



Update on Non-Culture-Based Diagnostics for Invasive Fungal Disease

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Abstract Diagnostic tests for fungi provide the mycological evidence to strengthen diagnosis of invasive fungal disease. Conventional microbiology and histopathology have their limitations. Recognizing this, there have been attempts at developing new methods to improve yield of diagnosing invasive fungal disease (IFD). The recent focus has been on non-culture-based antigen detection and molecular methods. The use of antigen detection of IFD through 1,3- β -D-glucan and galactomannan assay have been expanded, followed by development of lateral flow assays, and in combination

with other diagnostic modalities to further increase diagnostic yield. The molecular diagnostic front has seen initiatives to standardize polymerase chain reaction methodologies to detect fungi and anti-fungal resistance, new platforms such as the T2Candida Biosystems and foray into fungal metagenomics. As these newer assays undergo stringent validation before incorporation into the diagnostic algorithm, the clinician needs to be mindful of their bedside utility as well as their limitation.

Keywords Beta glucan · Galactomannan · T2Candida · Lateral flow · PCR

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Introduction

Invasive fungal diseases (IFD) are conventionally encountered in hosts with altered immunity, including patients with haematological malignancies, acquired immunodeficiency syndrome, stem cell or organ transplant recipients and those on long-term immunosuppressive therapy. More recently, additional susceptible cohorts have been identified, such as patients in intensive care units or recipients of novel immunotherapies. Presenting as localized or disseminated infection, IFD cause significant morbidity and mortality. Due to the altered capacity to mount an immune response in at-risk patients, the clinical presentation may be varied. Furthermore there can be difficulty in obtaining representative specimens for sampling; hence diagnosis can be challenging. The consensus

case definitions on IFD by the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC/MSGERC) [1, 2]—while not intended for use in directing patient management—underscore how making a diagnosis of IFD is not always straightforward. Supporting mycological diagnostics play a pivotal role in strengthening likelihood of the diagnosis, determining whether cases can be upgraded from a “possible” to either “probable” or “proven” IFD. For this reason, a specific diagnostic test is adopted into the EORTC/MSGERC case definitions as supporting mycologic evidence only after detailed validation. In this review we give a brief update on the recent development of IFD diagnostics, focusing on antigen-detection and molecular methods.

Antigen Detection

Optimizing Existing Assays

1,3-β-D-Glucan

The utility of the pan-fungal biomarker 1,3-β-D-glucan (BDG) has been well-covered in multiple reviews [3, 4]. BDG is a major cell wall constituent of many pathogenic fungi, excluding *Cryptococcus* species, *Blastomyces* species, and *Mucorales*. It is now used in the diagnosis of various IFD, in particular invasive candidiasis, aspergillosis, as well as *Pneumocystis jirovecii* pneumonia (PJP) [5, 6]. Briefly, serum BDG has a sensitivity and specificity of 96% and 84% respectively for the diagnosis of PJP, and an overall 80% sensitivity 82% specificity for the diagnosis of IFD [6]. Having been conventionally assayed in serum samples, BDG measurement in bronchoalveolar lavage (BAL) and cerebrospinal fluid (CSF) is being explored. Possibly because of its plurality in a range of pathogenic fungi and environmentally, recent systematic reviews found BDG measurement in BAL and CSF to be a marginal or non-specific fungal marker, necessitating additional organism-specific testing such as molecular diagnostics in conjunction to aid specific diagnosis of IFD [7].

Beyond its value for diagnosis, serum BDG might also have a role as a prognostic marker. Giacobbe et al. in a retrospective study showed that an initial serum BDG of > 287 pg/mL in candidaemic patients had a 4.4 times higher 28-day mortality compared to those

who did not [8]. Persistently negative serum BDG level in candidaemic patients was associated with a better clinical outcome and prognosis compared to patients who presented with any positive BDG test [9]. Notably, decreases in serum BDG levels during anti-fungal therapy were predictors of successful treatment outcomes in invasive candidiasis and aspergillosis [10, 11].

Aspergillus Galactomannan

While primarily used in diagnosis of invasive aspergillosis, galactomannan (GM) is present as cell wall moiety in a variety of fungi. As such some non-*Aspergillus* fungal infections such as fusariosis, histoplasmosis, talaromycosis and blastomycosis can yield elevated GM levels [12]. In the appropriate setting, the clinician needs to be mindful to consider such fungi in their differentials beyond *Aspergillus*. Guidelines and reviews on GM and its role in the diagnosis of invasive aspergillosis (IA) have been published [12, 13]. Conventionally, GM measurement is performed on serum and bronchoalveolar-lavage samples using the Platelia *Aspergillus* ELISA (Bio-Rad Laboratories). Serum GM testing permits interval surveillance of at-risk patients but its sensitivity is generally lower, in excess of 70% in neutropenic patients, as compared to BAL GM. In non-neutropenic patients however, the sensitivity yield from serum GM is further reduced to less than 40%. In both serum and BAL, the specificity of the test approaches 90% [14, 15]. The sensitivity of GM testing might also be reduced if the patient is receiving mould-active prophylaxis [16]. Extending from its utility as a diagnostic tool, serial galactomannan kinetics can be used to prognosticate IA treatment outcomes [17].

An alternative commercial assay is the CE-marked *Aspergillus* Antigen ELISA developed by EUROIMMUN. This new *Aspergillus* galactomannoprotein (GP) ELISA is based on an alternative JP5 antibody (in contrast to BioRad's EB-A2 antibody) active against extracellular glycoprotein secreted by actively growing *A. fumigatus*, and is thought to be more specific against *Aspergillus* over other non-*Aspergillus* fungi and potential contamination by beta-lactam antibiotics [18]. In a comparative study on sera specimens between the Bio-Rad GM and Euroimmun GP assays [19], both had similar sensitivities and

specificities; the newer GP assay though did not outperform the GM assay.

New Platforms for Antigen Detection

Lateral Flow Devices for Aspergillus and Cryptococcus

Recently, two lateral flow assays (LFA) have been developed for the diagnosis of IA—the AspLFD (OLM Diagnostics, Newcastle Upon Tyne, United Kingdom), which detects an extracellular mannoprotein antigen secreted during active growth of *Aspergillus* spp, and the LFA-IMMY (IMMY, Oklahoma, USA), which detects *Aspergillus* galactomannan. The advantage of such LFA platforms lies in their field-deployability as point-of-care kits over traditional laboratory-based ELISAs and molecular diagnostics. The sensitivity and specificity of the AspLFD from BAL fluid ranged from 71–82% and 86–96%, respectively, for diagnosis of IA, whereas that of LFA-IMMY were 92% and 91%, respectively, for BAL, and 96.9% and 98%, respectively, when tested on serum samples [20–22]. In head-to-head comparisons, the LFA-IMMY was found to have slightly higher sensitivities (83–87% vs 69–73%), identical specificities though with better negative predictive value (89–91% vs. 81–82%) than the AspLFD [23, 24]. Use of digital readout further improved the diagnostic performance of both tests. As promisingly, a non-invasive galactofuranose-based monoclonal antibody LFA using urine for detection of pulmonary IA yielded sensitivity of 80% and 92% specificity [25].

Cryptococcosis is associated with pulmonary disease and meningitis, the latter especially in the HIV-infected population. Obtaining a microbiological diagnosis has, till now, been largely dependent on culture, India ink microscopy, and cryptococcal antigen (CrAg) testing on clinical samples. CrAg historically has been identified using latex agglutination, but in more recent years, the LFA has also become the default test in view of its increased sensitivity, ease of use requiring a minimal set-up, quick turnaround time, and cost effectiveness. In a validation study, the CrAg LFA was found to perform best compared to culture and latex agglutination, with a sensitivity and specificity of 99.3% and 99.1%, respectively, [26]. High blood antigen titres are also predictive of cryptococcal

meningitis and mortality, especially in HIV-patients, although there is no correlation between titre trends and clinical response to treatment, and hence serial monitoring of antigen titres provides limited prognostic value [27, 28].

Talaromyces Immunoassay

A novel enzyme immunoassay (EIA) for the detection of *Talaromyces marneffe* yeast cell wall galactomannoprotein, the Mp1p antigen, has shown promising results in a retrospective study with improved sensitivity (86.3% vs. 72.8%) and time-to-diagnosis (6 h vs. 6.6 days) compared to blood cultures [29]. Established antigen testing assays for diagnosis of other endemic mycoses have not been included within the scope of this review.

Molecular

Aspergillus

The incorporation of the *Aspergillus* PCR as a component of the mycological evidence in the latest EORTC/MSGERC case definition had been the product of the European *Aspergillus* PCR Initiative (EAPCRI) [2]. The EAPCRI had sought to optimize test replicability through standardizing *Aspergillus* PCR methodologies for specimen types (whole blood, serum/plasma, bronchoalveolar lavage fluid), including defining sample volume, extraction and elution conditions, and amplification processes [30]. The commercial *Aspergillus* PCR assays have variable sensitivities but their specificities are generally high approximating above 80% [31, 32]. Between performing one or two tests, studies have reported sensitivities and specificities in the range of 76.8–88% and 75–94.5%, respectively, for a single positive PCR test; two positive tests significantly improved the specificity from 76 to 95% [33, 34].

Cryptococcus

Recently, a multiplex PCR performed directly on CSF samples, the FilmArray meningitis/encephalitis panel (Biofire, Salt Lake City, Utah), of which *Cryptococcus*

is a target, has been utilized in the diagnosis of cryptococcal meningitis. In a systematic review, the PCR was found to have a high positive predictive value for detecting cryptococcal disease, although it had a high proportion of false negative determinations which was deemed due to patients who were already on treatment [35]. Where there is high clinical suspicion, cryptococcal antigen testing should still be performed even if the PCR is negative.

Mucorales

Mucormycosis typically affects severely immunocompromised patients, typically patients with haematological malignancies, solid organ transplants, and poorly controlled diabetes mellitus. It is difficult to diagnose and associated with high morbidity and mortality. By the bedside, diagnosis and empirical treatment are conventionally made based on clinical suspicion and radiological evidence as histopathological- and culture-based diagnoses have their limitations. MucorGenius® (Pathonostics, Maastricht, The Netherlands) is a newly developed *Mucorales* multiplex real-time PCR assay which detects the Mucormycetes *Rhizopus* spp., *Mucor* spp., *Lichtheimia* spp., *Cunninghamella* spp., and *Rhizomucor* spp., from BAL fluid, biopsy and serum samples. In a small retrospective with 10 patients confirmed with pulmonary mucormycosis, the MucorGenius®PCR had a sensitivity and specificity of 90% and 97.9% from pulmonary samples [36]. In another retrospective study by Mercier et al., the assay was also able to detect circulating *Mucorales* DNA in the blood of patients with invasive mucormycosis with an overall sensitivity of 75% [37]. More importantly, a positive result could be detected in patients up to 81 days (median 8 days, IQR 1.75–16.25) before the first positive culture result, and up to 24 days (median 3 days, IQR – 0.25–8.5) before radiological evidence of invasive disease.

Candida

Blood cultures have been the gold standard for diagnosing candidaemia as in the intensive care unit (ICU), but is limited by its sensitivity and turn-around time [4]. The *Candida* nucleic acid detection test, T2Candida (T2 Biosystems, Lexington, MA) is a fully automated, novel PCR-based system which hybridizes the genetic material to superparamagnetic nanoparticles

with detection via T2 magnetic resonance [38]. The detection limit is said to be up to 1 colony forming unit per mL of blood. The platform detects the major pathogenic *Candida* spp. (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*; and more recently, *C. auris*) directly from whole blood samples within 5 h [39]. From a recent meta-analysis, the pooled sensitivity and specificity of the T2Candida system were 0.91 (95% CI: 0.88–0.94) and 0.94 (95% CI: 0.93–0.95), respectively [40–42]. Importantly, T2Candida was shown to be advantageous in the setting of patients already receiving anti-fungal treatment, yielding T2Candida-positive but blood culture-negative results [41]. Its high negative predictive value in excess of 98% could also be a means for exclusion of *Candida* infection. A new, research-use only T2 Magnetic Resonance assay has been shown to detect *C. auris* on clinical skin swab samples with a sensitivity and specificity of 89% and 98%, respectively [43]. While the T2Candida system has been shown to be superior to conventional diagnostics including blood culture and BDG [44], its main drawbacks are the requirement for hardware setup and its current high cost per test.

Detection of Resistance

The antigen detection and molecular methods available are useful for determining the presence or absence of invasive fungal infections. However, one limitation is that most do not identify presence of resistance. Identification down to species level of some yeasts and moulds may be useful to pick up the species-associated intrinsic resistance, but not on acquired resistance. Acquired anti-fungal resistance can vary across individual institutions. Probably because culture-based anti-fungal susceptibility testing are most well-established for *Candida* [45], there are currently no commercial kits for identification of resistance traits such as ERG and MDR in *Candida* spp. Among moulds, acquired azole resistance has been most extensively described in *A. fumigatus*. At least two commercial assays are available, targeting mutations in the *cyp51A* gene: AsperGenius® (PathoNostics, Maastricht, Netherlands) and MycoGenie® (Ademtech, Pessac, France) [46–48]. However, their performance in terms of sensitivity in identifying the various mutations has been variable [47, 48].

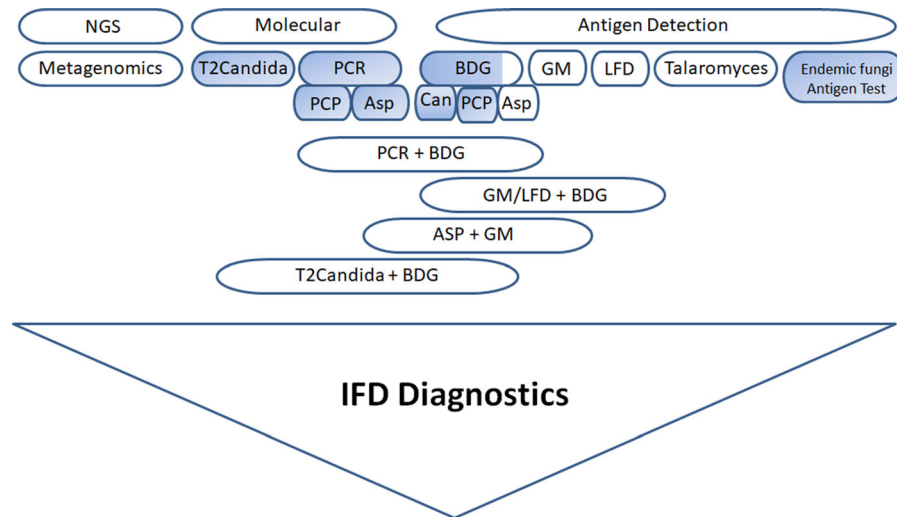


Fig. 1 Non-culture, non-histological diagnostic modalities for invasive fungal diseases (IFD). Shaded: test recommended by EORTC-MSG as mycological evidence for IFD. NGS next generation sequencing, PCR polymerase chain reaction, PCP

pneumocystis jiroveci, asp aspergillus, can Candida, BDG beta-D-glucan, GM platelia galactomannan, LFD lateral flow devices, T2Candida T2Candida biosystems

Combination Testing

An array of investigations are commonly deployed by the bedside to optimize diagnosis of IFD. A biomarker-based strategy combining blood *Aspergillus* galactomannan and PCR testing (versus conventional culture/histology-based or galactomannan alone) to guide empiric treatment in transplant and leukemia patients had led to significant reduction of anti-fungal usage [49, 50] or earlier diagnosis of IA [50]. Data looking at BDG, PCR, GM or Asp-LFD testing in various combination have been varied and less clear-cut [51, 52], purporting possible negative predictive utility for GM, though this may be queried in the practice of mould prophylaxis. In the setting of invasive candidiasis or intra-abdominal candidiasis whereby even T2Candida sensitivity may be compromised, diagnostic yield may be enhanced in conjunction with antigen testing (BDG, mannan) and conventional culture [44, 48, 53].

Metagenomics

Next generation sequencing (NGS) technology will inevitably find its role in diagnostic microbiology. The capability to elucidate the fungal mycobiome from clinical specimens may greatly enhance the non-

culturable diagnostic sensitivity. A proof-of-concept study using internal transcribed spacer (ITS) amplicon sequencing and custom analysis pipeline demonstrated that it was technically possible to detect *Blastomyces*, *Talaromyces*, *Aspergillus* and other fungi from BAL [54]. The predominance of *Candida* and/or *Aspergillus* had been detected in the BAL of ICU or lung transplant patients [55, 56]. However their presence did not portend invasive disease and that the colonizing and invasive *Candida* strains from candidaemic patients did not always match. Upon detection, distinction between colonization and infection would still be subject to clinical discretion at this point. Ahead, our ability to further discriminate and interpret metagenomics returns, reduce cost and shorten turnaround time of clinical specimens will determine when NGS will eventually establish its foothold in the clinical diagnostic laboratory.

Summary

Culture-based methods of fungal detection remain the gold standard in the diagnosis of IFD. However, this is being progressively supplemented by an increasing diagnostic array of surrogate markers of fungal infection, including antigen and molecular assays, as well as in combination (Fig. 1). Newer assays ought to

be rigorously validated before their incorporation into the diagnostic algorithm. Clinicians will need to keep abreast of the clinical utility and advantages of these assays, as well as their limitations. Of importance, the utilization of these tests and interpretation of their results should always be in conjunction with clinical and radiological assessments of the patient.

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