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Authors

Jenks, Jeffrey

White, P

Kidd, Sarah

et al.

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An update on current and novel molecular diagnostics for the diagnosis of invasive fungal infections

Jeffrey D. Jenks[1,2,*], **P. Lewis White**[3], **Sarah E. Kidd**[4,5], **Tyler Goshia**[6], **Stephanie I. Fraley**[6], **Martin Hoenigl**[7,8,ǂ], **George R. Thompson III**[9,10,11,ǂ]

[1]Durham County Department of Public Health, Durham, North Carolina, USA

[2]Division of Infectious Diseases, Department of Medicine, Duke University, Durham, North Carolina, USA

[3]Public Health Wales Microbiology Cardiff, UHW, Cardiff, United Kingdom and Centre for trials research/Division of Infection/Immunity, Cardiff University, Cardiff, UK

[4]National Mycology Reference Centre, SA Pathology, Adelaide, South Australia, Australia

[5]School of Biological Sciences, Faculty of Sciences, University of Adelaide, Adelaide, South Australia, Australia

[6]Department of Bioengineering, University of California, San Diego, La Jolla, California, United States of America

[7]Division of Infectious Diseases, Medical University of Graz, Graz, Austria

[8]BioTechMed, Graz, Austria

[9]University of California Davis Center for Valley Fever, Sacramento, California, United States of America

[10]Department of Internal Medicine, Division of Infectious Diseases, University of California Davis Medical Center, Sacramento, California, United States of America

[11]Department of Medical Microbiology and Immunology, University of California Davis, Davis, California, United States of America

Abstract

Background: Invasive fungal infections cause millions of infections annually, but diagnosis remains challenging. There is an increased need for low-cost, easy to use, highly sensitive and specific molecular assays that can differentiate between colonized and pathogenic organisms from different clinical specimens.

[*]**Corresponding Author: Jeffrey Jenks**, Division of Infectious Diseases, Department of Medicine, 315 Trent Drive, Hanes House, Durham, North Carolina, 27710, USA, jeffrey.jenks@duke.edu.

[ǂ]These authors are joint senior authors on this work

Author Contributions

JDJ, MH, and GRT conceived and designed the study. JDJ, PLW, SEK, TG, SF, MH, and GRT wrote the initial draft. JDJ, PLW, SEK, TG, SF, MH, and GRT provided critical comments. All authors read and approved the final manuscript.

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Areas covered: We reviewed the literature evaluating the current state of molecular diagnostics for invasive fungal infections, focusing on current and novel molecular tests such as polymerase chain reaction (PCR), digital PCR, high-resolution melt (HRM), and metagenomics/next generation sequencing (mNGS).

Expert Opinion: PCR is highly sensitive and specific, although performance can be impacted by prior/concurrent antifungal use. PCR assays can identify mutations associated with antifungal resistance, non-*Aspergillus* mold infections, and infections from endemic fungi. HRM is a rapid and highly-sensitive diagnostic modality that can identify a wide range of fungal pathogens, including down to the species level, but multiplex assays are limited and HRM is currently unavailable in most healthcare settings, although universal HRM is working to overcome this limitation. mNGS offers a promising approach for rapid and hypothesis-free diagnosis of a wide range of fungal pathogens, although some drawbacks include limited access, variable performance across platforms, the expertise and costs associated with this method, and long turnaround times in real-world settings.

Keywords

Molecular diagnostics; polymerase chain reaction; PCR; digital PCR; metagenomics; next generation sequencing; high resolution melt; invasive fungal disease

1. Introduction

Invasive fungal infections (IFDs) cause millions of infections and account for an estimated 1.6 million deaths annually, twice the number of annual deaths caused by HIV/AIDS [1,2] and similar to deaths from tuberculosis [3]. In addition, the population at risk from IFDs is increasing for a number of reasons, including an increasing number of hematopoietic stem cell transplants (HSCT) [4–8], solid organ transplants (SOT) [9], and individuals receiving systemic immunosuppressive drugs and immunomodulatory therapies such as glucocorticoids [10], TNF-α blockers and small molecule inhibitors such as ibrutinib [11,12]. In addition, infections such as invasive aspergillosis (IA) are becoming increasingly recognized in non-immunosuppressed individuals [13], such as in those living with solid organ malignancy [11,14], in the intensive care unit (ICU) [11,15,16], with severe influenza infection (influenza associated pulmonary aspergillosis (IAPA)) [17–19], and coronavirus disease 2019 (COVID-19)-associated pulmonary aspergillosis (CAPA) [20,21], with the latter being at risk from other fungal infections (e.g. COVID-19-associated mucormycosis (CAM)) [22,23].

Early diagnosis and prompt initiation of appropriate antifungal therapy is an important predictor of survival in individuals with IFD [24], yet diagnosis and subsequent targeted treatment remains challenging, particularly during early stages of infection [15,25]. Culture-based approaches suffer from low sensitivity [26–28] and long turnaround times [29]. Conventional fungal biomarkers, such as galactomannan (GM) and 1,3-β-D-glucan (BDG) are increasingly used but are limited by accessibility, variable turnaround times, and decreased sensitivity, particularly for individuals on mold-active prophylaxis or treatment [30–32]. For the diagnosis of *Pneumocystis* pneumonia (PcP), BDG has higher sensitivity and specificity in persons living with human immunodeficiency virus (HIV) compared

to those without HIV [33]. Combining biomarkers, such as GM and BDG, has been shown to increase the sensitivity and specificity, such as for the diagnosis of invasive aspergillosis [34] The sensitivity of other diagnostic tests, such as the *Aspergillus*-specific Lateral Flow Device (LFD) (OLM Diagnostics, Newcastle upon Tyne, United Kingdom) and the *Aspergillus* Galactomannan Lateral Flow Assay (LFA) (IMMY, Norman, Oklahoma, United States) are also lower for individuals on mold-active treatment and those without neutropenia [30,35,36]. Lastly, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) is a rapid and cost-effective method for the diagnosis of IFD, with pooled sensitivity and specificity of 91% and 95%, respectively, compared to culture [37]. One major drawback, though, is that detection is made from culture on solid media, so like culture these assays suffer from low sensitivity overall and long turnaround times. Non-culture tests are also lacking to diagnose non-*Aspergillus* mold infections, such as those caused by Mucorales, *Fusarium* spp., *Lomentospora prolificans*, and *Scedosporium* spp. [26,38].

There is an increased need for molecular assays that have both high sensitivity and specificity, are able to detect co-infections, can differentiate between colonization and invasive infection, and are able to be used on different specimens such as body fluids, tissue, and blood, while being low cost, easy to maintain and update. Here we review the current state of molecular diagnostic tests for IFDs, focusing on novel molecular tests such as PCR/digital PCR (dPCR), metagenomics next generation sequencing (mNGS), and high-resolution melt (HRM)/universal HRM.

Lastly, over the past decade there have been numerous changes to fungal taxonomy attributed to the use of molecular technologies to resolve evolutionary relationships between fungi, as well as changes to the rules governing fungal naming conventions. As a result, many clinically important species have undergone name changes, which were recently reviewed with guidance on managing name changes in the clinical setting [39]. Some laboratory identification system databases are now utilizing updated names, and as a result laboratories have progressed to reporting new names alongside the previous name for clarity. In support of this progress, updated species names will be used herein utilizing both the new and old names.

2.0 Preparation of Clinical Specimens

DNA extraction is critical to the success of polymerase chain reaction (PCR)-based detection of fungal pathogens [40]. Extensive evaluation by the international Fungal PCR Initiative (FPCRI) has standardized DNA extraction from whole blood, serum and plasma [40–42]. Ensuring adequate volume of sample (≥3 mL whole blood; ≥0.5 mL serum/plasma) and a small volume elution (<100 μL) are consistent requirements irrespective of sample type, while removal of human DNA (potentially a PCR inhibitor) and mechanical lysis of fungal cells to release DNA are required to improve whole blood extraction efficiency. Whole blood processing remains labor-intensive and a range of automated commercial kits for the extraction of *Aspergillus* spp. DNA from serum and plasma provide sufficient analytical performance [41,42]. The methodology to isolate DNA efficiently from bronchoalveolar lavage fluid (BALF) is already optimized. For detection of *Pneumocystis*

jirovecii, nucleic acids extraction processes yielding whole nucleic acid (WNA) are preferred as the detection of WNA has been demonstrated to be superior to detection of DNA alone [43]. Given the size of the human genome, it is important to remove human DNA prior to performing mNGS or risk host DNA overwhelming the DNA components of the fungal pathogens, consuming unnecessary sequencing space and reducing the sensitivity of the assay [44].

For the molecular detection of many fungal pathogens the optimal specimen type remains to be determined. Obviously for respiratory pathogens, targeting BALF appears sensible, and the use of less invasive respiratory samples has, apart from *Pneumocystis* pneumonia, received less clinical validation. When investigating sepsis caused by a range of fungi, various blood fractions have been used to varying degrees of success [45]. When the organism causes fungemia, then targeting the fungal cell as the source of DNA appears rational, although the testing of serum/plasma (targeting free DNA (DNAemia)) and using generic nucleic acid extraction platforms has been successful to aid the diagnosis of invasive candidiasis, invasive aspergillosis, and mucormycosis [41,46,47].

Detection and/or identification of fungal pathogens in fresh or formalin-fixed paraffin embedded (FFPE) tissue may be useful for the identification of fungi visualized on histological investigation but where culture is negative or has not been performed [48,49]. The fungal DNA yield from FFPE is dependent on collection of tissue containing a significant fungal load. In FFPE tissue, DNA yield is often poor due to formalin cross-linking and DNA degradation. Optimization of DNA extractions from tissue is underway and while there is currently no standard for DNA extraction from these specimens, commercially available tissue extraction kits offer some utility and standardization.

3.0 PCR

Candida

Standardization of molecular methods for invasive candidiasis (IC) is advancing through the efforts of the FPCRI. Optimal sample choice (serum, plasma, or whole blood) is yet to be confirmed, complicated by the transient presence of candidemia associated with the various forms of IC, which define the likely sources of available *Candida* DNA (DNAemia vs intracellular) [47].

The T2 Candida Panel (T2 Biosystems, Lexington, MA, United States) represents the pinnacle of commercial molecular tests for IC, with meta-analytical review of performance generating excellent performance with a sensitivity of 91% and specificity of 94%, although sensitivity can be limited by the prior or concurrent use of antifungal therapy or absence of candidemia in certain forms of invasive candidiasis [50–52]. In addition, like most *Candida* PCR assays the T2 Candida Panel can only detect a handful of *Candida* spp. so would miss infection caused by a less commonly encountered Candida species. The T2 Candida panel is now included as a diagnostic option for IC under the current European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC/MSGERC) guidelines [48]. The meta-analytical performance of other *Candida* PCR assays for the detection of candidemia is also excellent with a sensitivity

of 95% and specificity of 92%, but in the absence of candidemia sensitivity can be compromised, although may be reflect suboptimal extraction procedures targeting DNAemia [47,53,54]. Other commercial *Candida* PCR assays are becoming available and have been extensively reviewed [55].

The optimal combination of fungal biomarkers for the diagnosis of IC is yet to be determined, but a recent evaluation of the *Cand*ID (OLM Diagnostics, Newcastle-upon-Tyne, United Kingdom) described a clinical algorithm incorporating serum PCR and (1-3)-β-D-glucan testing, where IC could be both confidently confirmed (post-test probability >80%) or excluded (post-test probability <1) dependent on the combination of test results [56]. Hopefully, the A-STOP trial (ISRCTN43895480) will provide insight for combining biomarkers to achieve an optimal IC diagnosis.

The performance of *Candida* PCR in pediatric patients was unclear until the BIOPIC trial (NCT02220790) investigated biomarker performance, including *Candida* PCR, in pediatric patients. In this study, the sensitivity and specificity of the T2 *Candida* was 80% and 97%, respectively, and combining the T2 with mannan antigen ELISA increased sensitivity to 86% while maintaining a specificity of 95% [47,57]. The use of molecular tests to identify potential resistant species (*C. auris*, *Nakaseomyces glabratus* (previously *C. glabrata*) or *Pichia kudriavzevii* (previously *C. krusei*)) with potential resistance to antifungal therapy is feasible using species-specific assays or assays that differentiate/identify the species of interest. Molecular tests to identify mutations in the *FKS1* and *FKS2* genes potentially associated with echinocandin resistance may be useful, but assays to detect mutations associated with azole resistance may not be feasible due to the range of potential mechanisms (e.g. efflux pumps, increased target site expression, etc) and next generation sequencing may provide a solution [58].

Pneumocystis

The detection of *Pneumocystis* DNA using quantitative PCR (qPCR) is very sensitive (>95%) for the diagnosis of PcP [45]. While this high sensitivity has the potential to cause false positive results, this has not been demonstrated in a meta-analysis of PcP PCR - where specificity remains around 90% and increased through the detection of higher fungal burdens [59–62]. PcP PCR on non-invasive samples (serum, induced sputum, nasopharyngeal aspirate and oral washes) has demonstrated acceptable sensitivity (77% for serum and oral washes to 99% for induced sputum) while maintaining excellent specificity (≥90%) across all sample types [62].

When PcP PCR positivity is indicative of lower fungal burdens, combination with other mycological tests such as serum (1-3)-β-D-glucan (BDG)) and clinical presentations/context remains paramount. PcP PCR has been included into consensus guidelines for defining probable PcP in patients with host factors, typical radiologic findings, and PCR positivity [48]. Currently, these definitions do not discuss the influence of fungal burden on the certainty of classification, and arguably patients with a low burden of PcP require additional mycological evidence, such as BDG, before classification. The inability to provide a threshold reflected the technical diversity across the many “in-house” assays, which also prevents PCR becoming the reference method for PcP diagnosis.

The FPCRI are attempting to standardize PcP PCR methodology, which is complemented by the availability of commercial PCR assays. A multi-center evaluation of in-house and commercial assays for detection of *Pneumocystis* from BAL fluid found that reverse transcriptase qPCR (RT-qPCR) assays targeting WNA were significantly superior to qPCR assays targeting DNA only ($p<0.001$), regardless of fungal load. In addition, targeting the mitochondrial small subunit (mtSSU) provided the lowest Cq values and superior analytical performance [43]. While antifungal resistance is currently uncommon in PcP, the molecular detection of mutations in dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) associated with resistance to sulfamethoxazole and trimethoprim have been identified but association with treatment failure is not always evident [45].

Aspergillus

Over the past decade *Aspergillus* PCR has undergone considerable standardization focused on the rate-limiting extraction protocol [45,63]. qPCR targeting the multi-copy ribosomal DNA region (e.g. 18S, Internal transcribed spacers, 28S), duplicate testing of DNA eluates, and inclusion of an internal control to monitor for inhibition improve performance, which is generally superior for the detection of *A. fumigatus* over other *Aspergillus* spp. [63,64]. In addition, high quantitative levels of *Aspergillus* DNA have been shown to be associated with high mortality rates at 90 days compared to those with lower *Aspergillus* levels (cutoff <150 copies/mL). Furthermore, a negative PCR level has been shown to be associated with higher survival rates compared to those with detectable *Aspergillus* PCR levels after 2 weeks of antifungal treatment [65].

Clinical performance varies across “in-house” and commercial assays (Table 1). A recent Cochrane systematic review and meta-analysis of *Aspergillus* PCR blood testing generated a sensitivity/specificity of 79% and 80%, respectively, comparable with galactomannan enzyme immunoassay (GM-EIA testing [66]. Comparative analytical testing as well as a clinical study have demonstrated plasma to be superior to serum for detection of *Aspergillus* DNA [41,67]. When testing blood, the presence of two consecutive positives increased specificity to 95%.

Meta-analyses of *Aspergillus* PCR on BALF provided sensitivities and specificities, ranging from 76.8–79.65% and 93.7–94.5%, respectively, with the high specificities providing confidence for confirming IA [68,69]. The high specificities corroborate the incorporation of *Aspergillus* PCR as a mycological criterion in the current EORTC/MSGERC guidelines for probable IA [48]. In blood, two or more consecutive positive *Aspergillus* PCR samples are required, in BALF two or more duplicate *Aspergillus* PCR positive results from BAL fluid, or one positive from each of whole blood/plasma/serum and BAL fluid may be considered as mycological evidence for aspergillosis [48]. While combined GM-EIA and PCR positivity is not considered in the current EORTC/MSGERC definitions, combining their use when testing of both BALF and blood appears optimal for both confirming and excluding IA [45]. The incorporation of *Aspergillus* PCR into the consensus definitions has increased the possibility for defining probable IA, although ambiguity regarding this classification for BALF testing has been identified, with the detection of higher fungal burdens potentially more indicative of disease [70,71].

Performance in the setting of hematologic malignancy has been well studied, but less so in other populations (e.g., critical care or chronic respiratory disease), but testing samples from the focus of infection may benefit performance [45]. In pediatric populations cancer or have undergone allogeneic hematopoietic stem cell transplant, PCR currently has a grade BII recommendation for the detection of aspergillosis from serum and a grade AII recommendation from tissue and BAL per the 8th European Conference on Infections in Leukaemia (ECIL-8) [72].

Commercial *Aspergillus* assays can also detect the most prevalent *cyp51A* mutations (TR34/L98H and TR46/Y121F/T289A) conferring resistance to triazoles (Table 1). Being a single copy gene, these assays typically have reduced sensitivity compared to the detection of the organism itself. The range of mutations identified as causal for azole resistance continues to increase, beyond the *cyp51A* gene, and methods with a broader range of detection, such as next generation sequencing (NGS) or pyrosequencing, but capacity for direct sample testing are required [73]. In a prospective, multicenter clinical evaluation, the Aspergenius® (Patho*N*ostics, Maastricht, Netherlands) identified mutations associated with azole resistance in *A. fumigatus* in 14% of patients with probable IA, with 75% having azole resistance confirmed through susceptibility testing of a culture isolate; patients with a GMI in BAL fluid ≥1.0 were more likely to generate conclusive PCR results [70].

Mucorales—Increased use of molecular techniques for detection of Mucorales DNA has improved in the diagnosis of mucormycoses [74], where culture has poor sensitivity and there are no serological tests. Several conventional and qPCR assays have been described for detection of Mucorales DNA from fresh or FFPE tissue, BAL fluid, and serum, largely targeting ribosomal DNA [74–79]. In addition, quantifying the Mucorales DNA level in serum has been shown to have prognostic potential, as patients with decreasing and negative DNA levels by qPCR had improved survival compared to patients whose qPCR level remained positive [80]. However, as with other fungi, efficient DNA extraction is critical to the success of the PCR. The FPCRI is attempting to standardize Mucorales PCR and the performance of Mucorales PCR was reproducible across assays when testing contrived samples [81].

The recently published MODIMUCOR trial investigated Mucorales qPCR for the diagnosis and follow-up of patients with suspected IFI [46]. The qPCR detected the most common genera (*Mucor*/*Rhizopus* spp., *Lichtheimia* spp. and *Rhizomucor* spp.) when prospectively testing serum samples from 232 patients (11.6% with proven/probably infection). Sensitivity was 85.2% and specificity was 89.8%. The first positive result was obtained a median of four days earlier than the collection of culture or histopathology positive samples and a median of one day before imaging was performed. qPCR typically became negative a median of four days following initiation of amphotericin B, and survival was significantly higher in patients where qPCR became negative within seven days of commencing therapy compared to those remaining positive. The authors recommended inclusion of qPCR as a mycological criterion for mucormycosis in future EORTC/MSGERC definitions [46].

The recent development of commercial qPCR assays should assist Mucorales PCR standardization and enable direct detection of pan-Mucorales DNA [82–85]. Clinical

evaluation of these commercial assays remains limited. Scherer and colleagues used oligonucleotides specific for *Mucor* spp., *Rhizopus* spp., *Rhizomucor* spp., and *Lichthiemia* spp. to detect DNA in 450 BAL fluid samples from 374 patients, 10/374 having proven/probable mucormycosis, 4/374 with probable aspergillosis and 8/374 with possible IFI. Sensitivity for Mucorales and *Aspergillus* PCR was 100% and 75%, respectively, and positive Mucorales PCR results were also obtained from serum in 90% of patients. Culture was only positive for 2/10 patients, providing support for including the PCR testing of BAL fluid and serum in the mucormycosis diagnostic pathway, subject to prospective clinical validation. It further suggests that *Aspergillus*-Mucorales co-infections may be relatively common [86].

The MycoGenie® Real-Time PCR kits (Adamtech, Pessac, France) can detect both *Aspergillus* spp. and Mucorales spp. Upon prospective testing in sera from 744 patients (35 with aspergillosis, 16 with mucormycosis, and four with *Aspergillus*-Mucorales co-infection), the MycoGenie® assay detected Mucorales DNA in the sera of 16/20 mucormycosis patients, with 100% sensitivity in sera from the 14 patients with disseminated mucormycosis. Both *Aspergillus* spp. and Mucorales DNA were detected in sera from all four patients with co-infection [84]. Interestingly, the above studies both noted *Rhizomucor pusillus* as the most frequently detected Mucorales fungus, which is uncommonly isolated in culture, suggesting that this species may account for more infections than previously thought.

A potentially useful target for Mucorales PCR may be the multi-copy *CotH* gene family, which encodes spore coating proteins required for tissue invasion, are unique to Mucorales fungi and have broadly species-specific target sequence [87]. Using a mouse infection model, a *CotH*-specific PCR assay detected DNA from a variety of Murorales species in plasma, urine, and BAL fluid with 100% specificity, and with 90% sensitivity from urine. Testing urine samples from four patients with proven mucormycosis was also successful, warranting validation on larger numbers of human samples [87].

Rare fungi—Individual assays specific to rare yeasts are limited by the relatively low incidence of non-*Candida*, non-*Cryptococcus* invasive yeast infections. Incorporating the detection, but more so the differentiation of these rare yeasts into multiplexed or pan-fungal assays will likely provide an optimal diagnostic strategy, but currently clinical validation is limited. The molecular detection of rare yeast species requires substantial advancement [47].

In a multicenter, retrospective study from France, 81 sera samples from 15 patients diagnosed with proven invasive fusariosis was tested with a pan-*Fusarium* qPCR assay. DNA was detected in 14/15 patients (sensitivity 93%), with detection made a median of 6 days prior to diagnosis by positive blood culture or biopsy. By comparison, serum GM was positive in 7.1% of patients and BDG positive in 58.3% of patients. qPCR was negative in patients with other IFD's and in patients without IFD [88]. Endemic mycoses

The diagnosis of endemic mycoses (e.g., *Coccidioides* spp., *Histoplasma* spp., and *Blastomyces* spp.) is dependent on microscopy and culture coupled with serological or antigen testing, and the development of PCR-based assays thus been slow [92–96]. PCR

assays for detection of *Histoplasma* spp. have utilized a range of targets with varied performance (sensitivity of 67-100% and specificity of 96-100%) compared to culture and lacking clinical validation [92]. A whole nucleic acid qPCR targeting the *mtSSU*, provided high sensitivity (97.7% in microscopy/culture proven *H. capsulatum* var. *capsulatum* or *H. capsulatum* var. *duboisii* cases). This PCR was positive in 43.3% of blood samples from proven cases, with blood positivity associated with progressively disseminated histoplasmosis [93].

A recently developed duplex qPCR based targeted the proline-rich antigen 2 (*PRA2*) gene for detection and differentiation of *C. immitis* and *C. posadasii* in clinical specimens and cultures but has yet to be fully validated in a clinical study [97]. Other assessments of PCR have shown comparable sensitivity to that of culture [98]. A small number of in-house assays have been reported for detection and/or identification of *Blastomyces* spp. from culture, clinical specimens, or soil [94,99,100]. More recently, a duplex PCR for differentiation of *Blastomyces dermatitidis* and *B. gilchristii* was developed, targeting the *BAD1* (*Blastomyces* adhesin) gene [96]. Retrospective testing on 33 clinical specimens (FFPE, bronchial washings, lung tissue, skin tissue, cerebral spinal fluid (CSF), bone marrow, blood, brain tissue, and sputum) from patients with confirmed blastomycosis detected *B. dermatitidis* in 5/33 [96].

In the absence of a pathogen-specific PCR assay, pan-fungal PCR may assist in identifying unknown fungal infections by use of universal fungal primers that amplify multicopy fungal DNA targets (typically ITS1/ITS2 rDNA) from clinical specimens. Sequenced amplified product may then be used to identify the fungus. A retrospective review of pan-fungal PCR tests performed on 138 samples (including tissue, CSF, BAL fluid) from 108 patients at one medical center found that a fungal product was identified in 41/138 (30%), but that only 19 (46%) of these were potential pathogens [89]. Pan-fungal PCR was positive in only 11/94 (12%) of samples with negative microscopy/histopathology. Performance of pan-fungal PCR is best in normally sterile specimens than respiratory tract specimens due to the presence of fungal colonisers/contaminants in the latter [89–91]. An audit of pan-fungal PCR requests in BAL fluid over a five-year period found that only 8.5% of tests yielded a clinically significant results, and in only 1.2% was pan-fungal PCR the only diagnostic test that led to the diagnosis of IFI [91]. The optimal use of pan-fungal PCR with sequencing is for identifying fungi in normally sterile specimens with fungal elements observed on positive microscopy. An alternative to sequencing for fungal identification is high resolution melt curve analysis, which has the advantage of being able to differentiate multiple species, as well as being faster and less expensive (discussed below).

4.0 digital PCR (dPCR)

Droplet digital PCR partitions the PCR process into thousands of individual PCR reactions, where post amplification end-point fluorescence is used to differentiate positive and negative partitions and estimate the DNA concentration in the sample obviating the need for a standard curve. This highly reproducible process can be multiplexed to detect individual pathogens and to screen for mutations potentially associated with antifungal resistance, has the potential for enhanced detection of low DNA concentrations, particularly relevant

to fungal infection and has increased tolerance to inhibition compared to qPCR [101]. Currently, digital PCR for the detection of fungal DNA is limited to *Candida* spp., *Aspergillus* spp., and *Pneumocystis jirovecii* and the presence of mutations potentially associated resistance to antifungal treatment (Table 2). However, clinical validations are limited by small sample size, uncertain case classification and lack of a control population. Extensive clinical validation of these tests is required.

5.0 Metagenomics Next Generation Sequencing

Metagenomic next-generation sequencing (mNGS) to detect microbial cell-free DNA (mcfDNA) in plasma might be a promising approach to diagnosing IFI's, potentially allowing for earlier detection and diagnosis of fungal infections when other biomarkers tests from blood are still negative, and hypothesis-driven targeted testing is not yet feasible [102,103]. An example of this is the diagnosis of *Fusarium solani* by mNGS in the ongoing outbreak associated with epidural anesthesia in Matamoros Mexico.

While multiple platforms exist (Table 3), most data to date has been produced for the Karius® Inc. (Redwood City, California, United States) assay, which is commercially available in the United States and can identify and quantitate plasma mcfDNA of approximately 1,000 clinically relevant pathogens, including fungi, bacteria, DNA viruses and eukaryotic parasites [104]. The limited invasiveness of blood sampling, the potential for faster diagnosis compared to conventional testing, and the possibility of identifying a wide range of infections has made this method an attractive add-on to the diagnostic armamentarium across microbiology [103], although performance may differ between different mNGS platforms [105]. Testing of cerebrospinal fluid (CSF) by mNGS is most frequently performed at the University of California San Francisco Center for Next-Gen Precision Diagnostics (San Francisco, California, United States). As another benefit, NGS tests usually give quantitative test results, and kinetics of these results may also hold significant value for outcome prediction and treatment stratification [106].

A recent two center cohort study evaluated the Karius® Inc. mcfDNA sequencing test in 218 plasma samples from a cohort of 114 patients with severe COVID-19 infection in the ICU found that mcfDNA sequencing held promise as a sensitive and highly specific test for the diagnosis of CAPA, with a sensitivity of 83% and specificity of 97% [106]. An important added benefit of mcfDNA sequencing is its hypothesis-free testing approach and potential for identifying other rare fungal pathogens. Indeed, the test was able to detect *Rhizopus microsporus* in a patient that had been suspected to have CAPA, but inconclusive mycological test results, and also *Candida* spp. and *Pneumocystis jirovecii* in patients that had clinical suspicion and mycological test results indicating invasive infections with these pathogens [106]. In another study, plasma mcfDNA sequencing detected the same fungus identified from the biopsy tissue at the genus level in 7 of 9 patients with proven mold disease, with sites of infection including lung, peri-pancreatic lymph node, heart, brain, sternum, and small bowel [102]. In addition, the test was able to detect *Aspergillus lentulus*, a cryptic *Aspergillus spp.* that is difficult to differentiate from *A. fumigatus* by conventional methods and requires alternative antifungal therapy to the treatment of A. *fumigatus* infections [102]. Among 36 mostly immunocompromised patients, mNGS detected one to

five organisms in 21 of these patients (14/21 bacteria, 8/21 fungi, and 6/21 viruses) [107]. Positive tests prompted therapy changes in 12 of 21 patients, and of note, seven of eight fungi identified were considered clinically pathogenic with disease sites including sinus, liver and lung [107]. Another recent analysis evaluated 82 Karius® mcfDNA tests from a mixed cohort of adults and children who were mostly immunocompromised and found the Karius Test® results led to a positive impact on clinical patient management in 6 (7.3%), and negative impact in 3 (3.7%) cases [103]. Of note, in 3 of the 6 cases where the Karius Test® had a positive clinical impact the test led to early diagnosis of fungal infections (including IC, mucormycosis, and IA) that had been missed by conventional methods, but were later confirmed [103]. In one additional mucormycosis case, the test was also able to identify the causative Mucorales genus, but also wrongly identified a number of bacterial pathogens leading to escalation of antibacterial coverage that was in retrospect deemed unnecessary, leading to negative assessment of the clinical utility [103]. In a case report, mNGS was able to provide a diagnosis of mediastinal aspergillosis within 24 hours from when the clinical specimen was obtained, which was 5 days before histopathological results were available and 2 weeks prior to culture results being available to guide treatment [108].

The utility of plasma mcfDNA testing to diagnose fungal CNS infections has also been described [109,110]. In these reports, *A. fumigatus* and *Cladophialophora bantiana* were found to be the causative pathogens of CNS infections. Although this may provide additional information regarding the etiologic agent responsible, in cases where mass lesions attributed to fungal organisms are observed, surgical resection is often a key component of management. Additionally, it is likely publication bias exists for these reports and prospective evaluation of plasma mcfDNA testing is needed for the sensitivity and specificity of testing for CNS infections to be determined.

mNGS testing of CSF samples is also commercially available and has been evaluated in a prospective multicenter study involving hospitalized patients with idiopathic meningitis, encephalitis or myelitis [111]. In this study, 214 patients were enrolled and results made available to the treating physician. Of 58 infections in 57 patients, 19 (33%) were diagnosed by conventional testing and mNGS, 26 (45%) by conventional testing only, and 13 (22%) by mNGS only. Overall, mNGS diagnosed 32 infections compared to 27 infections with conventional testing only. Among the fungal diagnoses in this study, mNGS of the CSF identified *Cryptococcus neoformans* and *Candida tropicalis*, although missed cases of disseminated aspergillosis, CNS mucormycosis, an angioinvasive fungal disease (not otherwise specified) identified on pathology samples, and cryptococcosis with a serum cryptococcal antigen titer of 1:8. These cases were likely missed due to the lack of organism present in the cerebrospinal fluid (e.g. mass lesions or non-CNS infections with encephalitis from other causes), however mNGS remains a promising testing modality for potential use. Subsequent case reports have observed positivity in CSF samples compared to negative results concurrently obtained from blood [112].

While these results outline the promise of NGS/metabolomic testing for the diagnosis of fungal infections, limited access to testing, cost and highly specialized expertise, and subsequent long turnaround time are still large barriers to implementing these tests into routine patient care. In addition, there is a lack of standardization of mNGS across assays,

including the bioinformatics analysis [113], and challenges with the removal of human DNA from clinical specimens, as previously discussed. This is particularly an issue with clinical specimens from non-sterile sites such as BAL. Interpretation of results may be easier from specimens obtained sterile sites such as serum, CSF, and tissue from deep biopsies [114].

Further studies are therefore needed to evaluate the potential real-world impact of NGS testing and to identify optimal ways of utilizing these tests alongside conventional microbiological methods. Improved access to testing is required to demonstrate clinical utility given that the management of deadly IFDs is time critical. Until faster turnaround time can be achieved, these tests may primarily serve a role in the evaluation of patients without a diagnosis despite intensive investigation. Testing may also be performed as adjunct/confirmatory test, however this conflicts with the significant associated cost.

It is important to recognize the limitations of mNGS testing. These prior studies have reported some cases of false-positive results deemed by adjudication panels involved in the patients' care as unlikely causes of the disease presentation. The results should thus be interpreted in the context of each individual patient to avoid unnecessary treatment and additional diagnostic investigation.

6.0 High-Resolution Melt/Universal Digital High-Resolution Melt

Real-time PCR is often combined with High-Resolution Melt (HRM) analysis to discriminate target organisms or resistance genes and distinguish true positives from false-positives [115–118]. HRM technology offers a rapid, simple, cost-effective, and highly-sensitive method for nucleic acid screening, making it an appealing option for profiling samples prior to or instead of more extensive sequencing analyses. HRM is closed-tube based analysis that does not require post-processing, thus avoiding external nucleic acid contamination, and easing requirements for test operators compared to NGS approaches that cannot currently be carried out in clinical laboratories on a large scale. Like NGS, HRM is highly-flexible and can use probe-free methods with the use of DNA intercalating dyes that produce sequence-specific melting temperatures (T_m) due to factors such as the melting rate, GC content, PCR buffer, and amplicon size. Additionally, while traditional approaches may require time-consuming cultivation or reliance on phenotypic characteristics, HRM can rapidly detect and differentiate fungal pathogens based on their unique genetic profiles in less than 4 hours, resulting in a swifter diagnostic turnaround time that enables clinicians to initiate appropriate treatment promptly compared to conventional testing [119]. HRM analysis is also capable of detecting mutations or single nucleotide variations (SNVs) and discriminating heterozygotes by comparing T_m shifts or changes in curve shape [120,121].

Despite the promise of filling a crucial clinical niche, to-date standardized fungal HRM diagnostic platforms are largely unavailable in the healthcare setting. Several of the previously mentioned commercially-available PCR-based assays employ HRM but are not yet FDA approved. However, Biofire® (bioMérieux) has two FDA-cleared assays utilizing HRM to differentiate yeasts (Table 4). The BioFire® FilmArray® Meningitis/Encephalitis (ME) Panel (BioFire Diagnostics, Salt Lake City, Utah, United States) can discriminate *Cryptococcus* (*C. neoformans/C. gattii*) from 6 other bacteria and 7 viruses from 0.2 ml of

CSF fluid in as little as an hour. Previous studies have shown an overall agreement ranging from 93–99% between the ME panel and conventional diagnostic testing, and has shown 98.2% agreement with clinico-laboratory assessment in a large scale 705 patient study [122]. The BioFire® FilmArray® Blood Culture Identification (BCID) Panel (BioFire Diagnostics, Salt Lake City, Utah, United States) is run on positive blood cultures and can differentiate *Cryptococcus* (*C. neoformans/C. gattii*), *C. albicans, Nakaseomyces glabratus* (previously *C. glabrata*), *Pichia kudriavzevii* (previously *C. krusei*), *C. parapsilosis, C. tropicalis,* and *C. auris* among 23 bacteria while simultaneously detecting bacterial antimicrobial resistance genes in nested format by HRM. This BCID panel has demonstrated 94-99% agreement compared to conventional culture methods, and the largest known study of 2207 positive blood culture samples had 99.2% sensitivity among all yeast isolates [123,124]. Despite concerns for false-positive and false-negative results these Biofire® assays have demonstrated high utility and offer real-time broad detection coverage results [125]. Substantial cost is also a consideration and further system improvements and research is necessary before routine HRM analytics for a broader span of fungal organisms can be performed.

In one study of 100 patients suspected of having IFD, blood cultures were positive in 57 cases and negative in 43 cases. HRM was positive in 14 cases, including *C. tropicalis* (4), *Nakaseomyces glabratus* (previously *C. glabrata*) (4), and *Pichia kudriavzevii* (previously *C. krusei*) (6). HRM sensitivity was 24.6%, specificity 100%, positive predictive value 100%, and negative predictive value 50% [126]. HRM analysis has been shown to be capable of distinguishing most clinically-important species of *Candida*, and in separate studies has shown discrimination of individual species in *C. parapsilosis* and *Nakaseomyces glabratus* (previously *C. glabrata*) complexes, and up to 23 different species of *Candida* simultaneously [127–131]. Another study demonstrated how HRM could detect mutations in the *ERG11* gene in *Candida* conferred with azole resistance [132]. The ability of HRM to rapidly differentiate species within the *Candida* complex, including *C. parapsilosis*, *C. metopsilosis*, and *C. orthopsilosis*, is of clinical note as each species in the complex is not easily phenotypically distinguishable and can exhibit a unique epidemiology, virulence, and antifungal susceptibility, and most current commercial tests do not discriminate between species within *Candida* complexes. These studies highlight the potential for rapid approaches of broad-based *Candida* detection and identification followed by specific loci targeting for subtyping or resistance identification, as well as the possibility to multiplex different targets in a single assay.

HRM has also been able to discriminate between *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii* species by analyzing ITS regions [133,134]. Another analysis of HRM was able to give conclusive identification and clade classification of *Pythium insidiosum* [135]. HRM studies assay have also been described for other high priority pathogens and endemic fungi as well. Specific assays as well as panfungal assays have been developed for *Fusarium* spp. and species complexes [136–138]. Another study used HRM to differentiate *C. immitis, B. dermatitidis, H. capsulatum,* and *Paracoccidioides brasiliensis* from other clinically-relevant yeast and molds in a single test [139].

There have been many studies evaluating HRM to differentiate *Aspergillus* spp. Facilitated by its high-throughput modality, many of the most frequent *Aspergillus* resistance mutations have been characterized by HRM as well [140–142]. The ITS1-5.8S region has been used to discriminate *A. fumigatus, A. flavus, A. niger,* and *A. terreus* from clinical and environmental isolates [143], and a probe-based HRM method has shown that the ITS2 region was sufficient to discern isolates from *A. fumigatus, A. flavus,* and *A. ustus*, but not *A. terreus, A. nidulans,* and *A. niger* from clinical specimens [144]. However, in other studies different ITS2 primers and a probe-free HRM assays were able to differentiate *A. niger, A. flavus, A. fumigatus,* and *A. terreus* [138,145]. In addition, HRM has been demonstrated to discriminate between *A. fumigatus, A. terreus,* and *A. flavus* from patient sera among clinically-relevant yeasts and molds using the complete ITS region through a combination of primers [134]. The Aversi ITS assay primers (Eurogentec, Liège, Belgium) showed high discrimination between *A. versicolor, A. creber and A. sydowii* [146]. HRM and barcoding assay was able to amplify two targets in the beta-tubulin gene to differentiate *A. fumigatus, A. lentulus, A. terreus, A. flavus, A. niger,* and *A. tubingensis* [147]. Collectively the above studies highlight HRM as a valuable tool for accurate and efficient species identification within the *Aspergillus* genus, but it remains to be seen whether there is a specific target and probe-free HRM methodology that can differentiate all clinically relevant *Aspergillus* spp. simultaneously by HRM.

While there are no standard molecular detection assays for mucormycetes, advances in HRM have allowed for identification at the species level. In one study, HRM was able to identify species of *R. arrhizus, R. microsporus, M. circinelloides,* and *Lichtheimia* species complexes [148]. HRM analysis was applied on amplicons generated from semi-nested real-time PCR mucormycete-specific18S rDNA primers to accurately identify *R. microsporus, R. oryzae, M. racemosus, M. circinelloides, R. pusillus,* and *L. corymbifera* [149]. A follow-up study retested samples identified as positive by HRM with novel species-specific primers and qPCR to boost the sensitivity and specificity to 100% and 98%, respectively, while exhibiting 99% negative predictive value [150]. The same group used ITS primers and HRM to differentiate *M. racemosus* and *M. circinelloides* among *Aspergillus* spp. and clinically relevant fungal pathogens [138]. In another study, Mucorales strains (8 genera, 11 species) were differentiated among non-Mucorales strains (9 genera, 14 species) by HRM, demonstrating 100% sensitivity and specificity rates with a limit of detection at 3 rDNA copy/qPCR reaction using degenerate primers targeting the 18S region of the rDNA [151].

The main limitation often cited for HRM is the breadth of taxa in the assay panels. However, many of the above studies have employed a variety of universal fungal primers which are capable of broad-based screening of multiple pathogens simultaneously. Furthermore, universal screening with HRM is not limited to a specific set of known fungal pathogens. Its ability to rapidly identify a wide range of known pathogens while also reporting unknowns makes it a valuable diagnostic tool across clinical settings. This is especially beneficial in populations where different pathogens can cause infection and in cases where less common or novel pathogens could emerge which are often the most problematic in terms of resistance or virulence. The issue lies in the single bulk-reaction constraints from typical PCR well-plate formats for melt analysis, which cannot detect low-level nucleic acid targets in heterogeneous samples and have the potential to produce complex melt curves that pose

challenges in interpretation [152]. As a result, to provide a comprehensive HRM diagnostic from the above studies and approaches carries the disadvantages of multiple assays having to be run on each sample which adds cost and labor or multiplexing specific targets which is widely associated with reduced sensitivity.

Different instruments as well as assay compositions can result in differing degrees of Tm variability [152–154], yet clustering analysis has shown high conservation between instruments which allows for high reproducibility and cross validation between institutions or multi-center studies even if different instruments are used [155]. Engineering controls can help overcome differences in pipetting, reagents, and DNA isolations kits used, which all can affect Tms to the point of incorrect clustering leading to false-positives [156]. Regardless, melt curve shapes may still be reliably identified irrespective of Tm variation [157–160]. The FDA approved BioFire ME and BCID assays have been evaluated by the FDA across test sites and instruments with reproducibility results showing high percent agreement with the expected results and Tm standard deviations of ≦0.5 °C in the ME panel and max observed range between Tms of ≦2.2 °C in the BCID panel[161,162].

Goshia et al. recently described a rapid long amplicon fungal universal HRM (U-dHRM) assay coupled with ML capable of detecting and identifying invasive mold infections in <5 hours, reporting findings such as identification of rare molds in culture-negative BAL samples [157]. The fungal U-dHRM assay also did not show a clear correlation between dHRM results and BAL galactomannan levels, which was a topic not discussed in the previous *Aspergillus* HRM literature. Advancing universal HRM to a digital platform where individual genomes are sequestered into separate picoliter-sized compartments overcomes these issues, has shown increased sensitivities compared to qPCR HRM, and can allow for multiple pathogens from co-infections to be detected simultaneously. HRM based internal controls also improve the absolute quantification accuracy of dPCR [118]. U-dHRM also intrinsically provides absolute quantification results which allows for monitoring disease progression and informing treatment options [153]. Machine learning (ML) has significantly enhanced the progression of U-dHRM towards becoming a vital high-throughput diagnostic tool as well [134,157–159]. ML pipelines for U-dHRM handle data preprocessing such as noise reduction and removing artifacts or background signals and then providing automatic analysis of potentially thousands of melt curves from each patient sample. By using classification and prediction models that identify specific melt curve features, complex or very similar melt curves can be discriminated with 99%-100% accuracy, allowing for infectious pathogens to be rapidly identified and reducing time-to-results. A rapid long amplicon fungal U-dHRM assay coupled with ML capable of detecting and identifying invasive mold infections in less than 3 hours was recently described, reporting findings such as identification of *Aspergillus* spp. in BAL samples from patients with probable IPA where qPCR had resulted in negative, rare molds detected in culture-negative samples, and multiple *Aspergillus* spp. within one BAL sample [163]. The fungal U-dHRM assay also did not show a clear correlation between dHRM results and BAL galactomannan levels, which was a topic not discussed in the previous *Aspergillus* HRM literature. This suggests that U-dHRM may serve as an independent method allowing for a combined diagnostic approach for *Aspergillus* IMI infections. These results indicate that U-dHRM possesses desirable

attributes for various profiling applications that demand a combination of speed, sensitivity, quantitative capabilities, and broad profiling capacity.

8.0 Conclusion

IFDs cause millions of infections annually but the diagnosis in many cases remains challenging. As outlined in this review, advances have been made in the field of molecular diagnostics for the diagnosis of IFDs, and each diagnostic test has advantages and disadvantages (Table 5). Of the diagnostic modalities discussed, PCR testing is the most commercially available. Standardization of these assays has been an issue across settings, although this is improving, particularly for the diagnosis of infections from *Candida* and *Aspergillus* spp. Both HRM and mNGS are rapid, highly-sensitive modalities that can identify a wide range of fungal pathogens, but performance of PCR can be impacted with concurrent antifungal use, and currently, no molecular assay can reliably differentiate between colonizing and pathogenic organisms when testing non-sterile clinical specimens, such as BALF, and where multiple organisms are part of the mycobiome. In addition, there are very few commercial HRM and mNGS assays available, so these technologies are currently unavailable in most healthcare settings. Importantly, despite advances in molecular diagnostics for IFDs, there continues to be a need for low-cost and easy to use diagnostics for IFDs, particularly in resource-limited settings where these assays may be too costly or access to equipment and laboratory personnel and space are limited. Truly point-of-care molecular tests that may be beneficial in these settings are still lacking.

9.0 Expert Opinion

Given the increasing population at risk from IFDs, emergence of antifungal resistance, and limited antifungals available to treat IFDs, improved diagnostic modalities are crucial for the timely diagnosis, accurate surveillance, and management of IFDs. Given the low sensitivity and long turnaround times of traditional diagnostic modalities such as culture, and decreased sensitivity of conventional fungal biomarkers for individuals on antifungals for prophylaxis or treatment, molecular assays can help fill a niche. Still, as discussed molecular assays have limitations, and in many cases the best approach may be to use these assays in combination with culture-based approaches and conventional biomarkers.

As the COVID-19 pandemic underscored, where a significant number of secondary infections due to *Aspergillus* spp., Mucorales, and *Candida* spp. were observed, there is a wide range of individuals with varied clinical presentations that are at risk for IFDs. In addition, occupational exposures to dimorphic fungi in endemic regions of the U.S. involving immunocompetent individuals has been well-documented [164–166], as well as occupational exposure to *Aspergillus* spp. [167] and non-*Aspergillus* molds [168]. IFDs following combat-related injuries, primarily from Mucorales, are increasingly recognized [169,170]. Traumatic implementation of contaminated environmental material has been associated with mucormycosis post environmental disaster and a nationwide outbreak of fungal meningitis occurred after patients received contaminated corticosteroids injections [171,172]. Particularly, for these non-*Aspergillus* molds, molecular testing is the only approach towards diagnosis, other than traditional diagnostics such as culture. Given the

recognition that a wide variety of individuals with diverse risk factors are at risk from IFDs, continued validation, standardization, and acceptance of these modalities is crucial.

Although PCR molecular testing is recommended by a number of international consensus guideline groups for the diagnosis of IFD [26,48,173], currently neither mNGS or HRM are recommended given a lack of data, standardization, and validation of these modalities. In addition, as previously discussed there are limited mNGS and HRM platforms that are commercially available and clinical validation is currently limited. There is the need for more inclusion of these diagnostic assays in clinical trials, so more rigorous evaluation can be done under standardized conditions where confounders and variability among patients and clinical settings is limited. We call on the Food and Drug Administration (FDA) and European Medicines Agency (EMA) to ensure that molecular diagnostic testing is included in more clinical trials, particularly given molecular methods for the detection of *Aspergillus*, *Pneumocystis* and *Candida* spp. are now included in the EORTC/MSGERC definitions [48].

In the near future, molecular diagnostics for the diagnosis of IFD will be available that are highly sensitivity and specificity, but more so are also low cost, easy to maintain and update, and can differentiate between colonizers and pathogenic organisms. Platforms will have standardized methodology and there will be a number of mNGS and HRM commercial assays able to diagnose non-*Aspergillus* molds, mixed infections and infections from dimorphic fungi and also identify potential antifungal resistance through the detection of a wide range of genetic mechanisms, allowing for more targeted treatment approaches with a wide range of antifungal classes approved by then [174,175]. The application of mNGS will permit syndromic diagnostic testing, limiting the need for pathogen specific assays, with the broad detection range providing an alternative to classical microbiology. The application of NGS to the host will have the potential to identify patients predisposed to IFD, permitting personalized patient management [176]. Importantly, true point-of-care (POC) molecular diagnostics will be available in settings in low and middle-income countries that will complement antigen tests such as the *Aspergillus*-specific LFD and the *Aspergillus* Galactomannan LFA that can be performed near patients in hospitals, clinic, or theatres or war. Clinical mycology societies will collaborate with the involvement of low and middle-income countries to determine and overcoming shortcomings in the molecular diagnosis of IFD, develop mycology guidelines, and disseminate information broadly also via social media [177] regarding the epidemiology, diagnosis, and treatment of IFDs for both health practitioners and the lay public.

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Declaration of interests

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on the overall scope of the project and its potential benefit to MelioLabs, Inc.; however, the research findings included in this particular publication may not necessarily relate to the interests of MelioLabs, Inc. The terms of this arrangement have been reviewed and approved by the University of California, San Diego, in accordance with its conflict of interest policies. **MH** received research funding from Gilead, Astellas, MSD, IMMY, Mundipharma, Scynexis, F2G and Pfizer – all outside of the submitted work. **GRT** received research and consulting fees from Astellas, Amplyx, Cidara, F2G, Mayne, Melinta, Mundipharma, Scynexis, and served on the DRC for Pfizer – all outside of the submitted work. All other authors declare no conflict of interest.

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Article Highlights

- Polymerase chain reaction (PCR) is a highly sensitive and specific molecular assay and can identify mutations associated with antifungal resistance, although performance can be impacted by prior/concurrent antifungal use.
- Droplet digital PCR (dPCR) can be multiplexed to detect individual pathogens and to screen for mutations potentially associated with antifungal resistance, has the potential for enhanced detection of low DNA concentrations, particularly relevant to fungal infection and has increased tolerance to inhibition compared to quantitative PCR, although current options are still limited.
- High resolution melt (HRM) is a rapid and highly-sensitive diagnostic modality that can identify a wide range of fungal pathogens, including down to the species level, but multiplex assays are limited and HRM is currently unavailable in most healthcare settings.
- Metagenomics next generation sequencing (mNGS) offers a promising approach for rapid and hypothesis-free diagnosis of a wide range of fungal pathogens, although some drawbacks include limited access, variable performance across platforms, the expertise and costs associated with this method, and long turnaround times in real-world settings.
- True point-of-care molecular diagnostics assays are still needed that are available in lower resource settings and that can complement culture and conventional fungal biomarkers.

Table 1.

List of commercially-available PCR-based assays for detection of fungal infections, and associated resistance mutations.

Product	Manufacturer	Method	PCR Target, species and resistance mutations* detected
Invasive Aspergillosis			
Affigene *Aspergillus* tracer	Cepheid, Rolling Meadows, IL, USA	Real-time PCR	Target unknown *Aspergillus* spp.
A. fumigatus Bio-Evolution	Bio-Evolution, Bry-sur-Marne, France	Real-time PCR	ITS1 region *A. fumigatus*
artus® Aspergillus diff. RG PCR	Qiagen, Düsseldorf, Germany	Multiplex real-time PCR	Target unknown *A. fumigatus, A. terreus, A. flavus*
AsperGenius® Species and AsperGenius® Resistance	PathoNostics B.V., Maastricht, Netherlands	Multiplex real-time PCR	28S rDNA *A. fumigatus* complex*, A. terreus, Aspergillus* spp. TR_{34}/L98H and TR_{46}/Y121F/T289A mutations
Aspergillus spp. ELITe MGB® Kit	ELITechGroup S.p.A, Turin, Italy	Quantitative real-time PCR	18S rDNA Aspergillus spp. (A. niger, A. nidulans, A. terreus, A. flavus, A. versicolor, A. glaucus)
*Asp*ID	OlmDiagnostics, Newcastle, UK	Multiplex real-time PCR	Target unknown *Aspergillus* spp., *A. terreus*
FungiPlex® *Aspergillus* and Fungiplex® *Aspergillus* Azole_R	Bruker Daltonik GmbH, Bremen, Germany	Multiplex real-time PCR	Target unknown *Aspergillus* spp. (*A. fumigatus, A. flavus, A. niger*), *A. terreus* TR_{34} and TR_{46} mutations
LightCycler Septifast	Roche Diagnostics, Mannheim, Germany	Multiplex real-time PCR	ITS region *A. fumigatus* (and *Candida* spp.)
Magicplex Sepsis Real-Time Test	Seegne, Seoul, South Korea	Multiplex real-time PCR assay	Target unknown *A. fumigatus* (and *Candida* spp.)
MycoReal Aspergillus	Ingenetix GmbH, Vienna, Austria	Real-time PCR with melt curve analysis	ITS2 region *A. fumigatus, A. flavus, A. nidulans, A. niger, A. terreus*
MycoGENIE® *Aspergillus* Species and MycoGENIE® *Aspergillus fumigatus* and resistance TR_{34}/L98H	Ademtech, Pessac, France	Duplex real-time PCR assay	28*S* rDNA *Aspergillus* spp., *A. fumigatus* TR_{34}/ L98H mutations
Pneumocystis jirovecii			
PneumoGenius®	PathoNostics B.V., Maastricht, Netherlands	Multiplex real-time PCR assay	mtLSU rDNA *Pneumocystis jirovecii* DHPS mutations codons 55 and 57
MycoGENIE® *Pneumocystis jirovecii*	Ademtech, Pessac, France	Duplex real-time PCR assay	mtLSU rDNA *Pneumocystis jirovecii*
EliTech InGenius PCR Assay	EliTech Group, Bothell, WA, USA	Duplex real-time PCR assay	mtLSU rDNA *Pneumocystis jirovecii*
Bio-Evolution PCR	Bio-Evolution, Bry-sur-Marne, France	Duplex real-time PCR assay	mtLSU rDNA *Pneumocystis jirovecii*
FTD-*Pneumocystis jirovecii* kit	Fast Track Diagnostics, Luxembourg	Duplex real-time PCR assay	mtLSU rRNA *Pneumocystis jirovecii*
RealStar Pneumocystis jirovecii PCR Kit	Altona Diagnostics, Hamburg, Germany	Duplex real-time PCR assay	mtLSU rDNA *Pneumocystis jirovecii*

Product	Manufacturer	Method	PCR Target, species and resistance mutations* detected
RealCycler® PJIR Kit	Progenie Molecular, Valencia, Spain	Duplex real-time PCR assay	mtLSU rDNA *Pneumocystis jirovecii*
RIDA®GENE *Pneumocystis jirovecii*	R-BiopharmAG, Darmstadt, Germany	Duplex real-time PCR assay	mtLSU rDNA *Pneumocystis jirovecii*
PneumoID	OLM Diagnostics, Newcastle Upon Tyne, United Kingdom	Multiplex real-time PCR assay	Target unknown
MycAssay *Pneumocystis*	Myconostica	Duplex real-time PCR assay	mtLSU rDNA *Pneumocystis jirovecii*
Invasive Candidiasis			
AusDiagnostics Sepsis panel	AusDiagnostics Pty Ltd, Mascot, NSW, Australia	Multiplex tandem PCR	ITS1 or ITS2 regions *C. albicans*, *N. glabratus*, *P. kudriavzevii*, *C. parapsilosis*, *C. tropicalis*.
*Cand*ID® and *Auris*ID®	OlmDiagnostics, Newcastle, UK	Multiplex real-time PCR assay	Target unknown *C. albicans, C. dubliniensis, N. glabratus, P. kudriavzevii, C. parapsilosis* and *C. tropicalis, and Candida auris*
FilmArray Blood Culture Identification (BCID) Panel[1]	BioFire Diagnostics, Salt Lake city, Utah, USA	Multiplex real-time PCR assay	Target unknown *C. albicans*, *N. glabratus*, *P. kudriavzevii*, *C. parapsilosis* and *C. tropicalis*
FungiPlex® Candida and FungiPlex® Candida auris	Bruker Daltonik GmbH, Bremen, Germany	Multiplex real-time PCR assay	Target unknown *Candida* spp. (*C. albicans*, *C. parapsilosis, C. dubliniensis, C. tropicalis*), *N. glabratus, P. kudriavzevii, and C. auris*
Magicplex Sepsis Real-Time Test	Seegne, Seoul, South Korea	Multiplex real-time PCR assay	Target unknown *C. albicans*, *N. glabratus*, *P. kudriavzevii*, *C. parapsilosis* and *C. tropicalis* (and *A. fumigatus*)
MycoReal Candida	Ingenetix, Vienna, Austria	Real-time PCR with melt curve analysis	ITS2 region *C. albicans*, *C. dubliniensis*, *N. glabratus*, *P. kudriavzevii*, *C. lusitaniae*, *C. parapsilosis* and *C. tropicalis*
SeptiFast Real-time PCR	Roche Diagnostics, Mannheim, Germany	Multiplex real-time PCR assay	Target unknown *C. albicans*, *N. glabratus*, *P. kudriavzevii*, *C. parapsilosis* and *C. tropicalis*
SepsiTest-UMD	Molzym Molecular Diagnostics, Bremen, Germany	PCR and Sanger sequencing	18*S* rDNA All fungal species
T2Candida	T2 Biosystems, Lexington, MA, USA	T2 magnetic resonance	ITS2 region *C. albicans*/*C. tropicalis*, *N. glabratus* complex/ *P. kudriavzevii*, and *C. parapsilosis* complex
Sepsis Flow Chip	Master Diagnostica, Granada, Spain	Multiplex PCR with automated reverse dot blot hybridization.	Target unknown *C. albicans*, *Candida* spp.
Mucormycosis			
MucorGenius®	PathoNostics B.V., Maastricht, Netherlands	Multiplex Real-time PCR assay	28S rDNA Pan-Mucormycete *Rhizopus* spp., *Mucor* spp., *Lichthiemia* spp., *Cunninghamella* spp., *Rhizomucor* spp.
MycoGenie® *Aspergillus spp./ Mucorales spp.*	Ademtech, Pessac, France	Duplex Real-time PCR assay	28S rDNA *Aspergillus* spp. and *Rhizomucor pusillus, Mucor indicus, M. circinelloides, M. plombeus, Rhizopus arrhizus, R. stolonifer, Lichtheimia corymbifera, L.*

Product	**Manufacturer**	**Method**	**PCR Target, species and resistance mutations* detected**
			glauca, Cunninghamella bertholletiae, and *Mycotypha* species
FungiPlex® Mucorales	Bruker Daltonik GmbH, Bremen, Germany		Target unknown *Rhizopus* spp., *Lichtheimia* spp. Cunninghamella spp., *Rhizomucor* spp., *Mucor* spp., *Actinomucor* spp., *Apophysomyces* spp. *Saksenaea* spp., *Syncephalastrum* spp.

DNA: Deoxyribonucleic acid; ITS: internal transcribed spacer; PCR: polymerase chain reaction; spp: species

*Resistance mutations where applicable

Of note, *Nakaseomyces glabratus* (previously *C. glabrata*) and *Pichia kudriavzevii* (previously *C. krusei*))

Table 2.

The use of digital PCR for the detection of Fungal DNA.

Target	Sensitivity	Specificity	Cases/Controls	Comments	Reference
Aspergillus spp.	94%	100%	16/4	Respiratory samples, Positivity rate of digital PCR superior to QPCR. Digital PCR more tolerant of the presence of PCR inhibitors	[178]
	100%	NA	10/NA	Serum, Free DNA fragments in serum are usually short, consequently digestion of DNA does not increase copy number	[179]
Candida spp.	94%	79%	20/25	Blood, Positivity rate and accuracy of digital PCR superior to QPCR	[180]
	56%	NA	16/NA	Blood, Combination testing using qPCR and dPCR detected 94% of cases	[181]
Pneumocystis jirovecii	92%	88%	19/35	BALF, DHPS and DHFR mutation detection	[182]
	62%	N/A	37/N/A	Sputum	[183]
	44%	N/A	82/N/A	BALF, included pts where a diagnosis of PcP was not conclusive	

BALF: Bronchoalveolar lavage fluid; DHFR: Dihydrofolate reductase; DHPS: Dihydropteroate synthase; DNA: Deoxyribonucleic acid; ITS: PcP: Pneumocystis pneumonia; PCR: polymerase chain reaction; spp: species

Table 3.

List of commercially-available Metagenomic next-generation sequencing assays for detection of fungal infections, and associated resistance mutations.

Product	Manufacturer	Method	mNGS target, Pathogens (# of species)
Karius®	Redwood City, California, USA	mNGS	*Aspergillus* spp. (43), *Candida* spp. (32) *Fusarium* spp. (24) *Mucor* spp. (5) *Penicillium* spp. (24) *Rhizomucor* spp. (3) *Rhizopus* spp. (3) *Scedosporium* spp. (4) *B. dermatitidis, C. immitis, C. gattii, C. neoformans, H. capsulatum, P. jirovecii* ITS2,
CosmosID®	Germantown, Maryland, USA	mNGS	
CD Genomics	Shirley, New York USA	mNGS	ITS1 and ITS2
Novogene	Sacramento, California, USA	mNGS	ITS1/ITS1–1F, ITS2, 18S
DISQVER®	Noscendo, Duisburg, GERMANY	mNGS	16 000 microbial species covering more than 1500 pathogens and can detect bacteria, DNA viruses, fungi, and parasites

DNA: Deoxyribonucleic acid; ITS: internal transcribed spacer; mNGS: metagenomic next-generation sequencing; spp: species

Table 4.

List of commercially-available High Resolution Melt assays for detection of fungal infections, and associated resistance mutations.

Product	Manufacturer	Method	HRM target fungal pathogens
Biofire® FilmArray® Meningitis/Encephalitis (ME) Panel	Salt Lake City, Utah, USA	HRM	*C. neoformans and C. gattii*
Biofire® FilmArray® Blood Culture Identification (BCID) Panel	Salt Lake City, Utah, USA	HRM	*C. neoformans, C. gattii, C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis,* and *C. auris*
LightCycler® Septifast	Roche Diagnostics, Mannheim, Germany	Multiplex real-time PCR	*A. fumigatus, C. albicans, C. tropicalis, C. parapsilosis, C. glabatra, C. krusei*
MycoReal *Aspergillus*®	(ingenetix GmbH, Austria)	HRM	*A. fumigatus, A. flavus, A. nidulans, A. niger, and A. terreus*
AsperGenius® Species and AsperGenius® Resistance	PathoNostics B.V., Maastricht, Netherlands	Multiplex real-time PCR	28S rDNA *A. fumigatus* complex, *A. terreus, Aspergillus* spp. TR_{34}/L98H and TR_{46}/Y121F/T289A mutations
MucorGenius®	PathoNostics B.V., Maastricht, Netherlands	Multiplex Real-time PCR assay	28S rDNA Pan-Mucormycete *Rhizopus* spp., *Mucor* spp., *Lichthiemia* spp., *Cunninghamella* spp., *Rhizomucor* spp.
Magicplex™ Sepsis Real-Time Test	Seegne, Seoul, South Korea	Multiplex real-time PCR assay	Target unknown *A. fumigatus* (and *Candida* spp.)

DNA: Deoxyribonucleic acid; HRM: High-resolution melt; PCR: polymerase chain reaction

Table 5.

Novel molecular tests to diagnose fungal infections: Pros and Cons.

Molecular Test	Pros	Cons
PCR/Digital PCR	High sensitivity and specificity Can detect mutations to antifungals Can identify a wide range of pathogens, including rare pathogens	Assays lack standardization across settings Specificity can be limited with concurrent antifungal use
Metagenomics/Next Generation Sequencing	Rapid diagnosis Can identify a wide range of pathogens, including rare pathogens Quantitative results	Variable performance across different platforms Limited access to testing modality Long turnaround times
High-Resolution Melt	Rapid diagnosis Highly sensitive and specificity Can identify a wide range of pathogens to the species level Detect mutations or single nucleotide variations	Largely unavailable in the healthcare setting Limited availability of multiplex assays

PCR: Polymerase chain reaction