

Review Articles

Update on the Laboratory Diagnosis of Invasive Fungal Infections.

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Competing interests: The authors have declared that no competing interests exist.

Published: January 4, 2011

Received: December 30, 2010

Accepted: December 31, 2010

Mediterr J Hematol Infect Dis 2011, 3: e2011002, [DOI 10.4084/MJHID.2011.002](https://doi.org/10.4084/MJHID.2011.002)

This article is available from: <http://www.mjhid.org/article/view/7438>

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Abstract: Recent advances in the management of patients with haematological malignancies and transplant recipients have paralleled an increase in the incidence of fungal diseases due to pathogenic genera such as *Candida* and *Aspergillus* and the emergence of less common genera including *Fusarium* and *Zygomycetes*. Despite availability of new antifungal agents these opportunistic infections have high mortality. Rapid and reliable species identification is essential for antifungal treatment, but detection of the increasing diversity of fungal pathogens by conventional phenotypic methods remains difficult and time-consuming, and the results may sometimes be inconclusive, especially for unusual species. New diagnostic techniques (e.g., 1,3-beta-d-glucan detection) could improve this scenario, although further studies are necessary to confirm their usefulness in clinical practice.

Introduction: Despite the development of new techniques and new antifungal agents, diagnosis of invasive fungal infection (IFI), which still relies upon a combination of clinical observation and laboratory investigation, remains a challenge especially for immunocompromised patients with haematological disease.¹ This has important clinical repercussions since delayed diagnosis and therapy contribute significantly to the high mortality rates associated with IFIs,² whereas early intervention with antifungal drugs may result in more effective management of high-risk patients.³ While superficial and subcutaneous fungal infections often produce characteristic lesions that suggest the diagnosis, a thorough knowledge of

potential causative organisms is yet required to aid the diagnostic process, mainly in situations where systemic fungal infection is suspected but the clinical presentation is nonspecific and then ascribable to a wide range of infections, underlying illnesses, or complication of treatments.⁴

The exact identification of the infecting organism is became essential in light of the increased use of prophylactic schedules that predispose the patient not just to fungal infection, but also to the selection of fungal species such as non-*albicans Candida* (e.g., *C. glabrata* and *C. krusei*), *Aspergillus terreus*, *Scedosporium* species, and *Zygomycetes*, many of

which are intrinsically resistant to the available antifungal agents.^{5,6}

Culture-based detection methods: Laboratory diagnosis of IFI remains based on conventional approaches, such as the direct microscopically detection of the etiologic agent in clinical specimen and the isolation and identification of the pathogen in culture, and non-culture based methods involving detection of a serologic response to the pathogen or other marker of its presence such as fungal antigens or metabolites.⁴ Visual examination of fungi in tissue samples allows presumptive identification based on cellular morphology and staining properties, but it should be appreciated that invasive procedures necessary to obtain biopsies may be precluded in haematological patients. It should be noted that microbiological cultures are often insensitive or of limited use, since even with modern blood cultures systems candidaemia can be transient and not detected, or *Aspergillus* cannot be cultured from a significant proportion of sputum or bronchoalveolar lavage samples from patients with invasive aspergillosis (IA).⁷

Although a variety of culture media and incubation conditions may be required for recovery of fungal agents, chromogenic primary isolation media (e.g., CHROMagar Candida medium) can be employed for the presumptive identification of the most medically important *Candida* species, including *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. parapsilosis*. Most yeasts isolated from clinical samples can be identified using one of the numerous commercial identification systems, such as API 20C AUX, VITEK 2, and RapID Yeast Plus.^{4,8} Although the kits are relatively easy to use, it should be remembered that additional morphologic-based tests are often required to avoid confusion between organisms with identical biochemical profiles.

Unlike pathogenic yeasts, filamentous fungi can be identified only by visualization of macroscopic (colonial form, surface colour, and pigmentation) or microscopic (spore-bearing structures) morphologic characteristics, following to sub-cultivation of a mould isolate to encourage sporulation,⁴ a process that takes days to weeks. In addition to the use of genetic probes for the culture confirmation of dimorphic systemic fungal pathogens (e.g., *Histoplasma capsulatum*), an alternative and useful approach to the detection and identification of fungi in clinical specimens involves a broad-range polymerase chain reaction (PCR) followed by nucleic acid sequencing, after which the nucleic acid sequence is compared with known sequence database and identification is based on DNA homology.⁹ However, these methods are expensive and

time-consuming, and they are not currently suitable for routine identification.

By contrast, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is becoming a reliable method for identification of microorganisms. The remarkable reproducibility of the methodology is based on the measurement of constantly expressed and highly abundant proteins such as ribosomal molecules.^{10,11} Some recent studies have shown the potential of the MALDI-TOF technique to identify fungal clinical species such as *Aspergillus* and *Fusarium*. Recently, the MALDI-TOF technology provided a fast and accurate identification of common and unusual species of *Aspergillus* when tested on 124 clinical and 16 environmental isolates.¹² With regard to *Fusarium* species, a recent case report supported the usefulness of MALDI-TOF analysis in diagnosing an infection due to *Fusarium proliferatum*, which is a very infrequent pathogen within this genus.¹³

Antigen-based detection methods: To facilitate early diagnosis of IFI, important advances have been made in the development of laboratory markers (e.g., galactomannan [GM] and 1,3-beta-d-glucan [BG] assays), which have led to the potential for newer paradigms regarding prevention and early treatment of IFIs.¹⁴ Among fungal markers, GM, a component of fungal cell wall that can be detected by a sandwich-type enzyme-linked immunosorbent assay (ELISA) in serum or plasma,¹⁵ bronchoalveolar lavage (BAL) fluid,^{16,17} and cerebrospinal fluid,¹⁸ has recently been approved by the US Food and Drug Administration at a serum cut-off of 0.5, as a diagnostic adjunct for IA.¹⁴ Together with clinical criteria, a positive serum or BAL fluid GM would strongly suggest probable IA, as defined by authoritative consensus criteria.¹⁹ However, this technique has shown contradictory results, in terms of sensitivity and specificity, due to several factors, including the impact of prior antifungal therapy on the levels of circulating fungal components,²⁰ the occurrence of false-positive results in association with some antibiotic treatments,^{21,22} and the different cut-offs of positivity among studies.¹⁴ Thus, a recently published meta-analysis of 27 studies showed an overall sensitivity of 71% and specificity of 89% for proven cases of IA when used for surveillance.²³ In addition, GM also correlates well with outcome.²⁴

In contrast to GM, BG is a cell wall constituent of several fungi, including *Aspergillus*, *Candida*, and *Fusarium*, a spectrum of pathogens that encompasses the majority of those emerging in neutropenic patients with either prolonged neutropenia or chemotherapy-induced mucositis, with some notable exceptions such as *Zygomycetes*, a rare but emerging cause of invasive

mycosis.^{25,26} Measurement of serum BG has been shown to be an aid in the diagnosis of fungaemia and deep-seated mycoses, including IA.^{27,28} Among commercially available assays, the Fungitell, which is also an ELISA technique, is widely used to detect serum BG concentrations as low as 1 pg/mL.²⁷ The cut-off for a positive result is >80 pg/ml. As with GM, variable results have been reported for BG assay, with a slightly higher sensitivity and specificity, ranging from 70% to 90%.^{28,29} When performances of both GM and BD tests were compared to determine their diagnostic usefulness for high-risk haematological malignancy patients, GM assay was significantly better for detecting non-*fumigatus Aspergillus* species, whereas BG was shown to have a higher sensitivity in detecting IA and other mould infections.³⁰

Non-culture based methods for diagnosis of candidiasis are of limited value because the levels of circulating antigens are low and the transient nature of the antigenaemia requires sensitive assays and frequent sampling of at-risk patients.^{1,31} However, the use of Platelia Candida, an ELISA that combines the detection of mannan antigen and anti-mannan antibodies in serum, led to earlier diagnosis of *Candida* infection when compared with blood cultures.³¹ In haematological patients with hepatosplenic lesions, assessing mannan/anti-mannan antibodies shortened significantly the median time of diagnosis of candidiasis when compared with imaging.³²

Molecular-based detection methods: A range of polymerase chain reaction (PCR)-based methods have been developed with the prospect of give highly specific, highly sensitive, and rapid means for fungal detection and identification.³³ Most of them have focused on *Aspergillus* and *Candida* species, using different specimens types (e.g., serum, plasma, or BAL fluid), even though pan-fungal PCR amplification

technology may be able to detect a broad range of fungal targets.³³ Although PCR has been studied for years, the lack of standardization and clinical validation has led to its exclusion from consensus criteria for defining IFI.¹⁹ Nevertheless, a recent prospective evaluation of serial PCR assays against³⁴ or along with GM and computed tomography³⁵ was carried out in haematological patients, thus showing acceptable sensitivity and specificity. In such one study, the combination of serial PCR and GM detected 100% of aspergillosis cases, with a positive predictive value of 75.1%.³⁵ Of note, in a systematic review and meta-analysis of *Aspergillus* PCR tests for diagnosis of IA,³⁶ the authors proposed that a single PCR-negative test is sufficient to exclude IA, whereas two PCR-positive results are required to confirm disease. Compared with *Aspergillus* PCR, only a few *Candida* PCR methods have received major clinical evaluation.³⁷ As confirmed by a national consensus evaluation,³⁸ performance of these tests is generally good, with sensitivities and specificities consistently >90%.³³ Although addressed to critically ill patients, a prospective clinical trial published in 2008 reported positive predictive values and negative predictive values of >90% for a PCR method that detects several species of *Candida*.³⁹

Conclusion: Molecular detection methods, combined with additional microbiological and clinical information, has the potential not only to accurately and rapidly identify fungal pathogens, but also to indicate whether the pathogen is likely to respond to conventional antifungal treatment.⁹ Inclusion of these methods in a diagnostic surveillance strategy to exclude IFI in high-risk patients with haematological malignancy⁴⁰ should result in improved clinical management, thus allowing more rational use of antifungal drugs.

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