The Role of Microbiology for Diagnosis of Fungal Infections

13

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The incidence of the *invasive fungal infections* (IFIs) has notably increased over the last few years, above all in critical and immunodepressed patients who account for 70–80 % of all the cases of opportunistic systemic mycosis following the increase in the number of risk factors that predispose for these infections [1].

The diagnosis of IFIs is often complicated following the absence of specific signs and symptoms, especially in the early phases of the disease. For these reasons the European Organization for Research in the Treatment of Cancer/Mycosis Study Group (*EORTC/MSG*) proposed and has updated the criteria for the definition of IFIs, considering various clinical, and diagnostic aspects [2]. According to EORTC, invasive fungal diseases (IFD) can be classified as certain, probable, or possible. As regards certain and probable IFDs laboratory investigations play an important role. In particular, the certain diagnosis of IFD still remains based on classic diagnostic methods that use microscope observation of fungal elements in host tissue or sterile material, isolation in culture from sterile sites, and the investigation of the antigen *Cryptococcus* in serum.

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13.1 Classic Diagnostic Methods

The diagnostic meaning of direct laboratory tests is closely correlated with the clinical sample and the fungus involved. In fact, considering the invasiveness of the procedure needed to obtain biopsies, very often these investigations are carried out on clinical samples in which microscopic observation and isolation of colonies of *Candida* or *Aspergillus* are not necessarily associated with invasive infections but often with colonization of the mucosa.

Classic diagnostic methods, such as direct microscopy, histopathology, and culture, have a limited sensitivity to detect IFIs, and their usefulness depends on the possibility of obtaining samples of deep tissues which, in many cases, cannot be taken due to the patient's condition. Therefore, these approaches must be considered as essential investigations to be performed when possible [3–6].

13.1.1 Microscopy

In direct preparations, fungal elements may be misinterpreted as artifacts when stained with Gram or hematoxylin–eosin. Hyphae are best visualized by 'special fungal' stains. Hence, bronchoscopic material or tissue biopsies should be examined with periodic acid–Schiff, Grocott's methenamine silver, or optical brighteners (e.g., calcofluor white). Body fluids from normally sterile sites must be obtained and collected aseptically and transported to the laboratory promptly. The use of optical brighteners is recommended for microscopical examination of unfixed specimens. *Microscopic examination* of body fluids from normally sterile sites, such as blood, can be a rapid and specific method for the diagnosis of proven IFIs. However, microscopic examination has a low sensitivity, requires expertise for interpretation, and morphology cannot be used for definitive identification.

13.1.2 Culturing Techniques and Fungal Identification

Blood cultures (BCs) are essential for diagnosing candidaemia despite their low sensitivity. The problems connected with low sensitivity are attributable to several factors: (i) the number of BCs and the timing to obtain them and the total volume to be taken, (ii) permanence of *Candida* in the blood, and (iii) the BC system used. A panel of experts of the European Fungal Infection Study Group (EFISG) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) undertook a data review and compiled guidelines for the recommendations for taking and processing of blood samples to ensure the optimal isolation of microorganisms [7]. In particular, the number of BCs recommended in a single session is 3 (2–4), with a total volume varying according to the age of the patient, 40–60 mL for adults, 2–4 mL for children under 2 kg, 6 mL between 2 and 12 kg, and 20 mL between 12 and 36 kg. The timing to obtain the BC is one right after

the other from different sites, and venipuncture remains the technique of choice. BC set comprises 60 mL blood for adults obtained in a single session within a 30 min period and divided into 10 mL aliquots in three aerobic and three anaerobic bottles. BC sensitivity varies depending on the species and system used. *Lysis-centrifugation* procedures show a higher efficacy when other emerging and rare yeast pathogens are found in the blood of patients with fungemia [8]. Despite these recommendations, the accuracy of BCs is not very high since candidaemia can be transient and go undetected.

All fungi recovered from sterile sites should be identified down to the species level. 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, this takes an additional 48−72 h to complete after the yeast-like fungal pathogens are observed in the blood culture media [9, 10]. Unlike pathogenic yeasts, filamentous fungi can be identified only by visualization of macroscopic or microscopic morphologic characteristics, following sub-cultivation of a mold isolate to encourage sporulation, a process that takes from days to weeks [9]. Various molecular techniques, including fluorescent in situ hybridization and pyrosequencing, have been developed to speed up the *identification* of blood-borne fungi, but they have not been implemented as routine techniques in the clinical microbiology laboratory [11, 12]. Matrix-assisted laser desorption ionization–time of flight mass spectrometry has been described as a useful tool for rapid routine identification of clinical yeast and *Aspergillus* isolates [13, 14]. Recently, the Matrix-Assisted Laser Desorption Ionization Biotyper System has been successfully applied for identification of yeast species directly from positive BCs, with important implications for appropriate management of patients suffering from fungemia [15].

13.2 Nonculture Methods

To facilitate early diagnosis of IFIs and to make an early therapeutic intervention in an attempt to reduce the high mortality associated with invasive fungal infections, alternative procedures based on the detection and quantification of fungal biomarkers have been developed. In particular, galactomannan [GM] and 1,3-beta-d-glucan [BG] were included in the EORTC/MSG diagnostic criteria for invasive fungal infections for all types of patients. In the EFISG guidelines the combined use of mannan and anti-mannan antibodies in serum is also recommended for the diagnosis of candidaemia, while the detection of other antibodies, the septifast test, and in-house PCR are not recommended because no data are available evaluating their usefulness for the clinical diagnosis of candidaemia.

13.2.1 *Aspergillus* Galactomannan Antigen

GM is a component of the fungal cell wall that can be detected by a sandwich type enzyme-linked immunosorbent assay (ELISA) in serum samples, bronchoalveolar lavage (BAL) fluid samples, and cerebrospinal fluid. The current cut-off for optical density index for positivity is 0.5, though results of the The Platelia™ *Aspergillus* Ag in BAL fluid samples between 0.5 and 1.0 index have a lower predictive value than BAL sample results >1.0 index values, hence the results between 0.5 and 1 index values should be reviewed and supported by other clinical, radiological, or laboratory evidence of invasive aspergillosis. The Platelia™ *Aspergillus* Ag is a test which, when used in conjunction with other diagnostic procedures such as microbiological culture, histological examination of biopsy samples and radiographic evidence can be used as an aid in the diagnosis of invasive aspergillosis. The sensitivity and specificity of the test is conditioned by several factors, including the impact of prior antifungal therapy, the occurrence of false-positive results in association with β-lactam antibiotics (e.g., piperacillin–tazobactam), and in patients receiving products containing *galactomannan*, either parenterally or orally in the presence of an alteration of the intestinal barrier.

13.2.2 1,3-beta-d-glucan Antigen

1,3-beta-d-glucan antigen (BG) is a cell wall constituent of several fungi, including *Candida*, *Aspergillus*, *Fusarium*, *Acremonium,* and *Pneumocystis jiroveci*. However, BG concentrations are usually low or absent in patients with cryptococcal infections, and BG is usually absent in patients with zygomycosis since these fungi do not produce BG. Currently, 3 photometric BG assays kits are commercially available for diagnostic use: *Fungitell* (Associates of Cape Cod, East Falmouth, MA, USA), Fungitec-G (Seikagaku Biobusiness, Tokyo, Japan), β-Glucan Test (Maruha, Tokyo, Japan). In Europe and America, the most used is Fungitell with a cut-off value of 80 pg/mL. A meta-analysis showed that the BG assay had a sensitivity and a specificity of 76.8 and 85.3 %, respectively [16]. The performance of BG testing for the detection of systemic *Candida* infection did not differ considerably from its performance for the detection of invasive aspergillosis [16]. The diagnostic performance of BG testing for IFIs seems to be similar to that of galactomannan detection used for the diagnosis of invasive aspergillosis, though few studies have performed direct comparisons of the 2 tests for the diagnosis of invasive aspergillosis in the same patients. The use of albumin, antibiotics such as amoxicillin-clavulanate or piperacillin-tazobactam, hemodialysis with cellulose membranes, presence of serious bacterial infections, use of surgical gauzes containing glucan, or severe mucositis are associated with false positives. Therefore, certain issues regarding the clinical usefulness of BG testing have not been well clarified, including the timing and the frequency of BG testing for at-risk patients, as well as the criteria. The currently used cut-off of 80 pg/ml appears to be acceptable however; it has been shown that values above 160 pg/ml are highly

indicative of candidemia [17]. Moreover, in a study of a systematic survey of BG levels in surgical ICU patients when the number of positive samples required to make a diagnosis was increased to two or three, the specificity increased without experiencing a decrease in sensitivity [18]. The BG assay seems to be reliable as an aid in IFI diagnosis. However, its positivity or negativity should be interpreted very carefully and compared with clinical data. The timing of the assay is critical to allow for an early start of antifungal therapy.

13.2.3 Mannan Antigen and Anti-Mannan Antibodies

Among *Candida* antigens, mannan is a highly immunogenic polysaccharide bound to the yeast cell wall. It appears to be one of the main biomarkers for the diagnosis of invasive candidiasis. Platelia™ *Candida* Ag Plus is a one-stage immunoenzymatic sandwich microplate assay, detecting the circulating mannan *Candida* antigen in human serum or plasma. Even in cases of invasive candidiasis, the mannan antigen is more difficult to detect in patients tested positive for the anti-mannan antibody. In a study that evaluated the use of the Platelia *Candida* antigen kit for the diagnosis of invasive candidosis in preterm infants a greater sensitivity of the test was highlighted, probably due to the absence of circulating anti-mannan antibodies in these patients [19]. It is important to note the low sensitivity for the detection of *C. parapsilosis* and *Candida krusei* infection. This difference in the sensitivity for antigenic detection of different species is consistent with the nature of the *Candida* mannose epitope recognized by the EBCA1 monoclonal antibody used in the test. The combined detection of *mannan and anti-mannan antibodies* is considered to be a method for specific detection of *Candida* spp. in serum samples [20].

13.2.4 *Candida albicans* IFA IgG for Germ Tube Antibody Detection

This test is based on the detection, by an indirect immunofluorescence assay, of antibodies against the surface of *C. albicans* germ tubes *Candida albicans* IFA IgG for germ tube antibody detection (CAGTA). The test provides a rapid and simple diagnosis of invasive candidiasis (IC) in the clinical microbiology laboratory [21]. The performance of the test has been studied in hematological patients (87.5 % sensitivity and 95.2 % specificity) [22], and recently in ICU patients [23–25]. In particular, in these publications a significant decrease in mortality in ICU patients with a CAGTA-positive result was shown especially in those with increasing CAGTA values who had been treated with antifungals [23, 24]. Also, previous surgery was identified as the principal clinical factor associated with CAGTA-positive results [25]. The CAGTA detection assay has been used in combination with (1→3)-β-D-Glucan, C-reactive protein, and procalcitonin for discriminating between *Candida* spp. colonization and IC in non-neutropenic critically ill patients with severe abdominal conditions and to establish a model for the prediction of IC

[26]. This study shows that BG levels greater than 259 pg/mL combined with CAGTA-positive results accurately discriminate *Candida* spp. colonization from IC in non-neutropenic critically ill patients with severe abdominal conditions [26]. Therefore, although this test is not recommended because few data are available, the detection of CAGTA may be important for the diagnosis of invasive candidiasis in hematological and surgical patients admitted in ICUs.

13.2.5 Molecular-Based Detection Methods

A range of polymerase chain reaction (PCR)-based methods have been developed with the prospect of giving highly specific, highly sensitive, and rapid means for fungal detection and identification [27]. There are some commercial molecular methods for fungal DNA detection. LightCycler® *SeptiFast Test* (Roche Molecular Diagnostics, USA), provides rapid detection and identification of bloodstream infections by real-time PCR directly from blood. In particular, SeptiFast detects up to 25 microorganisms including five *Candida* species (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*) and *Aspergillus fumigatus*. Another commercial molecular method for fungal DNA detection is *MycAssay kits* (Myconostica Ltd, UK), and in particular one detects *Aspergillus* DNA (15 species) in serum or BAL, and another detects *P. jiroveci* DNA in respiratory samples. Also in-house PCR combined with classical diagnostic methods can improve early diagnosis of IFIs. In particular, in a study performed on preterm neonates in neonatal ICUs with suspected candidaemia the detection of fungal DNA directly from blood culture Isolator 1.5 microbial tubes, without prior cultivation, has been a promising approach for the rapid detection of *Candida* sp. [28].

However, several questions need to be addressed before the molecular methods can be adopted in daily clinical routine; these include (i) which DNA targets are best for commercial kits used in routine diagnostic laboratories, (ii) what the optimal methods are for extracting *fungal DNA* from clinical specimens obtained from various sites, and (iii) which detection methods are best for routine use. Molecular diagnostic methods may not distinguish individuals who are colonized from those who are infected. However, molecular detection methods, combined with additional microbiological and clinical information, have 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.

13.3 Conclusions

The role of microbiology in the diagnosis of IFIs is becoming ever more strategic in relation to the typology and clinical conditions of the patient as well as the clinical samples that can be obtained for laboratory tests. In the clinical samples where the presence of fungi indicates infection, microscopy and isolation in culture are both necessary even with the above mentioned limits. The microscopic

examination is rapid and low cost, isolation in culture identifies important epidemiological and therapeutic implications following identification of the etiological agent, and detection of virulence factors and the state of in vitro sensitivity. Thanks to the introduction of non-culture methods using biomarkers in association with the patient's clinical data, today it is possible to make an early diagnosis of IFIs that overcomes the limits connected with the low sensitivity of the culture method and the difficulty of obtaining bioptic samples not routinely available from patients who have serious risk factors. The use of an *integrated diagnosis* that associates conventional methods with the use of biomarkers seems to be important for a rapid and accurate diagnosis of IFIs. In fact, thanks to the advances in the study of pathogenesis, to the introduction of rapid and sensitive diagnostic tests, today it is possible to establish appropriate criteria for the selection of patients to undergo a specific diagnostic workup that allows a rapid, early diagnosis of invasive fungal diseases and favors diagnostic-driven strategy.

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