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ADVANCES IN DIAGNOSIS OF INVASIVE FUNGAL INFECTIONS (O MORRISSEY, SECTION EDITOR)

Nucleic Acid Tools for Invasive Fungal Disease Diagnosis

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Abstract

Purpose of Review This review has incorporated the knowledge and experience of the leads of each of the laboratory working parties of the fungal PCR initiative in order to provide up-to-date information on the performance and developments of PCR methods for the detection of fungi that commonly cause invasive fungal disease (IFD).

Recent Findings Molecular diagnosis of IFD enhances the current repertoire of mycological investigations. Providing superior sensitivity and turn-around-time over classical approaches, yet maintaining the benefits of classical tests (e.g. species level identification and identifying resistance). Standardization for *Aspergillus* PCR is almost complete; the recent release of commercial PCR assays for a wide range fungi (*Aspergillus*, *Candida*, *Pneumocystis*, Mucorales and Pan-fungal) and availability of external quality control schemes (e.g. Quality Control of Molecular Diagnostics for *Aspergillus*, *Candida*, *Pneumocystis*) means that fungal PCR testing is robust and ready for use, globally.

Summary Further work is needed to ascertain the utility of PCR in routine practice and to determine whether combining it with other biomarkers is an optimal strategy. PCR for detecting Mucorales sp. and on tissue, together with direct antifungal resistance detection in body fluids, may increase its diagnostic value across the board. This and the ability to diagnose Pneumocystis pneumonia and invasive candidiasis would go a long way towards attaining the long-held ambition of medical mycology to provide a comprehensive range of tests that can be relied upon to diagnose, at least, the common IFD. In short, PCR has a clear future and is close to achieving its full potential in our laboratories.

Keywords *Aspergillus* · *Candida* · *Pneumocystis* · Mucorales · Pan-fungal · Tissue · PCR

Introduction

Despite the tremendous efforts and success achieved in the past decade to improve the overall prognosis of patients with malignant diseases, invasive fungal diseases (IFD) remain as severe complications with high frequency, morbidity and mortality [1] The reasons are as numerous as the underlying fungal pathogens. In former days, prior to the availability of

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immunomodulatory therapy, IFD was restricted mainly to allogeneic stem cell transplant recipients and patients with haematological malignancy [2]. However, advances in transplantation medicine and immunomodulatory therapy and the consequent increase in the number of immunocompromised patients have led to a continual increase in IFD rates. This includes many other patients receiving kinase inhibitors, monoclonal antibodies, anti-calcineurin inhibitors or cytokine blockers [3]. In parallel, the diversity of the underlying diseases has been increasing, including a variety of newly identified autoimmune diseases and congenital immune disorders [4]. Manifestations of IFD are often subtle, atypical and diverse, comprising, e.g. pneumonia, meningitis, sinusitis, osteomyelitis and enteritis [5] . Furthermore, the spectrum of opportunistic fungal pathogens is increasing and includes species and genera previously considered as rare or cryptic, often associated with limited antifungal susceptibility (e.g. *Lichtheimia* spp., *Rhizopus* spp., *Scedosporium* spp., *Fusarium* spp., *Trichosporon* spp. and *Candida auris*), underlining the necessity for specific diagnostic tests to identify the most appropriate antifungal therapy [6–8].

Despite the fact that molecular diagnostic assays should be optimized locally, by each laboratory to suit its individual needs, reliable standardization is a mandatory prerequisite [9]. This includes standardization across the various clinical specimen types (e.g. blood, bronchoalveolar lavage fluid (BAL) and tissue) but also the large variety of clinically relevant fungal species. As the human body is challenged daily with ubiquitous fungi, including environmental spores from *Aspergillus* spp. or gut commensal pathogens such as *Candida* spp., PCR false positivity due to contamination is a major concern, and multiple sources have been identified [10]. In addition to requiring high specificity to limit false positivity, fungal PCR assays need to be highly sensitive to reliably exclude disease. The fungal DNA copy number is extremely low in many clinical specimens and for most of the fungal species. Consequently, these assays often perform close to or at their limit of detection [11].

The large variety of clinical entities and underlying diseases, the various specimen types and the broad range of clinically relevant fungal species made standardization overdue. In 2006, the European *Aspergillus* PCR Initiative (EAPCRI), (recently rebranded as the Fungal PCR Initiative (FPCRI)) was founded in an attempt to address this issue. Involving more than 60 centres across Europe, Australia, and the USA, 10 different working groups aimed to develop standards for fungal PCR methodologies and provide validation through clinical trials (www.fpcri.eu). Laboratory and clinical-translational working groups for *Aspergillus*, *Candida*, Mucorales, *Pneumocystis* PCR and the PCR-based detection of fungi in tissue were developed. For *Aspergillus* PCR, both working groups have been active for almost 15 years resulting in published recommendations for DNA extraction and PCR amplification [12–14]. EAPCRI / FPCRI standardization trials have led to the inclusion of *Aspergillus* PCR in the second revision of the European Organization for the Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) guidelines for defining IFD (in press).

This review has incorporated the knowledge and experience of the leads of each of the laboratory working parties of the FCRI in order to provide up-to-date information on the molecular diagnosis of IFD.

PCR Methods for the Detection of Specific Pathogens

Aspergillus PCR

A. fumigatus remains the most common cause of aspergillosis, but with the emergence of potentially resistant cryptic species within the Fumigati complex and geographical differences in epidemiology (e.g. *A. terreus* in Austria, resistant to amphotericin), it is important that molecular assays can detect more than just the predominant species. Knowing the analytical specificity (detection range/cross reactivity) of any *Aspergillus* PCR assay is important, and yet the detection of *A. fumigatus* DNA remains superior to that of other *Aspergillus* species [15].

Clinical performance has been critically assessed when testing blood and BAL fluid samples, but data on other samples (e.g. tissue biopsies, cerebrospinal fluid (CSF)) is more limited [16]. When testing blood samples, meta-analytical reviews generated sensitivity and specificity values of 84–88% and 75–76%, respectively [17, 18]. Specificity can be improved by requiring two samples (ideally consecutively, minimally within the same period of risk) to be PCR positive, although this will compromise sensitivity, which is optimal when a single positive threshold is applied. Comparison of *Aspergillus* PCR with antigen (e.g. Galactomannan-ELISA (GM) and (1–3)-β-D-Glucan (BDG)) testing of blood show the specificity of both antigen tests is significantly higher than for PCR (P <0.0001–0.012), but sensitivity of PCR is significantly higher than for BDG (P <0.0001–0.0477) [19•]. In a recent Cochrane review of *Aspergillus* PCR testing of blood samples, it was concluded that the sensitivity of PCR confers sufficient negative predictive value (NPV) to allow a diagnosis of invasive aspergillosis (IA) to be excluded, which, given the low prevalence of IA, is the optimal way to apply testing [20••]. PCR specificity in blood can be enhanced by repeat testing, where consecutive positivity generates a specificity of 96.2%, sufficient to commence therapy, and trigger a diagnostic workup (e.g. high-resolution chest computed tomography/bronchoscopy) to confirm IA [21].

Deep respiratory samples, such as BAL fluid, are usually taken to confirm clinical suspicion of disease. Subsequently,

specificity/positive predictive values (PPV) are critical. Meta-analysis of *Aspergillus* PCR testing of BAL fluid generated sensitivity and specificity ranging from 76.8–79.65 and 93.7–94.5, respectively, and is comparable to other biomarkers [22–24]. The high specificity generates a positive likelihood ratio (LR + tive) > 10 (meta-analytical range: 12.4–13.9), sufficient to confirm disease when positive and reduce concerns regarding the generation of false-positive results associated with *Aspergillus* airway contamination and/or colonization. When comparing meta-analyses of GM and PCR testing of BAL fluid, the specificity of PCR is significantly greater (P <0.0001–0.0019) [19•].

Aspergillus PCR testing of CSF enhances the diagnosis of fungal meningitis or encephalitis, generating sensitivity and specificity of 75% (9/12) and 98% (59/60), respectively [25, 26]. Using an *Aspergillus*-specific nested PCR increased sensitivity to 100% (8/8), with specificity remaining high (92%, 23/25) [27]. While it is feasible to perform multiple genus/species-specific PCR on tissue specimens, the range of potential pathogens combined with usually limited sample means a pan-fungal PCR is preferential.

Molecular tests offer the only alternative culture to determine antifungal resistance. Through the molecular detection of mutations (e.g. single-nucleotide polymorphisms or tandem repeat sequences), it is possible to predict whether *Aspergillus fumigatus* possesses genetic mechanisms that drive resistance. Common, environmentally driven, mutations (e.g. L98H/TR43 in the CYP51 gene) have been increasing associated with azole resistance in *Aspergillus fumigatus* infections. Commercial real-time PCR assays have been developed to detect these mutations from culture, but more importantly, direct from the clinical sample [28•, 29, 30].The origin of resistance (e.g. clinically versus environmentally driven) usually dictates the range of different mutations encountered, with a more diverse range of mutations being associated with prolonged clinical use of azoles [31]. Molecular tests are limited to frequent, but specific mutations cannot exclude the possibility of resistance caused by the ever-increasing range of Cyp51A mutations. In a recent comparison of the PathoNostics AsperGenius PCR assay, capable of detecting TR34/L98H and Y121F/T289A mutations, with an "in-house" method utilizing PCR sequencing of the Cyp51A gene, the latter showed greater positivity, particularly when testing biopsies, but the commercial assay provided a significantly better time to result, suitable for efficient patient management [29, 32]. The AsperGenius assay has also been used to differentiate three species (*A. fumigatus, A. lentulus* and *A. felis*) within the *Aspergillus fumigatus* complex, which is important given the intrinsic azole resistance in the cryptic species [33].

A multicentre evaluation of the AsperGenius assay when testing BAL fluid from 201 haematology patients generated sensitivity and specificity of 84% and 80%, respectively. Azole treatment failure was 75% in patients with TR34/L98H/ Y121F/T289A mutations, and 6-week mortality was 2.7-fold greater [34•]. Successful application of this test to less invasive samples (e.g. blood) could enhance the application, but sensitivity will be compromised. In one study, seven cases (50%) of IA had at least one genetic region potentially associated with azole resistance successfully amplified, although no resistance markers were detected [35]. The performance of the AsperGenius assay when testing plasma was less successful, albeit case numbers were limited [36].

Aspergillus PCR testing has been extensively standardized over the past decade. [13, 14, 37]. Methodological recommendations for the *Aspergillus* PCR testing of whole blood (WB), serum and plasma have been published by the EAPCRI/FPCRI. An independent meta-analysis of *Aspergillus* PCR methods showed that these recommendations were associated with a trend towards improved sensitivity and a significant improvement in specificity when testing blood (i.e. WB, serum/plasma) [17]. These recommendations, together with the availability of commercial assays, have led to the inclusion of *Aspergillus* PCR in the second revision of the European Organization for the Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) guidelines for defining IFD [38]. An international *Aspergillus* DNA calibrator has been developed and is being used to generate and international PCR standard to compare performance of different PCR assays across centres [39].

Candida PCR

The diagnosis of invasive candidiasis (IC) by conventional or real-time PCR remains an investigational approach in most centres [40•]. Differences in PCR performance between studies due to a lack of standardization of assays and study design hinder acceptance [41]. Nevertheless, performance is excellent, and a meta-analysis of *Candida* PCR for the diagnosis of IC, including 54 studies (4694 patients), reported a pooled sensitivity of 95% (95% CI: 88, 98) and specificity of 92% (95% CI: 88, 95) [42]. Improved PCR performance was associated with testing from whole blood, detection of rRNA or P450 gene targets and a high analytical sensitivity (limit of detection (LOD)) < 10 CFU/ml [42]. *Candida* PCR also had a significantly higher positivity rate (85%) compared with blood culture (38%) for patients with proven or probable IC [42]. Despite the findings of the respective meta-analysis, guidelines for defining IFD do not recommended PCR, due to limited standardization, compounded by the sparsity of well-validated commercial tests and subsequent lack of clinical evidence of an optimal process [43]. The MICAFEM study group aimed to evaluate serum PCR and blood culture in a prospective noninterventional multicentre study. The *Candida* detection rate by PCR (9.1%) was not significantly different to blood culture positivity (8.0%) [44]. The authors

acknowledge that optimization of diagnostic tests is required to improve patient outcomes [44].

To date, there are no multicentre clinical trials validating *Candida* PCR for clinical use [41]. Hopefully, this will be addressed by the A-STOP multicentre trial (ISRCTN study number 43895480), due to complete in 2021, which will assess the diagnostic performance of two *Candida* PCR assays for the diagnosis of IC, including the T2Candida assay [45••]. The lack of methodological standardization of *Candida* PCR is being addressed by the FPCRI, aiming to identify factors contributing to optimal PCR performance. Initial studies have focused on analytical specificity, and preliminary data showed that detection of the five main causes of IC (i.e. *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*) was excellent (Personal communication: Rebecca Gorton).

In the absence of extensive clinical validation and with only limited standardization, the most significant development for *Candida* PCR is the release of the T2Candida assay. T2Candida is the first FDA cleared and CE-marked test that combines the nuclear magnetic resonance and PCR molecular assay to directly detect and define the five most common pathogenic species, [46] grouped according to their antifungal susceptibilities as *C. albicans/C. tropicalis, C. parapsilosis,* or *C. krusei/C. glabrata* [47]. T2Candida is fully automated, directly testing ethylenediaminetetraacetic acid (EDTA)-whole blood. It amplifies the internal transcribed sequence 2 (ITS-2) DNA and uses magnetic resonance to detect and identify within 5 h and at a LOD of 1–3 colony-forming unit/m [46].

In the initial validation, the T2Candida demonstrated a sensitivity of 91.1% and specificity of 99.4% in the DIRECT trial (IC incidence 14.3%). However, only 7 clinical specimens from proven IC were available, with the sample population being enriched with 250 contrived positive samples [46]. Prospective T2Candida testing in the ICU setting, with a proven/likely disease prevalence of 13.5%, resulted in a sensitivity and specificity of 59% and 96%, respectively [48]. False negative results were observed in patients with deep-seated abdominal ($n = 3$) and pleural infection ($n = 1$) [48]. False negative results were also observed in a second study whereby 20% (4/5) of patients with pleural/abdominal deep-seated IC had negative results [49]. This highlights the importance of evaluating PCR in a representative patient cohort to understand test limitations. Further trials are required to understand performance of T2Candida for the diagnosis of deep-seated IC.

T2Candida demonstrates a high NPV ranging from 100 to 96% across studies (IC prevalence 0.4–15%) [46, 48, 50, 51]. Care should be taken when interpreting this value, as it is significantly influenced by prevalence (pretest probability) of IC, which is usually low. Nevertheless, a negative T2Candida has allowed earlier discontinuation of anidulafungin, median duration of therapy of 1 (1–2) versus 2 days (1–5) $P < 0.01$ [52]. T2Candida has also been shown to be more sensitive (59%) than blood culture (29%) for diagnosing IC and in determining candida clearance from follow-up specimens in the DIRECT2 (45% vs 24%) and STAMP (41.9 vs 12.9%) trials, respectively [48, 53•, 54]. In the MADRID study, the specificity of the T2Candida assay was excellent, but the sensitivity was compromised. Assay positivity was a good indicator of future disease and a poor prognosis [55]. A positive T2Candida within the first 5 days of diagnosis was associated with a 37-fold increased risk of complicated infection [55]. Results must be interpreted with caution as PCR positivity post-treatment initiation may be due to detection of non-viable circulating Candida [56].

When T2Candida is accurately costed (including taxes, staffing, wastage and overheads), the cost per test exceeds US$200 [48]. This combined with the need to purchase specific equipment means strategies to optimize use are essential. Targeting high-risk patients to exclude disease and monitoring clinical clearance post-diagnosis could potentially reduce antifungal costs and improve clinical outcome [57].

Whether molecular assays alone will find the "missing 50%" of IC infections is yet to be shown. It is more likely that molecular tests will be used alongside other rapid tests (e.g. BDG) or to complement existing conventional microbiological investigations. Combination of PCR and blood culture can improve the diagnosis of IC. In the only non-single-centre prospective study in a high-risk ICU setting combining T2Candida and blood culture performance increased the sensitivity from 59% and 29% respectively to 65%, without impacting on the specificity/PPV [48]. Nguyen et al. also reported improved diagnosis of IC when PCR was combined with blood culture, resulting in a sensitivity of 98% [54].

Mucorales PCR

The use of molecular techniques has improved the therapeutic management of mucormycosis, a rare but serious fungal infection associated with high mortality rates, especially in haematological patients. Distinguishing this from IA is of utmost importance, since the antifungal treatment for each is different. Early diagnosis and early initiation of directed treatment are essential to improve patient outcome [58]. Molecular techniques may help to provide fast, effective treatment by accurately identifying the causative fungi.

PCR amplification and sequencing have been applied to better identify isolates grown from cultures of biopsies or BAL samples collected in patients with Mucorales infection [59]. Molecular techniques have also been used to identify the fungus directly from the tissue samples when cultures were negative. The first approach in tissue samples used pan-fungal primers, targeting ITS regions, followed by sequencing [60, 61]. The second approach used Mucorales-specific primers. Diverse PCR techniques were tested and were successful, and

several studies confirmed that PCR results were better in fresh/ frozen samples than in formalin-fixed paraffin-embedded samples [62–64]. Molecular detection of fungi on hyphal positive biopsy samples is now recommended by the European ESMID and ECMM joint clinical guidelines [65, 66•]. Recent studies confirmed that Mucorales quantitative PCR applied on BAL fluid could lead to earlier initiation of specific antifungal therapy, improving outcomes of pulmonary mucormycosis patients [67].

These tools require invasive sampling (biopsy, BAL), which is not always feasible in seriously ill patients in haematology or intensive care units. PCR to detect Mucorales DNA in non-invasive samples such as plasma or serum have proved successful in diagnosing mucormycosis early (up to 8 days before mycological diagnosis and 3 days before imaging in patients with haematological malignancies) [68–72]. The test is applicable to all patients, especially when biopsy or bronchoscopy is not possible. The Mucorales qPCR performed on serum or plasma is becoming essential to the management of at-risk patients. It can be performed using a combination of several genera-specific qPCR assays, targeting the most frequent genera involved in human diseases (*Mucor/ Rhizopus, Lichtheimia; Rhizomucor; Cunninghamella*) [70•, 71, 72, 73]. Another option is a probe-based Mucorales-specific qPCR assay, with an additional step of sequencing to identify the genera [74]. Efforts from the FPCRI Mucorales Lab working party are ongoing to improve standardization, following the EAPCRI methodology [12].

The technique of whole-genome sequencing (WGS) is not currently commonly used to investigate Mucorales infection. However, a pan-genomic approach was used to identify the species *Mucor velutinosus* in a patient with acute myelogenous leukaemia who developed invasive mucormycosis [75]. WGS analysis was recently applied to study an outbreak of invasive wound mucormycosis in a French hospital, to investigate the link between different strains and understand transmission patterns [76] .

Diagnosis of Mucorales infection remains difficult, compounded by the absence of serological assays and limitations of conventional diagnosis. The development of qPCR tests and the availability of commercial assays should improve the rates of diagnosis and provide an accurate determination of the incidence of this devastating infection.

Pneumocystis PCR

Pneumocystis jirovecii is an unusual ubiquitous fungus in terrestrial mammals whose specific environmental niche has never been identified [77]. This pathogen is regularly inhaled early in life and can persist at the surface of the alveolar cells, mainly on Type I pneumocytes, causing pneumonia (PcP) in immunocompromised hosts. It replicates asexually via binary fission of trophic forms and sexually resulting in cyst formation (ascus) containing 8 ascospores [77]. *Pneumocystis* species are host-specific with *P. jirovecii* specific to humans and *Pneumocystis murina* or *Pneumocystis carinii* specific for mice or rats, respectively, and this has implications when designing specific molecular tests [78].

Until the recent revision of the EORTC/MSG definitions of IFD, PcP PCR testing of respiratory samples such as BAL fluid or induced sputa was not accepted as a mycological criterion for PcP, a result of limited standardization. Standardization of PcP PCR is progressing through FPCRI Pneumocystis PCR Laboratory working group. The gold-standard diagnosis remains microscopic observation of the fungus using conventional staining and/or immunofluorescence (IF) of respiratory samples. The detection of BDG in serum is also an interesting diagnostic tool in specific patient populations, providing excellent sensitivity and NPV (> 90%) [79].

Recent meta-analyses of PcP PCR provide excellent pooled performances, showing pooled sensitivity and specificity in BALF of > 98.3% and > 88%, respectively [80, 81].However, the PCR methods combined in the meta-analyses were heterogeneous ($n = 16$) [80, 81]. They include methods using end-point analysis of the PCR products involving gel electrophoresis, with or without southern-blot hybridization and both single-round PCR and nested-PCR. These initial formats are prone to methodological false positivity, due to contamination and were progressively replaced by qPCR, where the PCR product is detected and quantified during amplification, excluding post-amplification handling and minimizing the opportunity for environmental contamination with previously amplified products. Subgroup analysis of the PCR format in the meta-analyses did not differ significantly from the overall analysis [80, 81]. Global clinical pressure for the standardization of diagnostic PcP qPCR is already evident [82–84].

Quantification is key; the ability of qPCR to quantify results is beneficial for diseases like PcP, where a threshold can be used to differentiate a colonized individual from someone with active infection. Standardized units for reporting quantitative results are lacking and vary, dependent on assay. If plasmids have been used to generate a standard curve, then results are often reported as copy number/unit volume. Since the nucleic acid targeted by PCR is often multicopy, results can be reported as microorganisms/volume of sample. This is useful for correlating qPCR results with the fungal load in immunofluorescent microscopy-positive respiratory samples, (where the number of cysts are often expressed as +, ++ or +++) [85, 86]. When qPCR results are concordant with IF, there is little question about the interpretation of the results. However, there is no direct association between burden and IF/PCR concordance, with some IF-/PCR+ samples harbouring a high fungal load, and conversely, some samples with a lower fungal load being IF+/PCR+. A qPCR threshold

corresponding to the limit of IF positivity is required, but only when qPCR and its reporting is standardized will this be possible. At that point, qPCR can be considered as a replacement reference method for the diagnosis of PcP.

All forms of microscopic investigation are reliant on the experience and technique of the individual, and sensitivity and specificity of IF investigations will vary between users and locations. Most IF assays only detect cysts, which represents only 10% of the microorganism present in clinical samples, compromising sensitivity [87]. Some laboratories have already replaced IF by qPCR, because of the subjective and technical issues associated with IF assays. Nucleic acid extraction is theoretically more efficient for detection of the more abundant trophic forms, with cysts potentially resistant to lysis due to the presence of the cell wall. Unlike IF, there is little doubt about the interpretation of the qPCR negative results. The NPV of PCR assays is consistently high, provided an internal control PCR that has been utilized to avoid reporting false negative results.

Interpretation of discordant IF-qPCR+ results remains difficult. PCR Thresholds have been proposed to discriminate active pneumonia from colonization, which may coincide with the point at which IF results fluctuate. However, it is doubtful as to whether a single PCR threshold will be suitable and some authors propose a grey zone [88, 89]. When results are later than the upper cycle threshold (late Cq value = low concentration), there should be confidence that the patient does not have PcP. Conversely, positives before the lower cycle threshold (early Cq value = high concentration) need to be linked with a high specificity for disease. It is important that these approaches provide sufficient distinction with upper cycle thresholds generating > 90% sensitivity and lower cycle thresholds generating > 90% specificity.

This distinction relies on the concept of infection versus colonization. Carriage or colonization refers to the presence of the fungus in the absence of clinical pneumonia. Since bronchoscopies are only performed for investigating symptomatic respiratory disease, the detection of *P. jirovecii* should not be regarded as simple carriage, even if in the presence of other pathogens (e.g. bacteria, viruses, other fungi, parasites) have been diagnosed. The issue remains whether an IF-qPCR+ patient warrants specific therapy, particularly when immunosuppression is ongoing. This is dependent on fungal burden and clinical presentation, including radiology, all of which can vary with the underlying disease and the intensity of host response to the presence of the fungus [90]. The validity of negative PCR results is unclear when patients are receiving prior prophylaxis, although a positive qPCR despite PcP prophylaxis is clinically concerning. Some IF-qPCR+ patients will recover without specific therapy, particularly when immune recovery occurs. These complexities will not be resolved without appropriate clinical prospective studies.

PCR for Testing of Tissue

The identification of fungi in diseased tissues is the prerequisite for proving IFD while providing subsequent aetiology [91]. While sampling of tissue may not be possible in some patients, it has been proposed that fungal identification by microscopy, culture and PCR from surgically resected tissue offers the best chance for curative treatments in patients failing antifungal therapy [92].

Beyond the potential benefits for the management of individual patients, formalin-fixed, paraffin-embedded (FFPE) tissues from patients with IFD stored in pathology archives may be used to identify the aetiology of fungal infections as a prerequisite for rational future prevention and management strategies. The yield of PCR from FFPE tissues is generally lower than from fresh tissues with DNA extraction, the amplification strategy used and PCR inhibition being critical steps [93]. Often, degradation of DNA is mentioned as a limiting factor, but crosslinking of DNA by the fixative may also be important, and there is evidence that this may in part be reversible [94]. Due to low content of amplifiable fungal DNA, multicopy targets, such as the ribosomal RNA genes, are usually targeted in FFPE tissues. Specific PCR assays with small amplicons offer the best chance to detect DNA of a species or group of related fungi. A combination of assays may be required to identify most causative agents and mixed infections [95, 96]. However, broad-range assays are also needed to identify rare or emerging fungi [97, 98]. Beside the potential for contamination, broad-range PCR assays present additional challenges. As fungal DNA often represents a minor part of total DNA present in a sample, primers need to amplify fungal DNA in a surplus of host DNA. To achieve this goal, primers need to bind to regions conserved in fungi but with significant mismatches to host DNA [99].

With progress in DNA extraction and understanding of whole genome sequences, non-ribosomal DNA regions are increasingly being targeted for PCR assays. They allow superior species identification by targeting single copy genes used for discrimination between fungi within a species complex, offer insights into the prevalence of resistance determinants and improve knowledge of the molecular epidemiology of fungal pathogens by molecular typing [100, 101].

While PCR offers sensitive detection of selected fungal DNA from tissue specimens, a bias into over-selective amplification might be introduced. Next generation sequencing platforms give a wider view of microbial DNA in a tissue sample. However, initial results suggest that background host DNA and diverse microbes present in a sample may interfere with the identification of causative agents [102]. Selected elimination of background DNA and pathogen enrichment might be needed for effective use of this exciting technology [103]. Introduction of fungal contamination needs to be carefully monitored, and molecular results should be interpreted

together with the histopathology (i.e. the fungal elements present in tissue). Histopathology may be enhanced by fluorescence in situ hybridization (FISH) targeting ribosomal RNAs. In contrast to PCR, hybridization takes place on the tissue fixed on a slide. Therefore, fungal identification due to hybridization with specific probes may be localized within an infectious process. This allows a distinction between colonizing invasive species and detection of mixed infections [97, 104].

Other Fungi and Pan-Fungal PCR

Infection by *Scedosporium* or *Fusarium* species are associated with high mortality rates due to innate antifungal resistance, compounded by the limitations of classical diagnosis. Real-time PCR assays for fusariosis and scedosporiosis have been reported and allow specific diagnosis of these manifestations [105–108]. Further molecular tests to detect the emerging pathogens *Exophiala dermatitidis*, *Rasamsonia argillacea*, *Trichosporon asahii* and *Alternaria alternata* have been described [109–112]. The rapid development of a specific assay for the detection of the dematiaceous mould *Exserohilum rostratum* was clinically important in the major US outbreak involving this organism [113]. Both commercial and in-house assays molecular methods for dermatophytes are available, improving time to result and sensitivity, but the extra cost of molecular testing restricts widespread use [114]. Molecular testing for the endemic fungi, *Histoplasma capsulatum, Blastomyces dermatitidis, Coccidioides immitis, Paracoccidioides brasiliensis* and *Talaromyces (Penicillium) marneffei* has been described [115–119]. Infection can be caused by a wide range of aetiologies, and sampling is limited (e.g. tissue biopsy) restricting the number of individual tests that can be applied. Pan-fungal PCR provides a syndromic approach with the aim to detect most, if not all fungi. Confirming the presence of fungi in a sample, but not identifying the genus/species, is of limited clinical use, lacking the ability to direct appropriate antifungal therapy. Contamination can occur during sampling or enter during the molecular process, generating false positive results, which may be recognized by confirming the species. In a clinical evaluation of a pan-fungal PCR utilizing a single all-encompassing probe, the PPV was poor [120]. The use of a single assay combining pan-fungal primers and multiple aetiologically specific probes is preferable to a truly pan-fungal assay with species differentiation through sequencing or using genera/species-specific primers and probes in multiplexed fashion, as this will compromise analytical sensitivity. It also has benefits over using multiple individual real-time PCR assays specific for different fungal pathogens, which is neither cost-effective nor time efficient, unless targeting the more common fungal pathogens (i.e. *Aspergillus, Candida, Pneumocystis*).

One real-time PCR approach utilized high-resolution melt curve analysis to differentiate PCR amplified internal transcribed spacer (ITS2) regions. When testing BAL samples from 104 haematology patients, 18 with IFD, the sensitivity and specificity of this approach was 67% and 100% [121]. The sensitivity was less than the coinciding species-specific real-time PCR assays (83%), likely a result of the assay co-amplifying DNA from commensal fungal species (e.g. *Candida*) and the inability to detect most of the Mucorales species [121]. Combining melt-curve analysis with specific molecular beacons and DNA sequencing improved sensitivity to 83% when testing mainly biopsy proven cases of IFD [122].

General Considerations

Given the huge diversity of the qPCR assays published, there are now proposed recommendations for validation of the qPCR assays [83]. The MIQE guidelines (minimum information for validation of publication of quantitative real-time PCR Experiments) insist on a methodological approach to analytical validation of the test, with a clear delineation of the LOD and the efficiency of amplification. An internal control (IC) of the amplification is also mandatory, ideally intended for monitoring the efficiency of extraction of each individual sample but also identifying inhibition of the amplification process, through the comparison of the Cq value of the IC from each clinical sample with that of a control sample; this is a crucial point to avoid false negative results. However, the use of IC PCR for confirming amplification efficiency/identifying inhibition is a minimum requirement. If this format is chosen, then it is essential that a positive extraction control (representative of a typical reproducible positive clinical sample) is included alongside a negative extraction control (which should always be included) to monitor for procedural contamination.

Any nucleic acid from fungus different to that being in the clinical sample can be used for an IC. Unfortunately, it is a common mistake to amplify a human gene. Since human DNA is often present in huge and variable quantity, there is no reference point to control for the presence of PCR inhibitors, although it does confirm that a sample has been tested. Depending on the microorganism and potential DNA sources, specific DNA extraction processes are required, which can be deleterious to the integrity of the IC target, if it is included prior to extraction, and loss of the IC signal may be indicative of methodological limitations rather than inhibition. Similarly, when the clinical sample contains huge quantity of human DNA, the DNA extraction process can become saturated, leading to a loss/delay of IC signal. While this effect may seem to be a product of the process, it can have clinical implications, as in general the amount of fungal DNA present in a clinical sample will also be less than the concentration of

human DNA. Any deleterious effects on the IC signal may also be applied to the detection of fungal DNA. It is imperative that the concentration of any IC target be similar to that of typical positives generated by the fungal PCR. In the event of a very strong fungal PCR positive signal, the IC signal may be deleteriously affected. This has no relevance when interpreting the result and is an artefact of competition for the PCR reagents. These possibilities require consideration before finalizing the interpretation of the result.

Contamination remains an important concern when interpreting fungal PCR results, particularly when performing pan-fungal PCR assays. Contamination may arise during sampling, whether this is individual airway contamination or contamination of sample vessel there is no way to identify this as a contaminant, although fungal burdens will likely be minimal [123]. Contamination of molecular biology reagents including proteinase K, DNA/RNA spin capture columns and lysis buffers and enzymes specifically used for fungal NA extraction processes (Zymolyase/Lyticase) has also been reported [124–127]. Procedural contamination with fungi or fungal DNA, entering molecular process, can occur and should be monitored for using negative control specimens [16]. It is not possible to monitor individual specimens for contamination, but the number of negative controls should be proportional to the number of clinical samples being tested (e.g. 1 control per 10 samples). The onset of qPCR has minimized the impact of amplicon based contamination; nevertheless, unilateral workflow should be maintained, and additional controls monitoring for contamination of the amplification process alone should also be used.

Meta-analyses and Systematic Reviews of Fungal PCR Methods

Of the topics covered in this review, there is only sufficient data to perform meta-analysis for *Aspergillus, Candida* and *Pneumocystis* PCR methods. As described, the respective sections meta-analyses for *Aspergillus* and *Candida* PCR have generated data that is at least comparable or superior to the performance of other biomarker assays and conventional mycological approaches. Meta-analysis of PcP PCR generates excellent sensitivity ≥ 97%, and PCR negativity is sufficient to exclude PcP (NPV ≥ 99%). Conversely, PCR positivity readily confirms disease LR + ≥ 10. [80, 81, 128]. Despite the influence of an inferior reference method for diagnosing PcP, meta-analysis of PcP PCR confirms it to be a useful test for the diagnosis and exclusion of PcP. For PCR for the diagnosis of Mucorales, pan-fungal PCR, including the testing of tissue biopsies, and the available literature is too limited, at present, to allow a quantitative analysis of clinical PCR studies.

A systematic review of *Aspergillus* PCR testing of blood-based specimens initially published in 2009 [18] was updated by the FPCRI and republished in the Cochrane library in 2015 [21]. Since new evidence is accumulating annually, the Cochrane review was further updated in 2018 and now includes 29 primary studies published between 2000 and 2018 [20••].The mean prevalence of proven or probable invasive aspergillosis (IA) in the included studies was 16.3%. Most patients had received chemotherapy for haematological malignancy or had undergone haematopoietic stem cell transplantation. Pooled data show that PCR has moderate diagnostic accuracy when used as screening tests for IA in high-risk patient groups. The sensitivity and specificity of PCR for the diagnosis of IA testing blood varied according to the interpretative criteria used to define a test as positive. The summary estimates of sensitivity and specificity were 79.2% and 79.6% for a single positive test result and 59.6% and 95.1% for two consecutive positive test results. Diagnostic odd ratios (DORs) were 14.8 for a single positive result and 28.8 for two positive results, and LR + tive /LR – tive were 3.8/0.26 for a single positive result and 12.2/0.42 for two positive results. Consecutive positives show good specificity for the diagnosis of IA and could be used to trigger radiological and other investigations or for pre-emptive therapy in the absence of specific radiological signs when the clinical suspicion of infection is high. Importantly, the sensitivity of the test confers a high negative predictive value (NPV) such that a negative test allows the diagnosis to be excluded. Like galactomannan antigen detection, PCR has good NPV for excluding disease, but the low prevalence of disease limits the ability to rule in a diagnosis. As these biomarkers detect different markers of disease, combining them is likely to prove more useful. Meta-analysis confirmed that if both PCR and GM-ELISA were consistently negative, then the sensitivity (99%) was sufficient to exclude to IA, whereas the specificity when both assays were positive was 98% [17].

Two other systematic reviews are currently being undertaken by the FPCRI. One evaluating the diagnostic accuracy of PCR tests for PcP in respiratory tract specimens of immunocompromised patients. As PcP occurs in a range of immunocompromised, the aim of the study is to evaluate the performance of PcP PCR test according to patient population, PCR method and respiratory specimen tested. The second systematic review is aimed at evaluating PCR testing for the diagnosis of invasive candidiasis using blood specimens. This review will include studies dealing with populations with significant risk factors for invasive candidiasis, such as non-neutropenic intensive care unit patients, preterm newborns, paediatric patients, neutropenic cancer patients, patients with solid organ or haematopoietic stem cell transplantation and patients with others risk factors for invasive candidiasis. Both these reviews have been registered in PROSPERO, the international prospective register of systematic reviews maintained by the

National Institute of Health Research of the University of York. (polymerase chain reaction on respiratory tract specimens of immunocompromised patients to diagnose pneumocystosis. Available from http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42018087812; and, polymerase chain reaction for the diagnosis of invasive candidiasis using blood specimens. Available from http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42018088037).

Conclusions

Given that PCR was first reported for detecting fungi in the late 1980s, it has taken a very long time indeed to reach a level of maturity. However, the body of evidence has grown sufficiently to indicate that PCR for screening and for confirming a fungal aetiology provides as good test result as other biomarkers. Indeed, the collaborative efforts of many researchers around the globe have led to the technique being incorporated into the latest revision of the EORTC/MSG consensus definitions of invasive fungal diseases [38]. This should lead to wider acceptance of PCR at least for conducting clinical trials and undertaking epidemiological studies, if not for routine practice.

In contrast to better known biomarkers such as GM and BDG, PCR is likely to continue to be done mostly in centres of excellence using their own platforms. However, their results are likely to align and prove reliable and reproducible if they each adopt the protocols proposed by the FPCRI. Up until now, there has been a singular lack of commercial platforms for a variety of reasons, though this may change now that the community understands better how, and when, to employ PCR. Clearly, further work is needed to ascertain the utility of PCR in routine practice and, importantly, to determine whether or not combining it with other biomarkers, particularly GM, is optimal. It is also our expectation that PCR for detecting Mucorales, antifungal resistance in body fluids such as blood and BAL, but also in tissue, may increase its diagnostic value across the board. This and the ability to diagnose PcP and IC would go a long way towards attaining the long-held ambition of medical mycology to provide a comprehensive range of tests that can be relied upon to diagnose, at least, the common IFD and even some of those that are considered to be emerging. In short, PCR has a clear future and is close to achieving its full potential in laboratories, worldwide.

Compliance with Ethical Standards

Conflict of Interest P. Lewis White reports personal fees from F2G, Gilead, MSD and BOPA, received funding for travel and meeting attendance from Gilead, Launch Diagnostics, BOPA and Bruker Diagnostics and received research funding from Bruker Diagnostics. Dr. White received payment from F2G for providing diagnostic services outside the submitted work. Alexandre Alanio reports non-financial support from Astellas and personal fees from Gilead science outside the submitted work. In addition, Dr. Alanio has a patent on a means for diagnosing, predicting or monitoring Pneumocystis pneumonia issued. Laurence Millon reports support for travel to meetings from Gilead, personal fees from Gilead, support for travel to meetings from Pfizer, personal fees from Pfizer, and support for travel to meetings from MSD outside the submitted work. Rosemary Barnes being treasurer and Steering Committee member of the Fungal PCR Initiative. Joseph Peter Donnelly reports personal fees from F2G, Gilead and Pfizer outside the submitted work. Mario Cruciani, Rebecca Gorton, Volker Rickerts and Juergen Loeffler declare no conflicts of interest relevant to this manuscript.

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

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