antifungal concentration at which the intensity does not change over time; see Figure 6F for characteristic intensity curves over time for fungal growth at varying voriconazole concentrations and corresponding optical micrographs. While this AFST approach is rapid (MIC determination within 12 h) compared to reference methods, it was only demonstrated

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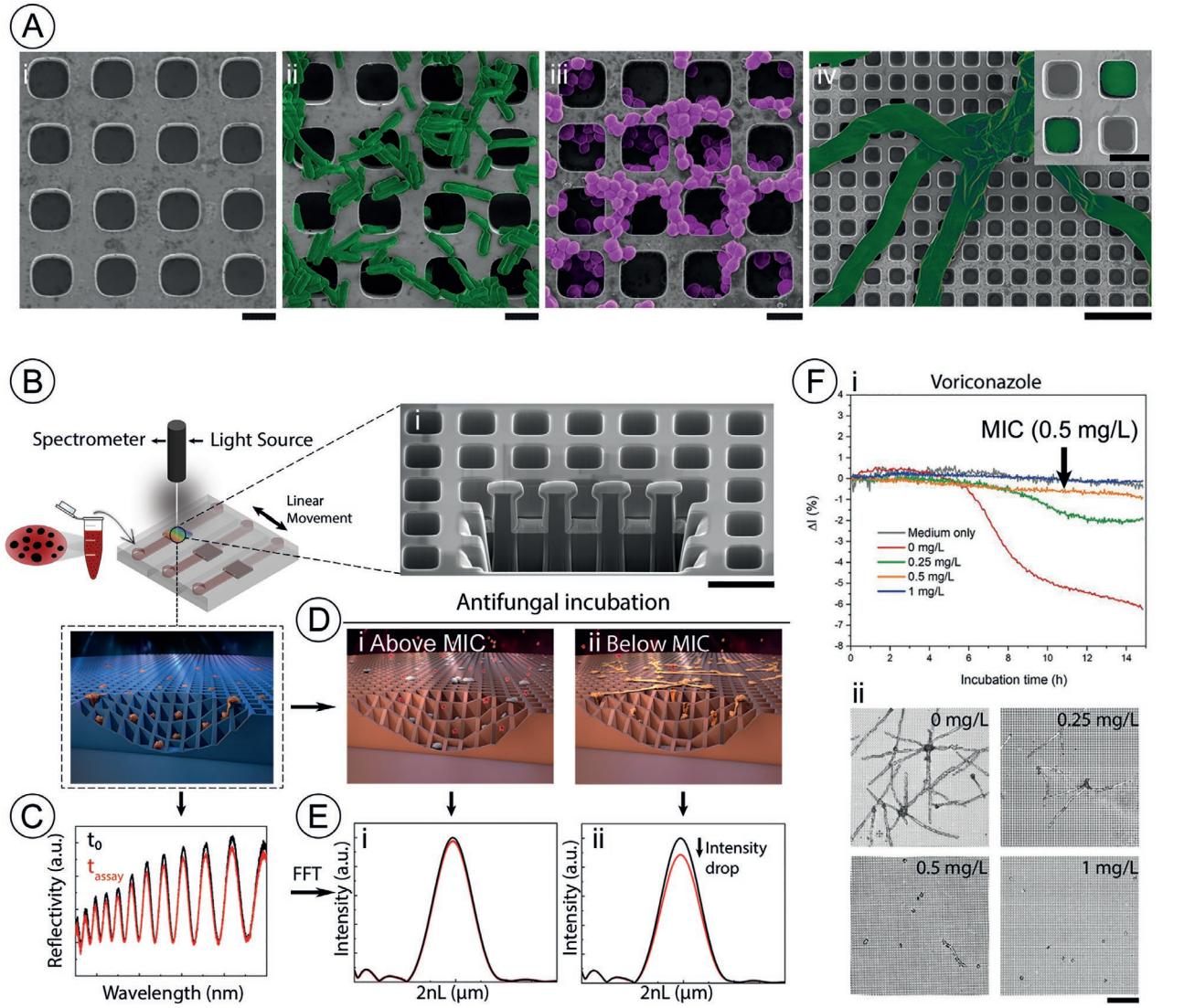

Figure 6. On-chip AFST on photonic silicon arrays. A-i) High-resolution SEM images of a silicon array consisting of microwells. These chips provide a unique surface for microbial colonization, for example of A-ii) *E. coli*, A-iii) *Staphylococcus aureus*, and A-iv) *A. niger*. The scale bars represent 2 µm A-i to Aiii-), 10 µm A-iv), and 3 µm (inset), respectively. B) The photonic silicon arrays entrap *A. niger* conidia while being illuminated by a white light source. B-i) A cross-sectional HR-SEM obtained by focused ion beam procedure depicts the periodic microwell structure with a width of ≈3 µm and a depth of ≈4 µm, creating C) interference fringes in the reflectance spectrum. D) Clinically relevant antifungals are introduced at varying concentrations after allowing the conidia to settle within the microwells resulting in D-i) growth inhibition and E-i) stable intensity values at concentrations above the MIC or D-ii) hyphal growth on top of the silicon array and E-ii) a resulting intensity decrease at subinhibitory antifungal concentrations. F-i) Characteristic intensity curves over time for *A. niger* growth at varying voriconazole concentrations and F-ii) corresponding optical micrographs. The scale bar represents 50 µm. Adapted with permission.[60] Copyright 2020, American Chemical Society.

for *A. niger* as a model species. Furthermore, the inoculum suspension adhered to the high cell number (10^5 conidia mL^{-1}) used in EUCAST reference protocols.[60]

6. Molecular Resistance Detection—Nonculture Methods

As previously discussed in this review, the main advantage of nonculture methods is their rapidity in comparison to most phenotypic techniques, which rely on monitoring growth. The major nonculture approaches are molecular analysis by MS, various genotypic techniques, and transcriptome analysis. In the following sections, we describe the concepts of these methods, focusing only on genotypic and transcriptome analysis. For MS, we refer the reader to Section 5.1. and note that while these MS-enabled AFST assays are nonculture by definition, extended time (up to 15 h) for microbial growth is required.[62,74]

6.1. Genotypic Antimicrobial Resistance Detection

Current phenotypic AFST methods and their preceding workflow (conventional and new) are still quite lengthy (2–6 days

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for diagnosis and identification[14,27] and mostly >10 h for AFST[12,15,46,62,64]); genotypic approaches can expedite resistance analysis to only a couple of hours.[17,33] These methods can identify pathogens and reveal their antimicrobial resistance by detecting the pathogens' DNA, including resistance-conferring genes or mutations by PCR, or employing whole genome sequencing for a comprehensive overview of resistance genes and mutations in a pathogen's genome.[11,38,86]

6.2. PCR-Based Assays for the Detection of Resistance-Conferring Mutations

For antifungal resistance in *Candida* species, most studies focused on detecting single nucleotide polymorphisms (SNP) in the glucan synthase encoding *FKS* genes.[17] Mutations in these genes alter the enzymes' amino acid structure and make it less affected by echinocandin-class antifungals.[1,3,17] Azole resistance is more challenging to detect in *Candida* species owing to the complexity of underlying resistance mechanisms. Yet, for *Aspergilli*, several mutations in the *CYP51A* gene associated with azole resistance have been identified.[18] Thus, classical genotypic approaches are limited to only predetermined genes, which reveal resistivity and cannot unveil susceptibility and determine MIC values.[11,33]

Most genotypic methods rely on PCR-based assays and allow resistance analysis within only a couple of hours.[87–89] For example, Dudiuk et al. have adapted classical PCR assays to detect mutations in the *FKS1* and *FKS2* genes, responsible for echinocandins resistance, in *C. glabrata*. Zhao et al. have combined asymmetrical PCR with molecular beacon probe-based melting curve analysis to detect mutated *FKS1* and *FKS2* genes in clinically collected *C. glabrata* samples within only 3 h. Furthermore, commercially available real-time PCR assays, such as the AsperGenius (PathoNostics, Netherlands) and MycoGENIE (Ademtech, France), were recently developed to detect *Aspergillus* DNA as well as few mutations that confer resistance to azole antifungals within 2.5 h.[90–92] A significant advantage of these genotypic approaches is their high sensitivity which enables species identification and resistance detection directly from body fluids such as sputum, bronchoalveolar lavage (BAL) fluids, or positive blood cultures,[33,93] thereby avoiding the prerequisite of obtaining high cell densities (10^3–10^6 cells mL^{-1}) in most phenotypic AFST methods.[12,15,46,63,64] For example, the AsperGenius assay is validated for testing directly from BAL fluids and has a limit of detection for *Aspergillus fumigatus* species identification and resistance detection of 10 and 75 genomes per sample, respectively.[93] Additionally, commercially available PCR-based assays for *Candida* species identification, such as the T2Candida panel (T2 biosystems, USA) and the SeptiFast assay (Roche Diagnostics, Germany), have recently entered the market.[94,95] Exemplarily, the T2Candida panel permits the identification of five clinically relevant *Candida* pathogens with high specificity (≈99%) and low detection limit (1–3 CFU mL^{-1}) within 3–5 h directly from blood.[94] While, to the best of our knowledge, there is no commercially available PCR-based assay providing resistance detection in *Candida* species from clinical specimens, these assays can still facilitate treatment decisions as they allow identification of fungal pathogens (e.g., *Candida krusei*) that feature intrinsic drug resistance to some antifungal agents.[96] We envision that these tests will be refined, and also the detection of resistance markers will be available in the near future.

6.3. Whole Genome Sequencing

A more holistic approach that does not only unravel resistance-conferring mutations in a single gene but instead analyzes the whole genome of a pathogen is termed whole genome sequencing (WGS).[97] **Figure 7** illustrates a general schematic of such genomic approaches for antimicrobial resistance detection. In such assays, the pathogenic microbes (bacteria or fungi) are isolated from clinical specimens; subsequently, the genomic DNA is cut into short fragments that are amplified and sequenced. Finally, a specialized software is used to assemble the sequencing results enabling detailed analysis and the detection of resistance genes and mutations.[11] WGS principally enables concurrent species identification, strain typing, and comprehensive detection of resistance-conferring genes and mutations.[98] Concerning AMR detection in fungal pathogens, WGS has been successfully applied to confirm numerous SNPs linked to resistance toward azoles, echinocandins, and 5-flucytosine in *C. glabrata*.[99] However, in the routine clinical praxis, the practicability of WGS is currently still limited as it is costly, slow (several days turnaround time), and requires complex and sophisticated software for data analysis and interpretation.[97,98,100,101] Furthermore, a standardized and open-access database with all known resistance genes and mutations for the entirety of pathogenic microorganisms is still lacking, as emphasized by the EUCAST.[98,102]

For a more comprehensive overview of genotypic AMR approaches, we would like to refer the reader to the recent articles of Boolchandani et al.[86] and van Belkum et al.[38] A more specific insight into nucleic acid-based and molecular strategies for resistance detection in fungi is presented by Sanguinetti and Posteraro,[17] and Kidd et al.[103] in their review articles. Rath et *al*. provide an overview of commercially available PCR assays for *Aspergillus* species identification and resistance detection.[90] To summarize, genotypic AMR techniques have a great potential to expedite the treatment of severe fungal infections by directing the physicians on which drugs should be avoided.[36]

6.4. RNA-Based Resistance and Susceptibility Detection—The Transcriptomic Approach

Molecular detection of antimicrobial resistance is not limited to proteome and DNA analyses, and messenger RNA (mRNA) profiling is emerging as a tool for resistance detection in microbial pathogens.[101,104,105] These methods are based on monitoring the expression signature of mRNA transcripts (transcriptome analysis by RNA sequencing) and leverage the fact that susceptible and resistant microorganisms have discernible mRNA profiles upon antibiotic exposure, allowing their classification into resistant or susceptible categories.

As an example, the GoPhAST-R assay[101] allowed unraveling the susceptibility of five different bacterial species for

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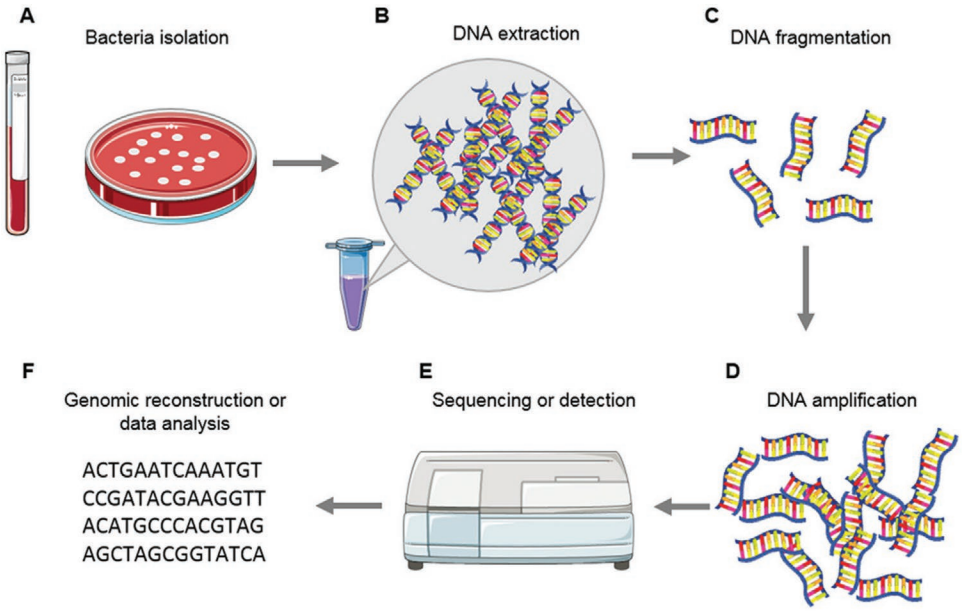

Figure 7. Workflow depicting genomic approaches for antimicrobial resistance detection in pathogenic microorganisms. A) Bacteria or fungi are isolated from clinical specimens. B) Subsequently, the cells are lysed, and the genomic DNA is extracted. C) Then, the DNA is cut into short fragments, and these fragments are D) amplified and E) sequenced. F) Finally, specialized software analyses and interprets the sequencing results and assembles the genome. Reproduced with permission.[11] Copyright 2018, American Chemical Society.

three clinically relevant antibiotics within 4 h, and its feasibility for direct analysis of positive blood cultures was demonstrated. Additionally, the GoPhAST-R assay allows analyzing the sequence of mRNAs to unveil essential resistance genes in the investigated pathogens. Combining this genotypic information with the phenotypic mRNA expression data enabled the classification of bacteria into 'resistant or susceptible' with 94–99% accuracy.[101] These transcriptomic approaches have been only demonstrated for bacterial pathogens, but it should be principally feasible to extend them also to fungal pathogens. Thus, we envision that such assays can become of great importance in the future and provide a novel way to detect antifungal susceptibility and resistance in fungal species.

7. The Potential of Microfluidics for AFST

Microfluidics has revolutionized the field of fungi research by enabling parallelization and high-throughput[106–108] processing of different fungal species, as single cells or populations,[107,109–112] while miniaturizing the experimental setup and reducing the required sample volume.[106] **Table 3** summarizes the main advantages that microfluidics-based techniques offer with respect to applications in fungal pathogen diagnostics.

Microfluidic systems also have the potential to integrate all steps (cell isolation from body fluids, detection and identification, and susceptibility testing) in a typical clinical flow, as schematically illustrated in **Figure 8**, and to simplify the current complex and lengthy procedures. For example, let us consider the case of candidemia, where the first functional microfluidic unit would allow *Candida* cell isolation from blood and fungal cell concentration. Subsequently, the resulting suspension could be directly processed by the 'identification unit'. The latter can include adapted conventional identification schemes (as discussed in Section 2) or more sophisticated devices, such as biosensors. Finally, a phenotypic AFST will be performed, and MIC values will be determined. While such integrated systems do not yet exist, we envision that in the near future, such schemes will be developed and successfully demonstrated for potential clinical applications. The following section describes emerging microfluidic-based techniques for each of the essential units (namely isolation, detection and identification, and phenotypic AFST) required. Specifically, we focus on microfluidic-based techniques for yeast pathogens, such as *Candida* and *Cryptococcus*.

7.1. Step 1: Isolation and Concentration of Fungal Pathogens from Blood

Several microfluidic approaches for isolation and concentration of fungal cells from blood rely on their morphological difference, namely cell size, and shape, from white blood cells (WBCs).[119,120] For example, inertial focusing of lysed blood in a spiral-shaped polydimethylsiloxane (PDMS) device (see **Figure 9**A-i) was used to separate *Candida* cells from WBCs and focus them into designated outlets as shown in Figure 9A-ii.[119] However, only ≈45% of *C. albicans* cells were actually focused into the correct outlet from lysed blood. This effect was ascribed to the higher viscosity of blood compared to a buffer solution used to establish the system.[119] Furthermore, the separation performance was less efficient for other *Candida* species (*C. glabrata*, *C. parapsilosis*, and *C. tropicalis*),[119] underscoring that such inertial focusing devices must be individually adapted for different-sized species.

Another approach is based on viscoelastic separation and concentration of *C. albicans* from WBCs using a hyaluronic acid solution as a viscoelastic fluid.[120] Lysed blood spiked with

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Table 3. Advantages of microfluidics for fungal pathogen diagnostic applications.

Advantage	Explanation	Ref.
Miniaturization	Miniaturization reduces the required sample and reagent volumes, as well as device size benefitting potential point-of-care applications	[106,113,114]
Parallelization and high-throughput	Fabrication of numerous microfluidic channels or the creation of microdroplets allows for parallelization and high-throughput screening	[106–108]
Integration of different unit operations	Integration of different unit operations (e.g., cell isolation and manipulation, species identification) into a single microfluidic device has been successfully demonstrated	[113,115]
Single-cell analysis	Microfluidic systems have been coupled with microscopy and imaging techniques for single-cell detection and analysis, reducing the absolute number of required cells	[107,113,115]
Controlled microenvironment	Microfluidic systems allow precise control over the microenvironment of cells and thus, are predestined to reveal the effect of the cellular environment on the cell's behavior	[117,118]

C. albicans cells and hyaluronic acid (at a concentration of 0.1%) was continuously introduced into the microfluidic device, as illustrated in Figure 9B-i. The larger WBCs (9–15 µm diameter) migrated toward the channel walls, and the smaller *Candida* cells (3 µm diameter) were focused in the microfluidic channel's center (see Figure 9B-ii depicting randomly distributed cells in the inlet and separated cell streams toward the outlets). Approximately 99% of yeast cells and 96% of WBCs were directed into their allocated outlet; furthermore, the microfluidic device allowed a 92-fold cell concentration using two sequential concentration processes.[120]

Even without being integrated into a microfluidic lab-on-a-chip device, as described above, these microfluidic cell isolation and concentration units can improve fungal pathogen diagnostics. Preconcentrating cells can help to reach sufficient cell numbers (gene copies) for successful PCR detection and save time in automated blood culture systems as higher cell numbers shorten the time to growth detection.[119,121] Furthermore, PCR assays' sensitivity can be improved by removing WBCs (before DNA extraction) as their excess DNA can inhibit PCR reactions applied to blood samples.[120,122] Finally, microfluidic processing could serve as an alternative to traditional centrifugation minimizing cell damage due to centrifugal forces.[123,124] Still, the applicability of such devices for processing 'real' clinical samples preceding PCR, blood culture, or AFST analysis should be demonstrated in the future.

7.2. Step 2: Microfluidics for Fungal Pathogen Detection and Identification

One approach for microfluidic detection and identification includes functionalizing microfluidic channels with species-specific capture probes such as antibodies.[125] Asghar et al. have created a microfluidic device with three channels and anti-*C. albicans* antibodies were immobilized onto the channels' bottom via protein G-based surface chemistry, as depicted in **Figure 10**A-i,A-ii, respectively. Green fluorescent protein (GFP)-expressing *C. albicans* at a concentration range of 10–10^5 CFU mL^{-1} were detected within 2 h (compared to days with conventional agar-based methods). This labeled proof-of-concept assay is only applicable for artificially derived GFP expressing yeasts; still, for wild-type strains, this approach could be potentially extended by employing fluorescently labeled antibodies or peptide nucleic acid-based fluorescence in situ hybridization.[125,126]

While only a handful of works demonstrated microfluidic-assisted identification of fungal species, there are numerous biosensing systems for fungal pathogens detection[127–130] which

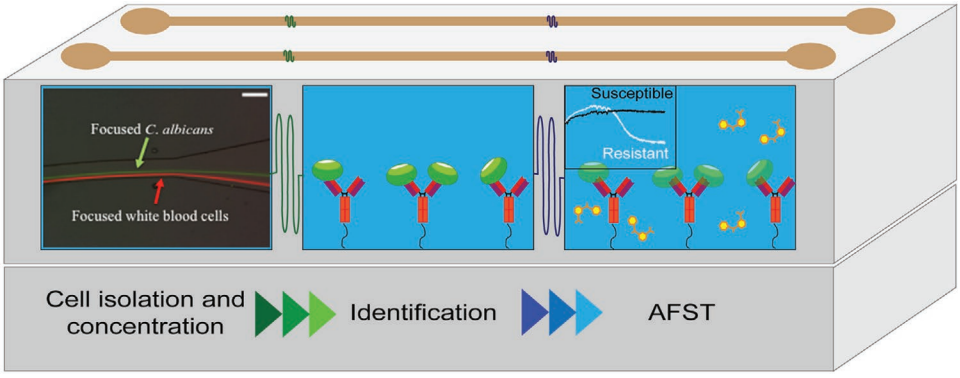

Figure 8. Ideal microfluidic lab-on-a-chip system for integrated cell isolation, detection, and identification, as well as phenotypic AFST of fungal pathogens from the blood. First, the *Candida* cells are separated from WBCs in lysed blood and concentrated before transferred to the "identification unit." This identification step is, for example, achieved by adapted conventional identification schemes or biosensors using species-specific capture probes such as antibodies. The subsequent introduction of antifungals potentially allows a phenotypic AFST and a determination of MIC values. The image depicting *C. albicans* and white blood cells focused within a microfluidic device is adapted with permission under the terms of the Creative Commons CC BY license.[119] Copyright 2019, the Authors. Published by Frontiers Media S.A.

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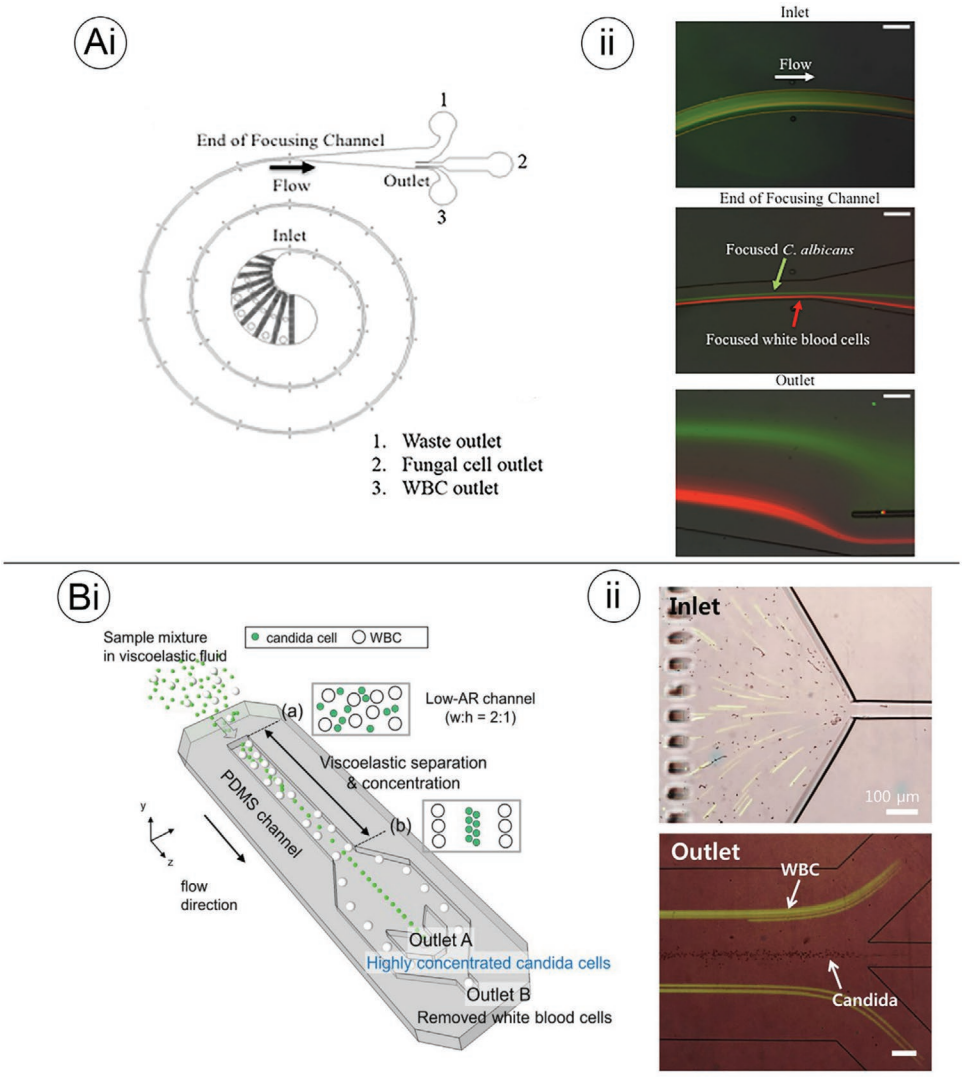

Figure 9. Microfluidic tools for isolation and concentration of *Candida* species. A-i) Schematic of a spiral-shaped microfluidic device for isolation and concentration of *Candida* cells from WBCs by inertial focusing. A-ii) *Candida* cells and WBCs are focused into separate streams as they flow through the device; cells are stained for clarity with fluorescein isothiocyanate (*Candida*) and calcein (WBCs). Adapted with permission under the terms of the Creative Commons CC BY license.[119] Copyright 2019, the Authors. Published by Frontiers Media S.A. B-i) Schematic of a PDMS-based microfluidic channel for size-dependent viscoelastic separation of *C. albicans* and WBCs. B-ii) Comparison of randomly distributed cells at the inlet and segregated *Candida* and WBC streams at the outlet. Reproduced with permission under the terms of the Creative Commons CC BY license.[120] Copyright 2019, the Authors. Published by Springer Nature.

have not yet been coupled with microfluidics. For example, immunosensors such as antibody-functionalized surface plasmon resonance (SPR)[129] and electrochemical impedance spectroscopy (EIS)[130] biosensors were employed for *Candida* cell detection. Thus, we envision that, in the future, the integration of these biosensors with available microfluidic devices[131] can be readily realized to advance the field.

7.3. Step 3: Phenotypic AFST

There are several microfluidic-assisted phenotypic AFST assays reported[60,108,132]; yet, some of these were only used for qualitative antifungals screening with no MIC determination. For example, a highly parallelized microfluidic system was developed for the screening of small molecule compounds that enhance the antifungal effect of amphotericin B against *C. albicans*.[108] In this assay, *C. albicans* cells expressing GFP only when alive were introduced into the microchannels, as shown in Figure 10B.[108] The cells were incubated in a liquid growth medium until the growing yeast cells filled in-line the microchannels and adhered to their surface. Subsequently, mixtures of amphotericin B and various small molecule compounds were introduced for 1 h, and fluorescence microscopy was applied to identify remaining fluorescent persister cells that were still alive.[108] From a library of around 50 000 compounds, 10 small molecules were identified whose addition to amphotericin B solution increased the antifungal effect.

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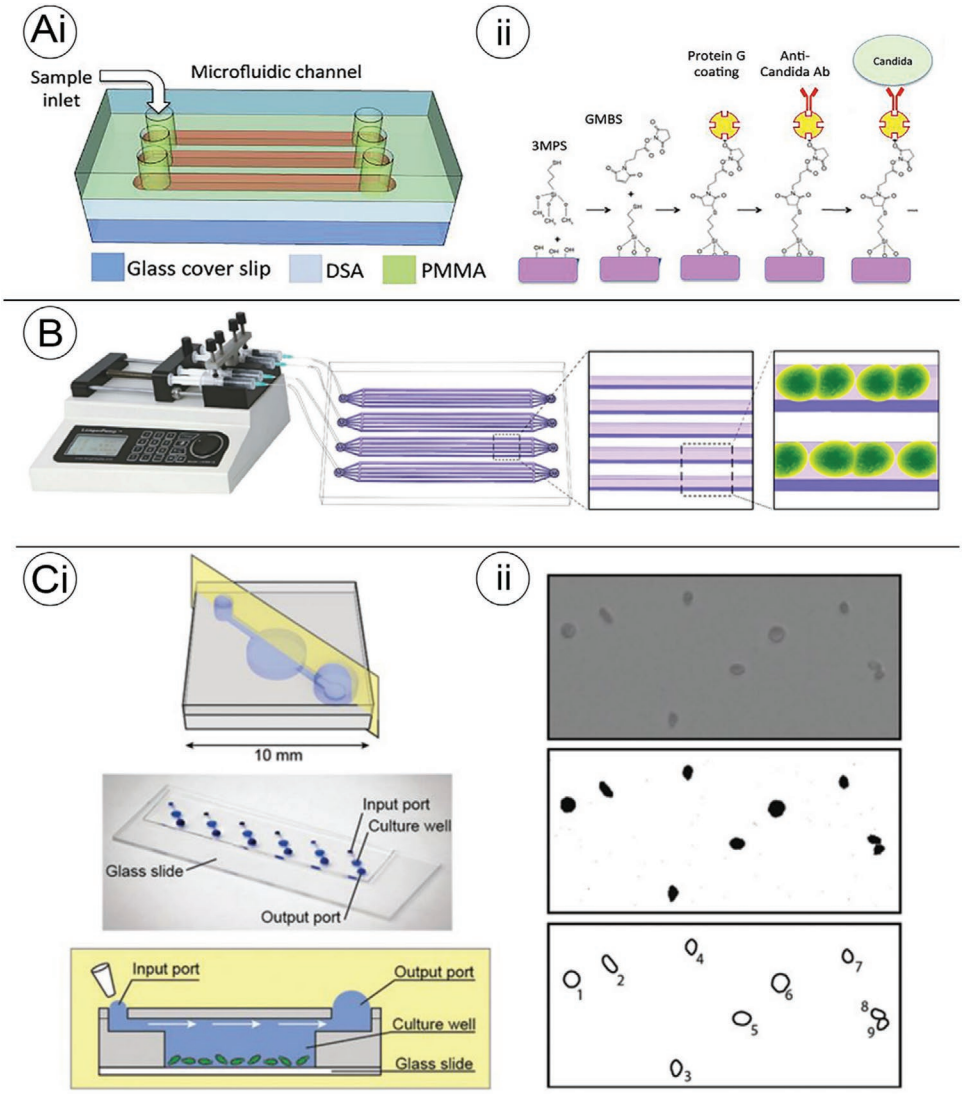

Figure 10. Microfluidic systems for fungi identification and phenotypic antifungal drug screening. A-i) Schematic of the microfluidic channel consisting of polymethyl methacrylate attached to a glass cover by a double-sided adhesive layer. A-ii) Functionalization of the glass slide with anti-*C. albicans* antibodies is achieved by using protein G-based surface chemistry and allows detection of bound *C. albicans* cells by fluorescence microscopy. Adapted with permission.[125] Copyright 2019, American Chemical Society. B) A microfluidic chip consisting of microchannels is used for high-throughput screening of antifungal drugs against *C. albicans*. Adapted with permission under the terms of the Creative Commons CC BY license.[108] Copyright 2019, the Authors. Published by Springer Nature. C-i) Schematic of the cell culture platform for *C. neoformans*. C-ii) Analyzing the germination of *C. neoformans* in the microfluidic device is performed by image processing and analysis. Adapted with permission.[132] Copyright 2016, Oxford University Press.

Another approach combined a PDMS-based microfluidic platform and an image processing unit to study the effects of two clinically relevant antifungals (amphotericin B and fluconazole) on germination and growth of *Cryptococcus neoformans* (*C. neoformans*).[132] Spore or yeast cells were seeded into microfluidic culture wells, exposed to the antifungal-containing growth medium at inhibitory drug concentrations, and incubated; see Figure 10C-i for image and schematic of the microfluidic cell culture device. Subsequently, the cells were imaged by light microscopy, and an image processing algorithm allowed to detect and analyze cells, as shown in Figure 10C-ii. This algorithm classified the cells into either spores or yeast, based on shape, cell area, and aspect ratio; vegetative growth was also assessed by measuring the number of cells in an image frame. Interestingly, both antifungals inhibited the yeast cell's vegetative growth but did not impair the germination process.[132] This method has the potential to be expanded for the identification and assessment of novel antifungal drugs targeting the germination process and could be extended to other species such as *Aspergilli* by adapting the image processing algorithm.

To the best of our knowledge, only the above-described optical on-chip assay in which *A. niger* conidia are introduced onto photonic silicon chips through microfluidic channels (see Section 5.5) demonstrated the employment of a microfluidic platform for AFST, including MIC determination. Other microfluidic AFST approaches, as presented in this section, will still need

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to demonstrate their applicability for a more quantitative AFST, including MIC determination for usage in clinical applications.

While microfluidic approaches specifically for AFST and MIC determination are still rare, microfluidic devices have been more frequently employed for AST of bacteria and have enabled MIC determination or differentiation between "resistant" and "susceptible" in a couple of hours. For example, Churski et al. have described a microdroplet system based on reducing resazurin to resorufin by living cells' metabolisms.[133] Avesar et al. further extended this principle to perform AST of small numbers of bacteria confined within nanoliter arrays allowing to reveal susceptibility within ≈5 h.[134] Baltekin et al. could even assess antibiotic activity by direct single-cell imaging and classify *E. coli* bacteria as susceptible or resistant within 30 min.[116] Although still in its infancy, the successful application of microfluidic systems for AST of bacteria emphasizes the potential use of microfluidics for point-of-care AFST.

8. Conclusion

The most crucial parameter in developing novel AFST methods is the assay time, which allows to promptly initiate a patient-tailored therapy, thus improving antifungal therapy outcomes and preventing the spread of antifungal resistance.[23,135,136] Yet, the development of rapid phenotypic growth-based AFST methods is particularly challenging as, in most cases, fungi are relatively slow-growing species, especially when compared to bacteria.[137] Thus, the main conclusion of this review is that despite the significant progress made over the last decade, the overall assay time for sample collection to AFST result is still too lengthy to provide a meaningful clinical advantage with respect to reference phenotypic techniques which require several days for completion.[12,16] For example, novel phenotypic approaches, such as fluorescence microscopy or flow cytometry (discussed in Section 5) reduce only the AFST assay time to a couple of hours,[61,63] but their preceding lengthy clinical workflow will remain unchanged (requiring typically 2–6 days). Therefore, the added value of nonculture molecular approaches, such as genotypic AMR detection methods which allow for both species identification and detection of resistance-conferring mutations within hours,[33,93] is superior to currently available phenotypic techniques. In practice, these genotypic detection schemes are rarely performed in clinical settings, especially as these methods only reveal resistance in selected cases and not susceptibility, and as such only guide physicians on which drugs should be avoided.[36]

We believe that only holistic solutions for the entire workflow (including cell isolation and concentration, species identification, and AFST) are likely to unravel the "time bottleneck" of fungal phenotypic disease diagnostics. This review reveals that the advancement of microfluidic techniques, in which integration of different functional units for cell isolation, identification, and phenotypic AFST is accomplished, will be a game changer and will also allow for parallelization and high-throughput screening[106–108]. We envision that in the near future, such holistic, integrated microfluidic schemes will be developed and successfully demonstrated for potential clinical applications to simplify and expedite the current complex and lengthy procedures.

The emergence and global spread of fungal pathogens further accelerated by the COVID-19 pandemic[138,139] necessitate a joint effort of health organizations, scientists from different disciplines, and clinicians to combat this neglected forefront of fungal pathogens pathogen diagnosis and monitoring.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

diagnostics, drug resistance, fungal pathogens, microfluidics, rapid antifungal susceptibility testing

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