Diagnosis of Invasive Fungal Infections – Current Limitations of Classical and New Diagnostic Methods

a report by

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Despite the availability of new antifungal drugs, the overall survival for immunocompromised patients with invasive fungal infections remains too low, with large variations according to underlying disease.[1–15] Although early diagnosis and subsequent early initiation of therapy improves outcome,[16–19] diagnosing invasive fungal infections can be difficult. The purpose of this article is to review the available armamentaria for the diagnosis of invasive fungal infections. A brief summary of the main clinical and epidemiological data for these infections is shown in *Table 1*.[3–7,12–15,20–56]

Diagnosis of Invasive Fungal Infection

Conventional Methods (Direct Microscopy, Culture and Histopathology)

All fungi obtained from sterile sites should be identified to species level by referral to a specialist laboratory. All bronchoscopy fluids from patients suspected of infection should be examined microscopically for hyphae and cultured on specialised media, and all clinical isolates of *Aspergillus* should be identified to species level.[57]

Current 'conventional methods' are very limited for the diagnosis of invasive fungal infections. Blood cultures have a low sensitivity for the diagnosis of candidaemia (~50%),[58,59] and cultures other than blood are non-specific and can take too long to became positive. Antifungal treatment is recommended following recovery of even one positive blood culture for *Candida*.[5] Identification of *Candida spp.* by culture requires the presence of viable organisms in blood or body fluids. In addition, several days may be required for blood cultures to become positive and, for non-*Albicans spp.* of *Candida*, additional subculturing is required to obtain pure cultures for use in subsequent phenotypic identification systems.[59]

Although the lungs are frequently involved in disseminated candosis, primary *Candida* pneumonia is a rare condition,[60,61] and benign colonisation of the airway with *Candida spp.* and/or contamination of the respiratory secretions with oropharyngeal material is much more common than true *Candida* pneumonia. Thus, diagnoses of *Candida* pneumonia that are based solely on microbiological data are often incorrect. In addition, debate persists about the significance of the isolation of *Candida* in the peritoneal fluid,[62] and the presence of *Candida* in the urine usually represents colonisation, despite its presence in 9% of hospitalised patients in the US.[63]

For the diagnosis of invasive aspergillosis, cultures of the respiratory tract secretions lack sensitivity. *Aspergillus* is grown from sputum in only 8% to 34%, and from broncho-alveolar lavage (BAL) in 45% to 62% of patients with invasive aspergillosis.[64] *Aspergillus* recovery from the respiratory tract usually represents colonisation in immunocompetent patients but may strongly suggest invasive disease in the immunocompromised host.[65,66] While confirmation of the diagnosis of invasive aspergillosis has typically required histopathologic evaluation, profound neutropaenia and thrombocytopaenia often preclude the pursuit of biopsies. Transbronchial biopsy or brushings are too often false negative. Biopsies of endobronchial lesions have been useful when such lesions are encountered. Blood, cerebrospinal fluid (CSF) and bone marrow specimens rarely yield *Aspergillus spp.*[33]

In contrast to disseminated aspergillosis, disseminated fusariosis can be diagnosed by blood cultures in 40% of patients.[12,15] The rate of positive blood cultures increases to 60% in the presence of disseminated skin lesions.[14] Microscopically, the hyphae of *Fusarium* in tissue resemble those of *Aspergillus*; the filaments are hyaline, septate and 3–8μm in diameter. They typically branch at acute or right angles. The production of both fusoid macroconidia and microconidia are characteristic of the genus *Fusarium*.[13] Skin lesions should be submitted to biopsy.

The diagnosis of zygomycosis is usually made histologically, and the demonstration of fungal elements from cytologic preparations is complicated by the difficulty of extracting fungal elements from invaded tissues.[11] The poor sensitivity of sputum culture (<25%) makes diagnosis of pulmonary

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Table 1: Clinical and Epidemiological Data for the Main Agents of Invasive Fungal Infection

	Main agents	Epidemiology	Major Risk Factors	Clinical Manifestations
Yeasts				
Candida[3–6,20–26]	C. albicans C. parapsilosis C. glabrata C. tropicalis C. krusei	*Increased incidence and shift to non-*Candida albicans spp. *Increased resistance to antifungal drugs.*	*CVC and renal support.* *Neutropenia, steroids, colonisation, antimicrobials, surgeries.*	*Non-specific.* *Sepsis not-responsive to antimicrobials.* *Typical fundoscopic lesions.*
Cryptococcus[7,27,28]	C. neoformans var. grubii. C. neoformans var. gattii.	*AIDS-defining disease;* C. neoformans var. grubii *is an opportunistic agent;* var. gattii *is a primary pathogen.*	*AIDS and other T-cell immunodeficiencies; steroids.* *Eucalyptus trees (*gattii*).*	*Meningoencephalitis.* *Pneumonia.* *Skin infection.* *Fever of unknown origin.*
Others[29–31]	Rhodotorula Trichosporon Pichia anomala Malassezia spp.	*Emerging infections in immunocompromised patients.* Pichia anomala *has been associated with outbreaks in paediatric ICUs.*	*CVC and parenteral nutrition (*Pichia anomala, Malassezia spp.*).* *CVC, cancer (Rhodotorula);* *Neutropenia, steroids.*	*Disseminated infection similar to that caused by* Candida spp.
Moulds				
Aspergillus[32–37]	A. fumigatus A. flavus A. terreus A. niger	*Leading infectious cause of death in leukaemia and BMT patients.* *Infection usually acquired by inhalation of spores.* *Increased resistance to antifungals.*	*Prolonged neutropenia.* *Transplantation, especially lung and BMT.* *HIV, steroids.*	*Usually starts as pneumonia or sinusitis.* *May disseminate, mainly to the CNS.* *Symptoms may be mild; a high index of suspicion is required.*
Mucorales[38–44]	Mucor Rhizopus Rhizomucor Absidia	*Rising incidence in some centres.* Mucorales *may enter the human host through inhalation, percutaneous inoculation or ingestion.* *Gastrointestinal and disseminated forms are rarely diagnosed antemortem.*	*Haematological malignancies, neutropenia.* *Immunosuppression, deferoxamine therapy.* *Diabetes mellitus (usually with ketoacidosis).*	*Rhino-orbito-cerebral zygomycosis (44% to 49%)* *Cutaneous (10% to 16%)* *Pulmonary (10% to 11%)* *Disseminated (6% to 12%)* *Gastrointestinal (2% to 11%)*
Scedosporium[45–47]	S. prolificans S. apiospermum *(*anamorph Pseudallescheria boydii*)*	S. prolificans *is resistant to practically all available antifungals.* *Colonisation in many patients with cystic fibrosis.*	*Prolonged and profound neutropenia.* *Surgery and trauma.* *Near-drowning (*S. apiospermum*).*	*Fever unresponsive to antimicrobials.* *Skin involvement.* *Dissemination to the CNS is very frequent.*
Fusarium[12–15,48–50]	F. solani F. moniliforme F. oxysporum	*Widely distributed (soil, plants, air).* *Main aetiology of fungal keratitis.* *High incidence of skin lesions (that should be sent to examination) and positive blood cultures.*	*Tissue breakdown from direct trauma.* *Onychomycosis caused by* Fusarium. *Neutropenia.*	*Disseminated infection may involve the skin (70% to 90%), and lungs and sinuses (70% to 80%).*
Others[31,51]	Paecilomyces Trichoderma Acremonium Scopulariopsis Microascus *Dematiaceous moulds*	*Emerging fungal infections in immunocompromised patients.* *Outbreaks of* Paecilomyces *infection associated with contamination skin lotion and intraocular lens.*	*BMT.* *Immunosuppression.* *Neutropenia.* *Use of CVC.* *Contaminated medical devices.*	*Ocular infection (*Paecilomyces*).* *Peritonitis in patients undergoing CAPD.* *Pneumonia, sepsis.* *CNS infection.*
Dimorphic Fungi				
Histoplasma[52]	H. capsulatum	*AIDS-defining disease.* *Endemic in certain areas of North and Latin America.* *Usually acquired by inhalation.*	*AIDS.* *Travel to endemic areas.*	*May be auto-limited.* *Pneumonia, sepsis, skin lesions, CNS.*
Coccidioides[53,54]	C. immitis C. posadasii	*Endemic in deserts in North-America.* *Usually acquired by inhalation of high number of spores.*	*Travel to endemic areas.* *AIDS.* *Haematological malignancies.*	*60-70% asymptomatic.* *Pneumonia.* *Disseminated infection.*
Paracoccidioides[55,56]	P. brasiliensis	*Restricted to Latin America;* *Affects mainly men (13:1).*	*Opportunistic infection in transplant recipients or AIDS patients.*	*May be auto-limited;* *Acute (subacute) juvenile form.* *Chronic adult form.*
Penicillium[31]	P. marneffei	*Endemic in South-east Asia.* *Bamboo-rats implicated in the epidemiology.* *Usually acquired by inhalation.*	*AIDS.* *Travel to endemic areas.*	*Low-grade fever, anaemia, weight loss.* *Skin lesions similar to molluscum contagiosum.*

Note: CVC, central venous catheters; AIDS, acquired immunodeficiency syndrome; ICU, intensive care unit; BMT, bone-marrow-transplant; CNS, central nervous system; CAPD, continuous ambulatorial peritoneal dyalisis.

zygomycosis challenging.[38] The yield of BAL is not higher,[38,67] but direct microscopy of BAL together with transbronchial biopsy may increase the yield.[43] A positive finding from BAL from a neutropaenic or immunocompromised host would be highly suggestive of infection, and should be treated as such.[43] Even though, on microscopy, Mucorales have been classically described as having broad (10 to 50μm), ribbon-like aseptate hyphae with right-angle branching, the hyphae are actually pauciseptate, and the angle of hyphal branching can vary from 45° to 90°, reinforcing the importance of obtaining material for culture.[68] About 80% of disseminated infections with *S. prolificans* are associated with positive blood cultures, but this proportion is much lower with *S. apiospermum* infections.[45]

All tissues from patients with suspected infection should be stained with fungal stains in parallel with regular stains. The practice of assessing H&E stains of tissues before deciding whether to use specialised stains for fungi frequently introduces fatal delays for patients. Reporting of specimens containing any fungal elements should always include the presence and absence of yeast forms, hyphae and whether or not they are septate, if it is possible to tell, and whether there is any melanin present.[57] However, while the demonstration of *Aspergillus* in tissue is the reference standard for diagnosis of invasive aspergillosis, a definitive diagnosis is possible only after identification of the fungus cultured from that tissue.[69] So, part of the biopsed tissue or other surgical specimen should be sent to the microbiology laboratory (not in formalin), in addition to the pathology laboratory. Immunohistological staining using polyclonal fluorescent antibody reagents can distinguish *Aspergillus spp.* from *Fusarium spp.*[70] *In situ* hybridisation may also help to distinguish *Fusarium spp.* from *Aspergillus* and *Pseudoallescheria* in tissue sections.[71]

New Diagnostic Tools

Galactomannan

Galactomannan is a cell wall polysaccharide released by *Aspergillus spp.* during fungal growth in tissue.[69,72,73] A commercially available sandwich ELISA (Platelia *Aspergillus*, BioRad) detects galactomannan by use of a rat monoclonal antibody. The test has been validated for serum specimens only and has a detection limit of ~1ng/mL, which is 10 to 15 times lower than the limit of the latex agglutination test used previously (Pastorex *Aspergillus*, Biorad).[73–76] Circulating galactomannan may be detected at a median of five to eight days before clinical manifestation of aspergillosis.[72,77–80] The concentration of circulating galactomannan corresponds with the fungal tissue burden[81,82] and may therefore be used to monitor the response to treatment.[77,78]

Studies evaluating the role of galactomannan assay in the diagnosis of invasive aspergillosis have largely been conducted with leukaemia patients or haematopoietic stem-cell transplantation (HSCT) recipients.[77,78,82–89] After initial clinical studies suggested a high sensitivity and specificity,[73,76,78,79,90,91] further studies revealed high rates of false-positive results among paediatric patients and neonates.[81,92,93] In one study, rates of false-positive results as high as 83% were observed[93] that may be related to cross-reactivity with *Bifidobacterium bifidum*, found in large inocula in the guts of breast- and formula-fed infants.[94] The presence of a damaged gut endothelium may increase the absorption of dietary galactomannan.[95] Specificity was also lower in adult allo-HSCT recipients than in adult auto-HSCT recipients or non-transplant patients.[92] The rate of false-positive results is high in the first 30 days following bone marrow transplantation and 10 days after starting cytotoxic chemotherapy.[96,97] The use of galactomannan as a surveillance tool in transplant recipients has been associated with a positive predictive value of only 10%.[92] The results also suggest that routine ELISA tests are not useful in patients with febrile neutropaenia with no clinical or radiological signs suggestive of a pulmonary infection.[92] Otherwise, all high-risk patients with a respiratory tract infection or suspected extrapulmonary aspergillosis should be repeatedly tested with galactomannan ELISA, as the predictive positive value of the assay was highest in these groups.[92]

Cross-reactivity of Platelia *Aspergillus* galactomannan with *Penicillium spp.* has been noted[96] but is deemed to be of little clinical relevance since *Penicillium spp.* are rarely pathogens in humans. In addition, drugs of fungal origin, such as antibiotics, may be associated with a false-positive test, including ampicillin-sulbactam, piperacillin-tazobactam, and amoxicillin-clavulanic acid.[98–102] The timing of collection of the sample may influence the test results, with reactivity being less likely in samples collected at trough levels or prior to the administration of the dose.[103] ELISA cross-reactivity has been observed with other fungi, including *Paecilomyces variotii*, and *Alternaria spp.*[92] False-positive reactions have also been seen in bacteraemic patients,[96] and in those with autoreactive antibodies.[64,88]

In a study of 3,924 serum samples in cancer patients, the overall sensitivity of the ELISA galactomannan test was only 29.4% (64.5% when only patients with proven invasive aspergillosis were analysed).[92] Low sensitivity for the ELISA assay has also been reported in allo-HSCT recipients (60%), lung transplant recipients (30%),[89] liver transplant recipients (56%),

and in patients with various other conditions including non-malignant diseases (52%).[83,93] While a positive test in a lung transplant recipient with a clinical illness compatible with invasive aspergillosis may be considered highly suggestive of this infection, a negative test does not rule out aspergillosis.[64] The sensitivity of the test has been typically lower in non-neutropaenic patients (15% to 30%) and may be related to lower circulating galactomannan levels.[64] The use of both prophylactic and empiric antifungals may also lower antigen levels by decreasing the fungal load.[64]

Overall, the galactomannan test seems to be a highly specific diagnostic tool (94% to 99%), even though sensitivity has ranged from 50% to 93% in patients with haematologic malignancy.[72,77,78] In order to improve these results, some authors have suggested that the recommended cut-off value of the test should be reduced from 1,500 to 1,000.[72,77] A further reduction to 0.700 was suggested for adults who have not undergone allogeneic transplantation,[92] and recent research suggests that an index of 0.5 is a more definitive threshold.[104] These modifications may increase the sensitivity of the test, with only a low decrease in specificity. Discussion persists about the best cut-off for galactomannan assays.

Because galactomannan is a water-soluble carbohydrate, it can be detected in other fluids.[69] Although the antigen can be detected in the urine,[73,74,105–107] little is known about its pharmacokinetics and clearance by the kidney. The effect of renal failure or dialysis on the clearance of galactomannan is also indefinite. Little is known regarding the correlation between galactomannan detection in urine and disease progression, and false-positive results may be an important drawback for this test.[69,73,74]

Because galactomannan is predominantly released by *Aspergillus* hyphae during growth and to a much lesser extent by conidia, detection of galactomannan in BAL fluid provides better evidence for aspergillosis than culture[108,109] or polymerase chain reactions (PCRs), which do not discriminate between contamination conidia and hyphae.[110–112] However, false positive results may occur in patients only colonised with *Aspergillus* when BAL is tested,[69] but this can be improved when one combines this method with the high-resolution computed tomography (CT) scan.[34,113]

Diagnosis of CNS aspergillosis is very difficult and even brain biopsies do not always result in a clear diagnosis.[69] Culture of CSF infrequently yields positive results,[94,114] and both chemical findings in the CSF and the CT results are often non-specific.[115–117] Galactomannan may also be detected in the CSF, with a sensitivity and specificity of 80% and 100%, respectively, according to a study involving only five patients with proven CNS aspergillosis.[117] Galactomannan has been detected in other clinical specimens as well.[69]

1,3-beta-D-glucan

1,3-beta-D-glucan is a cell wall component of yeast and filamentous fungi, which is detectable in the blood during most invasive fungal infections. Factor G, a coagulation factor, is a sensitive natural detector of this antigen.[118] The reported sensitivity and specificity for the assay have ranged from 67% to 100%, and 84% to 100%, respectively.[118–122] The test does not detect cryptococcosis, and it is also not positive in cases of oral candidosis or fungal colonisation.[121] However, in addition to invasive aspergillosis and candidosis, it detects infections caused by species of *Fusarium*, *Trichosporon*, *Saccharomyces* and *Acremonium*, which are less common but equally important fungal pathogens, especially in immunocompromised hosts.[121] False positive tests have been reported in patients undergoing haemodialysis, patients with cirrhosis, recipients of antitumour polysaccharides, and patients immediately following abdominal surgery.[64]

PCRn

Although PCR is at lest 19 times more sensitive than culture for *A. fumigatus*,[123] PCR-based molecular diagnostic tests for aspergillosis are not commercially available and remain largely unstandardised. A sensitivity of 79% to 100% and a specificity of 81% to 93% have been documented, depending on the methodology used.[124–126] Such assays, when performed on blood or BAL samples, have shown a negative predictive value for invasive aspergillosis ranging from 92% to 99%. However, PCR-based assays performed with BAL samples have shown low positive predictive values that are likely to reflect respiratory tract colonisation.[127]

Markers for Invasive Candidosis

Mannan is a cell wall surface carbohydrate that circulates during infection with *Candida spp.*,[128] and studies have shown a correlation between detectable mannanemia and tissue invasive in patients with candidaemia.[129] However, mannan is rapidly cleared from the blood and occurs in low levels, necessitating frequent sampling for detection.[130] In addition, this is a very expensive test. D-arabinitol is a metabolite of certain species of *Candida* that accumulates in the urine of patients with invasive candidosis. Assay sensitivity for D-arabinitol is only ~50%, and does not detect *C. krusei* and *C. glabrata*.[131,132] Enolase is perhaps the antigen with the greatest promise for the diagnosis of invasive candidosis.[133,134] The sensitivities

of assays for enolase have ranged from 54% to 75%, and higher sensitivity may be achieved with serial testing. Furthermore, the enolase antigen is highly specific for *Candida spp.*, and is not present in superficial *Candida* colonisation.[133]

Experimental models and clinical studies have shown PCR to be more sensitive than culture for detection of candidaemia. The sensitivity of the *Candida* PCR assay was 95.0% in one study, compared with a sensitivity of 75.0% for the *Candida* ELISA aiming to detect mannan (a difference not statistically different).[104] The specificities of the *Candida* PCR and ELISA were the same at 97.0%. In 45% of these patients, the PCR method detected the infection earlier than the ELISA. Newer realtime PCR assays such as TaqMan and the Light Cycler require no post-amplification manipulations and can potentially be automated for all steps from DNA extraction to final PCR amplicon detection and quantitation.[59]

It should be noted that there have been several head-to-head comparisons of the various assays for detection of the *Candida* antigens discussed earlier. Unfortunately, none of the assays have performed well enough or has good enough predictive value at this point to be able to recommend its routine use in a clinical laboratory. Perhaps a combination of two assays may increase the accuracy of diagnosis of invasive candidosis.[130]

Markers for Other Invasive Fungal Infections

The amplification of gene sequences unique to fungi is conceptually appealing, offering the potential for rapid and sensitive diagnosis of invasive fungal infections. In general, assays targeting multicopy genes have better detection limits than those targeting single copy genes.[135] Although PCR may become a diagnostic modality to identify *Mucorales*,[136–138] *Scedosporium*,[139–141] and several other fungi,[142–151] no commercial test is available to date. These techniques hold promise, but they are not yet standardised or readily available in most clinical laboratories. Also, large clinical trials to determine the sensitivity and specificity of such molecular tests are non-existent.

Role of the CT Scan

The 'halo sign' refers to a zone of ground-glass attenuation surrounding a pulmonary nodule or mass on computed tomography (CT) images.[152] The presence of a halo of ground-glass attenuation is usually associated with haemorrhagic nodules.[153] In severely neutropaenic patients, this suggests infection by an angioinvasive fungus, most commonly *Aspergillus*. The halo sign is documented in 33% to 60% of patients with invasive aspergillosis and is short-lived.[154] To be useful for the diagnosis of aspergillosis, the CT scan must be performed within five days of the onset of infection, because ~75% of the initial halo signs disappear within a week.[155] The 'air crescent' sign does not appear until the third week of the illness, and its appearance may be too delayed to be helpful in the diagnosis of invasive aspergillosis.[154]

Although usually regarded as an indication of haemorrhagic nodules, the halo sign may also be present when tumour or inflammatory cells infiltrate the lung parenchyma.[153,155–159] The halo has been described in patients with eosinophilic pneumonia, bronchiolitis obliterans organising pneumonia,[160] and tuberculosis,[161] and in patients infected by *Mycobacterium avium* complex,[155] *Coxiella burnetti*,[162] cytomegalovirus, herpes simplex virus,[153] and myxovirus.[160] Patients with post-transplantation lymphoproliferative disorders[163] and *Wegener granulomatosis* may develop the halo sign,[153] as well as some of those who have undergone transbronchial biopsy.[155] Although it is less common, the halo sign may also be observed in non-haemorrhagic nodules, in which case either tumour cells or inflammatory infiltration account for the halo of ground-glass attenuation.[153] Nonetheless, in the appropriate clinical setting, the halo sign is considered to be early evidence of pulmonary aspergillosis even before serologic tests become positive,[164] and it warrants the administration of systemic antifungal therapy.[165]

Future Perspectives

Invasive fungal infections constitute a major challenge for the management of immuno-compromised patients, mainly haemato-oncology patients, transplant and allo-HSCT recipients. In order to improve the survival for these infections, an early diagnosis is required. As shown in this article, conventional microbiological, histological and radiological techniques remain the cornerstone of diagnosis but are insensitive and have a limited impact on clinical decision-making. There is an urgent need for new and efficient diagnostic methods. These tests should be fast and highly sensitive. In addition, the recognition of the causal agent should be very precise. With the advance of molecular tools, new fungal species and new mechanisms of resistance will be clarified. DNA- and RNA-based methods hold promise for improved sensitivity and specificity, but these methods will require extensive validation in clinical studies. Finally, costs are also very important in selecting an appropriate diagnostic test for invasive fungal infections. ■

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