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FUNGAL INFECTIONS IN TRANSPLANTATION (S SHOHAM, SECTION EDITOR)

Diagnostics for Fungal Infections in Solid Organ Transplants (SOT)

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Abstract

Purpose of Review This review examined the literature on the diagnosis of invasive fungal disease (IFD) in patients undergoing solid organ transplants (SOT) to describe the diagnostic options available for this cohort.

Recent Findings The tools available for the diagnosis of IFD in SOT patients are similar to those for patients undergoing stem cell transplants. These include (1) direct visualisation by radiography or histopathology, (2) antigenic tests using ELISA or lateral flow devices for fungal antigens, and (3) PCR-based assays that are commercially available for the two primary IFD affecting SOT patients, aspergillosis and candidiasis. Testing recipients and donors for IFD susceptibility may lead to improved prediction of IFD in SOT.

Summary The organ being transplanted has a strong bearing on the risk of IFD and the fungi that will cause disease. No single methodology can yield a definitive diagnosis so combinations of diagnostic tests targeted to the specific patient can indicate the probability of IFD.

Keywords Diagnosis · Invasive fungal disease · qPCR · Solid organ transplant · Invasive aspergillosis

Introduction

Immunosuppression is the greatest risk factor for the development of potentially fatal invasive fungal infection (IFI). Immunosuppression is usually medically induced to enable stem cell transplants (SCT) or solid organ transplants (SOT) to be undertaken; however, genetic disorders, e.g. chronic granulomatous disease, or prolonged use of corticosteroids are also among the risk factors for IFI [1–3]. The incidence and epidemiology of IFI in SOT have been reviewed elsewhere [4–7]. The most common cause of IFI in SOT is *Candida* spp. with the most common mould being *Aspergillus* spp. [8•]. An issue for IFI in SOT is that the infection may develop > 1-year post-transplant; this creates challenges for treatment and diagnosis [8•]. In this short review, the focus will be on the breadth of diagnostic methods available for IFI in SOT, not all of these would be available in community health care when the disease may present an extended period after the transplant.

Unlike SCT, there is a greater diversity of SOT; those of primary interest include lung, heart, kidney, and liver [4]. The type of transplant is the first indicator of the probable cause of IFI since *Candida* spp. are the most frequent cause in all transplants except for lung transplants where invasive aspergillosis (IA) is more common [8•]. Timing of IFI can also be instructive because infections caused by *Candida* usually occur earliest followed by *Aspergillus*, then *Pneumocystis* pneumonia; IFI > 1-year post transplants also include zygomycosis, cryptococcosis, and infections by endemic fungi [9]. The timing to onset of IFI can be affected by antifungal prophylaxis leading an increasing incidence of later development of these infections [10].

The most important causes of IFI are species of the genera *Candida* and *Aspergillus* (in the 2010 TRANSNET study, 53% of IFI were invasive candidiasis and 19% were IA [8•]); however, there are also increasing reports of *Scedosporium*, *Fusarium*, *Mucorales*, and *Pneumocystis* infection in SOT [2, 4]. The numbers of species from *Candida* and *Aspergillus* that cause IFI are also increasing with several being relatively drug resistant, and antifungal drug resistance has been emerging across these genera, e.g. azole resistance in *Aspergillus fumigatus* [11•, 12,

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13•]. This diversity of fungi that can cause IFI in SOT is a challenge as species identification and detection of drug resistance are also desirable.

The diagnostic methods and samples used for SCT and SOT are relatively similar with a focus on blood, blood derivatives, and bronchoalveolar lavage (BAL); some diagnostic strategies utilise urine or exhaled gases [14, 15]. In all non-culture methods, the aim is to detect evidence of fungal infection in the host; these methods include imaging (computerised tomography (CT) scans), polymerase chain reaction (PCR), fungal antigen detection by enzyme-linked immunosorbent assay (ELISA), or lateral flow devices [16••, 17]. These methods span a wide range of fungus- or host-derived molecules, yet few are able to give rapid and definitive diagnosis of IFI.

The absence of definitive diagnostics has created a situation where directed treatment is relatively rare, creating an overuse of antifungal drugs through empirical therapy and widespread use of antifungal prophylaxis. This also complicates diagnosis since the use of antifungal prophylaxis interferes with the performance of diagnostic assays [18•, 19].

Diagnosis

There are several challenges related to the diagnosis of IFI in SOT; the incidence of disease varies between different transplant types, the immunosuppressive regimen being employed, and the use of prophylaxis are but a few [4, 20•]. The great variability in patients susceptible to IFI means that diagnostic strategies should be tailored to the individual case rather than a generic 'one size fits all' approach. Clinical information on the patient allied to diagnostic tests provide the greatest probability of accurate diagnosis.

Diagnosis of IFI in transplant patients relies on a range of tests and patient observations that yields a probability of infection; this has been described in the European Organization for Research and Treatment of Cancer and the Mycoses Study Group (EORTC/MSG) system for classification of IFI [16••]. In the absence of a single test, we must consider a range of options from culture or direct observation of the fungus in a patient sample to molecular methods [21]. Given the need for early detection to improve treatment outcomes [22], the time to diagnosis cannot be reduced if methods need to have visible signs of fungal infection; this creates a need for sensitive molecular diagnostic methods.

Medical Imaging

One of the key factors to take into consideration when diagnosing IFIs in SOT recipients is their immunocompromised state, as such the use of any diagnostic modality should be minimally invasive. Medical imaging plays a key role in these clinical situations. While various imaging techniques (X-ray, ultrasonography, CT, and MRI) are available, radiography is usually the first step in any diagnostic workflow. Unsurprisingly, radiological findings can vary significantly due to a variety of host factors [23••]. However, any deviation from a normal healthy state in the area undergoing radiography should be followed by computed tomography (CT). CT is the modality of choice due to its higher resolution and sensitivity at detecting abnormalities [24]. The role of CT imaging has been a key tool since the 1980s in the early detection of IFIs [25]. A number of CT studies have been carried out on smaller numbers of SOT recipients with both proven and probable IPA diagnoses to help define the 'typical' imaging signs [23••, 26•]. It has been found the most common CT finding to be consolidation or mass, which tended to present 1-month post-transplantation, and the second was large nodules, followed by ground-glass attenuation commonly referred to as 'halo sign' [23••]. Similarly, irregular lung nodules surrounded by the 'halo sign' were present in 80% of SOT recipients confirmed with IPA diagnosis [26•]. The same study also noted the presence of regular nodules, patchy consolidation, and cavity. While these studies were undertaken to help define 'typical signs' of infection, these are not always present and vary depending on whether a patient is neutropenic or non-neutropenic [23••, 27]. Furthermore, the same signs can also be associated with other infectious agents as well as non-infectious disease [28]. Regardless of their non-specific nature, these signs should raise suspicion of IFI in this patient group which can be followed up with further diagnostic examination.

Visualisation and Culture

As mentioned above, diagnosis of IFI is guided by the EORTC/MSG classification system which serves to categorise a given case as 'proven', 'probable', or 'possible' [16••]. Classically, the requirements for a case to be defined as 'proven' include the visualisation of fungal fragments, such as hyphae, within tissues via microscopy and the recovery/culture of the etiological agent from a sterile site within the SOT recipient. When direct microscopic observation does occur, identification can often be inconclusive due to the high level of similarity shared amongst

a number of the filamentous fungi such as *Aspergillus*, *Fusarium*, and *Scedosporium* for example. An ideal clinical outcome would be the recovery of a fungal isolate on culture media enabling a number of other tests (antifungal susceptibility testing, PCR, qPCR, or mass spectrometry [MS]) to be carried out enabling definitive identification. However, even this can be complicated by the fact that many SOT recipients suffering IFI such as invasive aspergillosis (IA) or candidiasis return negative cultures in up to 20–50% of case [20•, 29]. It is also important to consider that there are a number of organisms such as *Pneumocystis jirovecii* that cannot be cultured [20•]. It is not uncommon for SOT patients, despite having active disease, to be negative for both microscopy and culture. As a result, meeting the 'proven' criteria is impossible; hence, the inclusion of the 'probable' and 'possible' criteria EORTC/MSG classification system requires alternate methods of detection. In terms of positive patient outcomes, timely diagnosis is key to enabling either the clinical implementation of antifungal therapy or the withdrawal of treatment should therapy be successful.

Sample Types for Molecular Detection

For the application of nonculture-based methods, it is important to determine the best sample type in a particular case. Blood, sputum, BAL, and urine are available with BAL being the most demanding on the patient and less easily obtained at a high frequency (usually twice weekly is recommended [30•]). Sputum and BAL are associated with pulmonary infection and so would be most beneficial in lung transplants; the lung also has a transient microbiome that can create the risk of false positive results [31•]. Blood is routinely collected in healthcare settings and gives access to fungal diagnostic targets that may originate from any transplanted organ. Studies have shown that both serological and PCR-based tests can be performed with excellent results from serum [32•] linking these tests to existing workflows within the healthcare setting.

Serological Methods

Detection of pathogenic antigens in serum or other blood-derived samples is well established in the detection of microbial pathogens. The detection of fungal galactomannan has been implemented since the 1980s [33] and is still being refined and *Cryptococcus* antigen detection has also been effective [34]. More recent developments such as lateral flow devices create the possibility of point of care diagnostic options.

Galactomannan (GM) is a component of the cell wall of *Aspergillus* spp. released by hyphae as well as germinating spores/conidia and can be detected by a number of commercially available assays and formats (ELISA and lateral flow). GM detection has become the mainstay of IA diagnosis and can be measured using a number of bodily fluids including peripheral blood and BAL, with BAL being the preferred sample type [35••]. The most widely adopted platform for detection is the Platelia™ *Aspergillus* enzyme-linked immunoassay (PA-EIA: Bio-Rad, France). However, despite its widespread use over many decades, there is still some conjecture within the literature as to the accepted threshold for GM test index positivity (0.5 or > 1.0) [36]. Another compounding factor is that it is not uncommon for SOT recipients with *Aspergillus* positive cultures to produce negative GM test results when using serum [37]. As a result, a negative GM test should not be used to rule our IA. A number of meta-analyses have been carried out on the detection of GM in BAL. The majority to these studies involved patients with underlying haematological conditions and demonstrated a sensitivity and specificity ranging from 61 to 92% and 89–98%, respectively [38–40]. A similar study by Husain et al. (2007) focused on lung transplant recipients, which resulted in a sensitivity of 60% and a specificity of 95–98% depending on GM cut-off index used (0.5 versus 1.0) [41]. The use of a lower GM index cut-off, particularly from pulmonary samples such as BAL, has the potential to complicate diagnosis due to the number of individuals naturally colonised by this fungus. Thus, distinguishing active infection from colonisation is aided by the adoption of a GM index > 1.0 [36]. As a result, the EORTC/MSG recently updated their GM cut-off definition for BAL to ≥ 1.0 [16••]. As with many diagnostic tests, GM testing is not without its limitations. Potential impacts to test results include the patient's immunological status particularly when using serum (neutropenic or non-neutropenic), the commencement of antifungal therapy, and extended turnaround times [29].

More recently, a novel *Aspergillus* GM lateral flow assay (LFA) (LFA; IMMY, Norman, Oklahoma, USA) has been developed enabling point-of-care (POC) diagnostic testing to be undertaken with good agreement between this the GM ELISA test (for review, see White et al. 2019) [35••]. In a study by Mercier et al. (2020), the IMMY LFA was tested using BAL samples from previous ICU patients and it was found that the LFA test had good sensitivity (88–94%) and specificity (81%) [42•]. The same authors suggested this methodology be used as a rapid screening diagnostic while awaiting other microbiological results.

The diagnostic repertoire specific for detecting *Aspergillus* has been further expanded by the introduction of another POC test (*Asp*LFD; OLM Diagnostics, Newcastle Upon Tyne, UK). This LFD test incorporates a monoclonal antibody, Mab JF5, used to detect an *Aspergillus*-specific antigen released by growing hyphae and can be applied to both serum and BAL [43, 44]. A meta-analysis by Pan

et al. (2015) of several studies for proven/probable versus no IA demonstrated a pooled sensitivity and specificity of 95% and 95%, respectively, for both serum and BAL for this test [45]. Both POC tests have the added advantage over the GM ELISA in that they require basic laboratory training and equipment, are cheaper, and have a more rapid turn-around time [35••]. These POC options greatly enhance diagnostic flexibility as many SOT recipients start to feel ill due to IFIs months to > 1-year post-transplantation. It is not unusual for their hometowns to lack the substantial diagnostic capabilities of the high-tech transplantation centres, making these tests vital alternatives. While the detection of GM remains a key test for *Aspergillus*, it cannot be used to detect a number of other clinically relevant fungal pathogens due to the fact that they do not produce this polysaccharide.

Candida is the most frequent fungal pathogen encountered in SOT recipients [6]. Blood culture plays a key role in the detection of candidiasis; however, it is not uncommon for cultures to be negative in up to 25–50% of cases [20•]. A key adjunct test to enable the detection of *Candida* spp. has been the β-D-glucan (BDG) test. 1,3 β-D-glucan is a cell wall component of most fungi and thus should be viewed as a non-specific test. Carried out on serum as opposed to BAL, it can detect several fungal species including *Candida* spp., *Aspergillus* spp., and *Pneumocystis* [4]. Meta-analysis of the utility of the BDG test in diagnosing IFIs from serum demonstrated a pooled sensitivity of 80% and a specificity of 82% [46]. Due non-specific nature, this test is often used as the first stage of a diagnostic pipeline and as with many tests also has its limitations [6].

Another POC test that has had a significant impact on diagnosis of cryptococcosis has been the cryptococcal antigen (CrAg) test [35••]. This has been an important advancement due to the low sensitivity of culture-based methods coupled with the fact that cryptococcosis is the third most common IFI in SOT patients [47]. As with other lateral flow devices described above, this test is easy to perform and can be used on both serum and cerebral spinal fluid (CSF). Critically, this test displays excellent sensitivity (99.3%) and specificity (> 99%) when performed on serum and CSF enabling clinicians to either rule in or exclude this fungal pathogen as a cause of disease [35••, 48, 49].

As is evident from above and the sheer number of reviews and articles written on this subject, the diagnosis of IFI remains a significant challenge. This is not helped by absence of stand-alone tests that can be utilised to provide a definitive diagnosis in many cases. When combined with the wide variation in organ transplant types, the clinical specimens used for diagnosis, and the diversity of potential causative agents coupled with the spectrum of clinical symptoms presented by SOT recipients, the challenge is immense. At present, diagnosis requires a combination of diagnostic tests [35••]. However, even this is not clear-cut strategy due to the extra costs involved in testing as well as the potential for introducing conflicting results further delaying the commencement of antifungal therapy.

Molecular Methods

An extension of the concept of detecting evidence of fungal infection is the detection of fungal nucleic acids. This has been an extremely fertile area of research and is still developing with the use of low-cost next-generation sequencing (NGS). Detailed reviews of molecular tests for diagnosis of IFI have recently been published [50•, 51•].

The promise of polymerase chain reaction (PCR)-based diagnosis of IFI has made it the subject of research studies for over 30 years [52]. An area where PCR is well-established for identification of moulds and yeasts is from tissue samples where amplification and sequencing are criteria for definition of proven IFI [16••]. However, this does not have the early detection benefits of qPCR.

It has taken extensive testing for qPCR-based diagnostics to be accepted as an appropriate diagnostic strategy for invasive aspergillosis (IA) [16••, 18•]. IA standardisation was critical since each lab using qPCR was employing different methodologies, standards, and sample types creating inconsistency in the results [52, 53]. This standardisation has created a framework that can be applied to other IFI and in the development of commercial assays.

qPCR has been included in the revised EORTC/MSG definitions for diagnosis of IA from blood, plasma, serum, and BAL and is also accepted for *Pneumocystis* infections [16••]. Commercial assays available for IA [54] or in-house assays should follow the recommendations of the FPCRI [18•, 30•]. Other moulds have fewer options; there has been research into diagnostic assays for the *Mucorales* but further research is required [55, 56]. Diagnosis of IFI caused by *Candida* spp. is potentially easier to detect than IFI caused by moulds since it can grow in blood culture; there are several diagnostics for invasive candidiasis including commercial assays [57]. An evolution of PCR detection is the T2*Candida* platform which combines target DNA amplification and magnetic resonance; this is an automated system that can identify up to five *Candida* spp. from EDTA-blood samples; the initial clinical trial indicated 99.5% specificity and 91.1% sensitivity [58]. Further research confirmed the utility of the assay compared to blood culture [59]; however, it will require continued research to validate this platform.

The majority of diagnostic tests focus on a single species or small groups of organisms that commonly cause IFI but the increasing number of fungi causing IFI means that panfungal approaches may be of the greatest importance for the future of IFI diagnosis in SOT. Development of assays that target greater numbers of species has been investigated with multiplex PCR strategies that have shown excellent

potential sensitivity and specificity but there is the challenge that these will be limited to a relatively small panel of target fungi [60]. There are issues of analytical specificity when targeting multiple fungi by PCR since there can be cross reactivity between assays and non-target species; this was apparent in efforts to develop pan-*Aspergillus* assays [61]. The use of PCR-based diagnostics presents a problem; qPCR is excellent at detecting minute quantities of target DNA in a sample but cannot provide accurate identification for a large number of target fungi. Using pan-fungal primers followed by sequencing of the qPCR product could be used to address this problem but would introduce time penalties associated with additional methodological steps. One solution to this is the use of high-resolution melt curves and panfungal primers similar to the multiplex PCR assay mentioned earlier [60]. A drawback with PCR-based assays is cost; a retrospective audit of the cost of panfungal PCR found a cost-per-diagnosis of AUD15,978 per year [62••]. Targeted use of molecular diagnostics to at-risk patient cohorts would be prudent given the financial implications of broad use of these methods.

A further diagnostic solution is the combination of PCR with mass spectrometry (MS); an assay for over 230 fungi was described that used the mass of PCR products linked to a database of expected masses to detect and identify fungal pathogens. The assay achieved 90.9% sensitivity and 82.3% specificity when tested in patient BAL specimens [63]. Though these values are adequate, the assay had a negative predictive value of 96.9%, so it may have potential to rule out the presence of IFI; this is the approach recommended for qPCR assays for IA [18•]. The use of MS for the identification of fungal pathogens is also being developed; however, it is generally reliant on having fungal biomass for organism identification [64]. An interesting development is the use of MS for the detection of a fungal-specific dihexasaccharide (DS); this was performed as well as standard tests for galactomannan and β-D-glucan, with the advantage that it could be used for candidiasis, aspergillosis, and *Mucor* mycosis [65•, 66]. The DS remained in circulation longer than fungal DNA after antifungal treatment; this could be an important consideration since prophylaxis and treatment have a negative effect on other molecular diagnostics.

Whole-genome sequencing in diagnosis for invasive fungal disease has been covered in a previous review [67]. It has similar applications in samples from SOT patients allowing identification of the fungus, virulence factors such as antifungal resistance mutations, and epidemiological analysis.

Host Factors

Another approach to diagnosis of infectious diseases is to look for host responses characteristic of infection. Assays such as QuantiFERON determine if the host has been exposed to a pathogen such as *Mycobacterium tuberculosis* through the production of cytokines (γ-interferon) based on the response to pathogen-specific antigens [68]. There has been a search for characteristic cytokine profiles indicative of IFI in at-risk patients. Initial studies of host response to IFI examined cytokine profiles in patients undergoing SCT; these looked at cytokines released into BAL and serum in patients with IA and those with no evidence of IFI [69, 70]. Increased IL-8 was identified as a potential biomarker for IA in BAL from at-risk patients. However, a further study in lung transplant patients found a different cytokine profile including IL-12 and IL-1RA; this indicates that the immune responses vary between SCT and SOT [71]. These studies focused on IA so other IFI may induce different cytokine profiles; further research is required before there can be cytokine-based diagnostics for IFI in SOT.

An indirect host factor that can help to predict the risk of IFI is host genetic susceptibility. There have been several genome-wide association studies (GWAS) to link infectious diseases to genetic variants in humans. Polymorphisms in genes such as *CARD9*, *TLR-4*, and *CXCR1* are associated with the development of IFI; *CARD9* mutations have emerged as important in susceptibility to fungal infection [72••]. Further polymorphisms in *TNFSF4* and *MAPKAPK2* were associated with an increased risk of IA [73]. Additional polymorphisms carrying a risk of IFD have been collected together in reviews on this topic looking at specific fungi, e.g. IA [74] and IFI in general [75, 76]. qPCR or genomic sequence screening of recipients and donors prior to SOT could be used to identify the presence of risk alleles, which would assist in patient management.

Conclusions

In the absence of definitive tests for diagnosing some of the most important fungal pathogens in SOT patients, there is a need to combine several strands of evidence to achieve a diagnosis. The choice of diagnostic tools will be dependent on the availability of expertise in some treatment centres. An approach that combines knowledge of the type of transplant, host and donor genotyping, visualisation (CT scan or histopathology), antigen testing, or qPCR may yield an effective diagnosis.

Declarations

Conflict of Interest The authors declare no competing interests.

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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