13 Diagnostics of Fungal Infections

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I. Introduction

Whereas invasive fungal infections were once a rare event, a constant increase has been observed for decades and fungi nowadays represent a major threat for hospitalized and immunocompromised patients all over the world (Leone et al. 2003; Lehrnbecher et al. 2010; Miceli et al. 2011; Martin 2012). This development is mainly related to the constant increase in patients with compromised immunity. The rise of human immunodeficiency virus (HIV), introduction of increasingly invasive therapeutic measures in intensive care medicine, and iatrogenic immunosuppression associated with solid organ transplants and bone marrow/stem cell transplantation has generated new – and constantly growing – risk collectives for invasive fungal infections. Newly arising forms of immunosuppression have also significantly changed epidemiology of fungal infections. The worldwide spread of HIV infection has resulted in a dramatic increase in opportunistic mycoses like *Cryptococcus* meningitis or *Pneumocystis* pneumonia. In fact, the latter was originally a disease entity known to affect malnourished or premature infants and has now become a classical infection in patients with defects in T cell responses, including AIDS patients (Vanek and Jirovec 1952). The increasing number of fungal infections is not solely due to

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an increase in well-known pathogenic fungi like *Candida albicans, Aspergillus fumigatus, Cryptococcus neoformans*, and *Pneumocystis jirovecii* (formerly known as *P. carinii*), although these species are still responsible for the majority of cases. In contrast, descriptions of infections caused by fungi that were previously considered apathogenic or of species that have never been observed before are now found in the literature in increasing numbers (Chakrabarti and Slavin 2011). The identification of these "novel" pathogens presents major problems for the diagnostic laboratories. In addition, the diagnosis of even the most common invasive fungal infections like candidemia and aspergillosis by conventional methods is fairly insensitive and often only available at late (sometimes too late) phases of the infection. This is especially true for invasive aspergillosis (IA), a disease entity for which the need for early specific therapy has unequivocally been shown. This chapter summarizes current diagnostic procedures in medical mycology, including classical culture- and morphology-based methods as well as novel molecular tools. These latter including protocols targeted at molecular species identification and molecular approaches to the diagnosis of fungal infections directly from patient samples and mass spectrometry. In a recent editorial in *Science*, it has been estimated, that "billions of people each year" are infected by fungi, with the majority being "relatively minor infections" (Brown et al. 2012).

However, exact numbers for invasive fungal infections are scarce and although studies have suggested a dramatic increase in the 1980s, incidence rates available have mostly been estimated for defined risk collectives in single centers (Wilson et al. 2002). Based on the analysis of large patient cohort data from hospital discharge surveys, Wilson et al. calculated incidences from candidiasis, aspergillosis, cryptococcosis, and histoplasmosis (Wilson et al. 2002). Based on these data, the authors estimated incidence rates of approximately 228 per million US population for candidiasis, 34 per million for aspergillosis and 30 per million for cryptococcosis. Accordingly, candidiasis accounts for 80% of all invasive fungal infections in the USA. Whereas, as expected, HIV-patients accounted for most cases of invasive cryptococcosis (79%) and cancer patients accounted for 42% of cases of IA, most patients hospitalized for invasive fungal infections were neither HIV-positive nor transplant or cancer patients (56%). Several studies from the USA and European countries have estimated the incidence of candidemia to be between 20 and 110 per million; however, other analyses reported numbers of up to 240 per million (Pfaller and Diekema 2005), which is in line with the above-mentioned study by Wilson et al. (2002).

II. Clinical Considerations in Diagnosing Fungal Infections

With the notable exception of endemic mycoses due to the dimorphic fungal pathogens and infection by *Cryptococcus gattii* (Byrnes et al. 2011), systemic fungal infection mainly occurs in immunocompromised patients. Consequently, the rapid evolvement of clinical importance is tightly linked to a growing number of immunocompromised patients. Major developments in the last 100 years that have contributed to this are solid organ transplantations, bone marrow or hematopoietic stem cell transplantation (BMT/HSCT), and modern intensive care therapy (see chapter "Invasive Aspergillosis in the Intensive Care Unit" by Meersseman). In addition to this, the HIV pandemia has resulted in a dramatic increase in immunocompromised patients in some parts of the world that are also predisposed to fungal infections. Whereas the type and degree of immunosuppression may not always be well defined (e.g., in surgical intensive care unit patients), it seems clear that a fully functional human immune system is largely protective against invasive mycoses. Knowledge about the specific immune status of a patient on the other hand allows predictions about the risk of fungal infections that may aid in both diagnosis and clinical management. Rather than providing an exhaustive review of known risk factors for all kinds of fungal infections, some defined clinical situations will be highlighted in the following section to underline basic concepts in protective immunity against fungal infection.

A. Fungal Infections in Chronic Granulomatous Disease

Several congenital immune defects are associated with an increased risk of invasive fungal infections. Among the most important conditions is chronic granulomatous disease (CGD), which is characterized by **dramatically reduced or absent formation of reactive oxygen intermediates due to a defect in the enzyme complex NADPH-oxidase**, responsible for generating superoxide in neutrophilic granulocytes. Invasive aspergillosis (IA) is one of the most common infections in these patients and frequently results in dissemination, with *Aspergillus* species being one of the most common causative agents of brain abscesses. These infections tend to have a high case fatality rate and IA is responsible for more than one third of all fatalities in CGD patients (Winkelstein et al. 2000; van den Berg et al. 2009; Antachopoulos 2010). In contrast to IA, the incidence of invasive candidiasis is only slightly elevated in CGD patients. Whereas this may indicate a higher functional redundancy of immune effector mechanisms against *C. albicans*, this finding may also be related to functional integrity of the gastrointestinal epithelial barrier, which prevents translocation of *C. albicans* from its natural reservoir (Koh et al. 2008). Recently, it has been shown that the genetic defect underlying CGD also prevents the formation of neutrophil extracellular traps (NETs) (Bianchi et al. 2009). Successful treatment of invasive infection due to *Aspergillus nidulans* was achieved by gene therapy and speculated to be related to restoration of NET formation in the patients' neutrophils (Bianchi et al. 2009), which may indicate that the role of NADPH-oxidase goes beyond the direct effects of reactive oxygen intermediates. CGD patients are also at risk of other mold infections, including many genera that are known to cause infection in humans (Antachopoulos 2010).

B. Fungal Infections in HIV/AIDS

The discovery of AIDS and its causative agent HIV resulted from descriptions of unusual occurrences of several opportunistic infections in young adult men. Among those infections was *Pneumocystis carinii* (now *Pneumocystis jirovecii*) pneumonia (PcP). PcP is still one of the most important AIDS-defining diseases in Western Europe and the USA. Before the HIV/AIDS epidemic, PcP was rather uncommon and had, in fact, been recognized initially as a pulmonal infection in malnourished low-birthweight infants (Vanek and Jirovec 1952). After the emergence of AIDS, PcP became the leading AIDS-defining diagnosis in the USA and in Europe (Huang and Hecht 2000). In addition to PcP, invasive cryptococcosis is of major importance in HIV-infected patients (Warkentien and Crum-Cianflone 2010) and has developed into one of the most important HIV-related opportunistic infections. Especially in Africa, cases of cryptococcal meningitis may outnumber cases of bacterial meningitis in countries with a high AIDS prevalence, and it has been estimated that each year one million cases of cryptococcal meningitis occur worldwide (Park et al. 2009). However, due to the introduction of highly active combination antiretroviral therapy (HAART), numbers of cryptococcosis have declined substantially in North America and Western Europe (Park et al. 2009). With regard to infections by *Candida* spp., oral candidiasis (thrush) is very common in HIV-infected patients. More importantly, esophageal candidiasis is one of the major AIDS-defining illnesses. Oropharyngeal candidiasis may be one of the first clinical signs of HIV infection, occurring in 50–95% of all HIV-infected patients (Rabeneck et al. 1993; Fidel 2006). In contrast, systemic candidiasis is much rarer in HIV.

C. Fungal Infections in Hematopoietic Stem Cell Transplantation

Since its beginnings, transplantation of bone marrow cells or peripheral blood hematopoietic stem cells (HSCT) has developed into a curative approach for several diseases. Congenital immune deficiencies, benign hematological disorders, autoimmune diseases, and hematological malignancies are the major fields of

application of HSCT (Jenq and van den Brink 2010). In the latter case, rather than just "replacing" a patient's own hematopoietic system, HSCT can also be considered one of the most effective immune therapies, as the newly transplanted immune system has been shown to attack remaining tumor cells in what has been termed the graft-versus-tumor (GVT) effect (Jenq and van den Brink 2010). Several factors contribute to immunosuppression in HSCT patients and increased risk of fungal (and other) infections, including the underlying disease, the conditioning treatment leading to more or less complete eradication of the patients own hematopoietic system, and (later in the course) the immunomodulatory treatment required for treating graft-versus-host disease (GvHD), a major complication of allogeneic stem cell transplantation. Consequently, several risk factors for developing invasive fungal infection in HSCT recipients, including the intensity of total body irradiation, the degree of donor human leukocyte antigen (HLA) mismatch, time to engraftment, corticosteroid therapy, and many others have been described. Fungal infections have been as high as 20% in allogeneic HSCT recipients and could increase to 40% in patients with severe GvHD (Jenq and van den Brink 2010). Initially, systemic candidiasis was the major fungal infection in allogeneic HSCT patients, with an incidence of about 11% and an associated mortality of 39% (Goodrich et al. 1991; Marr 2008). Both *Candida* sepsis (acute invasive candidiasis) and chronic disseminated candidiasis (typically with hepatosplenic involvement) were observed in HSCT patients. However, invasive *Candida* infections have decreased considerably following the implementation of azole prophylaxis, which has proven to be highly effective in preventing these infections (Marr 2008). The introduction of new conditioning regimens with less myeloablation and less damage to mucosal barriers has further decreased the incidence of invasive candidiasis (Fukuda et al. 2003). During the 1990s, invasive infections caused by *Aspergillus fumigatus* were noted in HSCT recipients and found to be associated with very high mortality rates of 80–90% (Marr 2008). Since then and despite the availability of new and more effective treatment regimens (Herbrecht et al. 2002) and antifungal prophylaxis regimens covering *A. fumigatus* (Cornely et al. 2007), **IA continues to be a major infectious complication in allogenic HSCT.** In recent years, increasing frequencies of other mold infections have been observed in many centers. Of particular importance are infections caused by zygomycetes (Vehreschild et al. 2013), but other fungal genera including *Fusarium* and *Scedosporium* have also been described as emerging fungal pathogens. Therefore, the epidemiology of fungal infections in allogenic HSCT patients has been constantly changing during the clinical development of clinical protocols and will most likely continue to do so, demanding epidemiological awareness and high-quality mycological diagnostics to be available at HSCT centers.

D. Fungal Infections in Solid Organ Transplant Recipients

The risk of infectious complications in solid organ transplant recipients is increased by the immunosuppressive therapy and by the potential introduction of pathogens through the transplanted organs. In addition, the underlying organ failure and the surgical intervention itself can add to the individual risk of infection (Grim and Clark 2011). Early infectious complications (generally defined as occurring up to day 30 post transplant) are often related to surgery. Maximal immunosuppression usually occurs after that period and up to 6 months after transplantation (Grim and Clark 2011). In most solid organ transplants, with the notable exception of lung transplantation, invasive candidiasis is the most common fungal infection. It is most common in transplants affecting the integrity of the gastrointestinal tract, including liver, small bowel, and enterically drained pancreas transplant recipients. In these patients antifungal prophylaxis is generally recommended. In lung transplant recipients, IA is the most common fungal infection, most probably due to the constant exposure of the transplanted organ to the ubiquitous spores of this pathogen. In addition, impaired ciliary clearance and decreased cough reflex may add to the risk of

aerogenic IA (Grim and Clark 2011). In recent years, other mold infections have increasingly been observed in these patients, including zygomycosis (Lanternier et al. 2012). Finally, infections with *P. jirovecii* have been frequently diagnosed in solid organ transplants, including several outbreaks in kidney transplant recipients. The underlying reasons for the observed increase in outbreaks of PcP in these patients remains unclear. As a result, trimethoprim-sulfamethoxazole prophylaxis has become a widely accepted practice incorporated into many kidney transplantation guidelines (de Boer et al. 2011). Outbreak situations of PcP, with up to 28 cases in a given setting, may require the use of molecular typing methods for unequivocal documentation of epidemiological coherence. These approaches are described in chapter "Molecular Epidemiology of Pneumocystis Outbreaks" by Hauser and Kovacs. Invasive cryptococcosis plays a minor role in solid organ transplant recipients (Osawa et al. 2010).

III. Diagnostic Criteria for Invasive Fungal Infections

The unequivocal diagnosis of invasive fungal infections (IFI) is still a major problem in the clinical setting. Patients at risk of IFI in general are also prone to a number of other infections as well as several syndromes or pathophysiologies that may cause **related or even identical symptoms.** It has been conceived that this diagnostic uncertainty leads to over-diagnosis and over-treatment of fungal infections in at-risk cohorts (Kibbler 2005) due to empirical treatment of suspected cases. Recognizing the practical problems arising from this situation – and most importantly a notable discrepancy between cases of IFI included in therapeutic studies (mostly fulfilling quite rigorous criteria for diagnosis of infection) and the average patient in clinical routine – the Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer (EORTC) and the Mycoses Study Group (MSG) of the National Institute of Allergy and Infectious Diseases established a **consensus effort** to standardize the definitions of IFI. This standardization was (and still is) explicitly targeted towards clinical research and notably was never designed to guide clinical decisions on therapeutic intervention – although the definitions may of course aid in these decisions (Ascioglu et al. 2002).

In the first version of the EORTC/MSG criteria, three levels of diagnostic certainty were defined: "proven", "probable," and "possible." Patients classified as "possible" according to the initial version of the definitions were considered to present enough information to warrant some form of empirical treatment but, at the same time, inclusion of these patients into clinical trials for antifungal drugs was discouraged due to the low specificity of the diagnosis (Ascioglu et al. 2002). Assignment to a level of diagnostic accuracy was based on a combination of "host factors," "clinical manifestations" (with two levels of evidence, termed major and minor, within this category), and "mycological results" (Ascioglu et al. 2002). Importantly, usage of the definitions was restricted to patients with cancer and to HSCT recipients, thus addressing two major populations at risk of IFI but clearly also excluding several other risk cohorts, e.g., patients in an intensive care unit (ICU).

In 2008, the EORTC/MSG consensus group published a revised version of these definitions (De Pauw et al. 2008). In the meantime, the original definitions had formed the basis for several large scale clinical trials of antifungal drug efficacy and several other studies including the formulation of clinical practice guidelines (Herbrecht et al. 2002; Walsh et al. 2002a, b; Cornely et al. 2007; Ullmann et al. 2007; De Pauw et al. 2008; O'Connell and Walsh 2008) and evidently gained considerable acceptance worldwide. In the revised definitions, now targeted towards diagnosis of invasive fungal disease (IFD) rather than IFI, only minor changes were introduced in the "proven" level, whereas the definitions of probable and possible cases were modified significantly. In the revised version, probable cases required the presence of host factor + clinical feature + mycological criterion, whereas possible cases were defined by the presence of host factor + clinical feature but absence of mycological evidence for IFD (De Pauw et al. 2008). The impact of the revision published in 2008 on classification of patients should not be underestimated: In a study analyzing 589 high-risk patients, as many as **81% of**

“possible” and 75% of “probable” cases according to the initial definition had to be regrouped as “non-classifiable” (Tsitsikas et al. 2012), indicating that the overall specificity was increased by the criteria modification. This was mainly due to the elimination of minor clinical criteria and the emphasis placed on specific computed tomography (CT) findings (Tsitsikas et al. 2012). One major problem of the revised definitions is the fact that patients with host-factor and mycological criteria but without defined clinical symptoms are grouped as non-classifiable. In addition, application of the EORTC/MSG criteria is still restricted to immunocompromised patients and therefore does not allow a general application, especially when regarding intensive care patients, which have become a major at-risk cohort (Martin et al. 2003; Meersseman and Van Wijngaerden 2007; De Pauw et al. 2008). Furthermore, it has to be emphasized, that the EORTC/MSG definitions are still intended for use in clinical and epidemiological research and are not meant to guide clinical diagnosis in individual cases (De Pauw et al. 2008). Despite this and beside facilitating our ability to test the efficacy of therapeutic regimens and strategies (De Pauw et al. 2008), these definitions also set the standards for evaluating diagnostic tools and provide a commonly accepted reference in the absence of a reliable gold standard for unequivocal diagnosis of invasive fungal disease.

IV. Radiology in the Diagnosis of Fungal Infections

Early detection of fungal infections is very important because the morbidity and mortality rates of the disease are high, especially in immunocompromised patients (Krowka et al. 1985; Soubani et al. 1996). The radiological diagnosis of intrapulmonary fungal infections, in particular, is sometimes challenging due to the multitude of either infectious or malignant differential diagnoses. Although the diagnostic value for the detection of fungal infections on chest radiographs (CXR) is poor (Korones et al. 1997), in cases of clinical suspicion of a pulmonary fungal infection CXR should be the first-line modality. In more severe cases, computer tomography (CT) needs to be performed, even if the CXR is normal. In contrast to radiography, **CT can give a picture of the whole extent of disease** and, in some cases, point to specific pathogens involved in the disease (Wah et al. 2003). Particularly in younger patients, CT should be performed at a low dose (LDCT). Magnetic resonance imaging (MRI) is an imaging method especially suited for detecting visceral mycotic lesions. In these cases, MRI is more sensitive in detecting, as well as in defining, the stage of the disease than is contrast-enhanced CT (Semelka et al. 1992). The most common pathogens of pulmonary fungal infections in immunocompromised patients are *Aspergillus* species, *Cryptococcus neoformans*, and *Candida* species. Pulmonary *Aspergillus* infections in immunocompromised patients can be grouped into **angio-invasive aspergillosis** (AIA) and the less common **broncho-invasive aspergillosis** (BIA). CT findings of AIA include a halo sign (defined as a ground glass opacity surrounding the circumference of a nodule or mass), infarct-shaped consolidations, and an internal, low-attenuation cavity or air-crescent sign (Figs. 13.1, 13.2, and 13.3). Typical CT patterns of BIA are small airway lesions, peribronchial consolidations, and/or bronchiectasis. In a late manifestation of AIA, an air-crescent sign can be observed, which is caused by an intracavitary fungus mass of AIA that moves when the patient changes position (Aquino et al. 1994; Shibuya et al. 2004). The air-crescent sign can also appear in saprophytic aspergillosis in immunocompetent and immunosuppressed patients when pre-formed cavities are colonized with *Aspergillus* spp. (Fred and Gardiner 2009). Pulmonary cryptococcosis is an emerging disease in neutropenic patients. The most common radiological features in pulmonary infection with cryptococcosis are poorly defined nodules (commonly between 7 and 20 mm) or patchy air space consolidations. Cavities, ground glass opacities, the halo sign, mediastinal lymphadenopathy, and pleural effusion are rarely seen in pulmonary manifestations (Chang et al. 2006). Common CT findings in patients with *Candida* infections

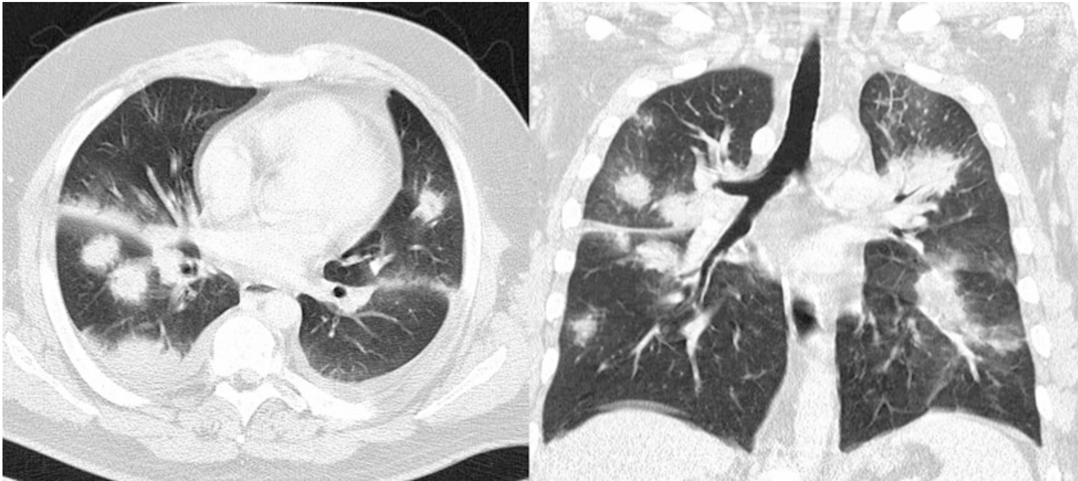

Fig. 13.1. Angioinvasive aspergillosis in a 35-year-old man with acute lymphatic leukemia. CT scan shows bilateral peribronchovascular nodular lung lesions with surrounding halo of ground-glass attenuation representing adjacent hemorrhage

include random bilateral nodules with a diameter between 3 and 30 mm. Consolidations and centrilobular nodules are rarely seen (Althoff Souza et al. 2006). In hematogenous fungal disseminations that affect the central nervous system and the hepatosplenic system, microabscesses can be observed by using MR imaging rather than contrast-enhanced CT (Semelka et al. 1992; Anttila et al. 1996). However, MRI is sometimes not available and the examination can last about 30–40 min, which, in many cases, is not feasible for the critically ill patient. Contrast-enhanced CT and ultrasound (US) are the routinely performed first-line modalities in the radiological assessment of hepatosplenic fungal abscesses.

V. Classical Laboratory Diagnosis

A. Pre-analytical Considerations in Diagnosing Fungal Infections

As with other microbial infections, the diagnosis of fungal infections depends upon a combination of clinical observation and the application of various laboratory techniques. Superficial fungal infections are often associated with suspicious lesions, which guide clinical diagnosis. In contrast, invasive fungal infections rarely show specific signs. As a consequence, the selection, collection, possible storage, and transportation of diagnostic material are of utmost importance in order to establish the diagnosis. During transportation, fungi must remain viable in order to be recovered on culture, and specimens should not be allowed to desiccate. Transportation to the laboratory should occur at room temperature. Specimens should be delivered within 2 h after sampling but should not arrive at the laboratory later than 24 h after collection. Specimen collections from the central nervous system should be processed as soon as possible. When immediate processing is not possible, the specimen should be held at ambient temperature (CLSI 2012).

To establish or confirm diagnosis, it is essential that the laboratory is provided with an adequate specimen. For the diagnosis of invasive fungal infections, specimens originating from sterile locations are preferred. Blood cultures should be performed whenever deep fungal infections are suspected. All *Candida* species or other organisms such as *Histoplasma* spp. have been shown to be reliably detected by the current available **blood culture** systems. For aspergillosis and mucormycosis, blood cultures are of limited utility. However, disseminated

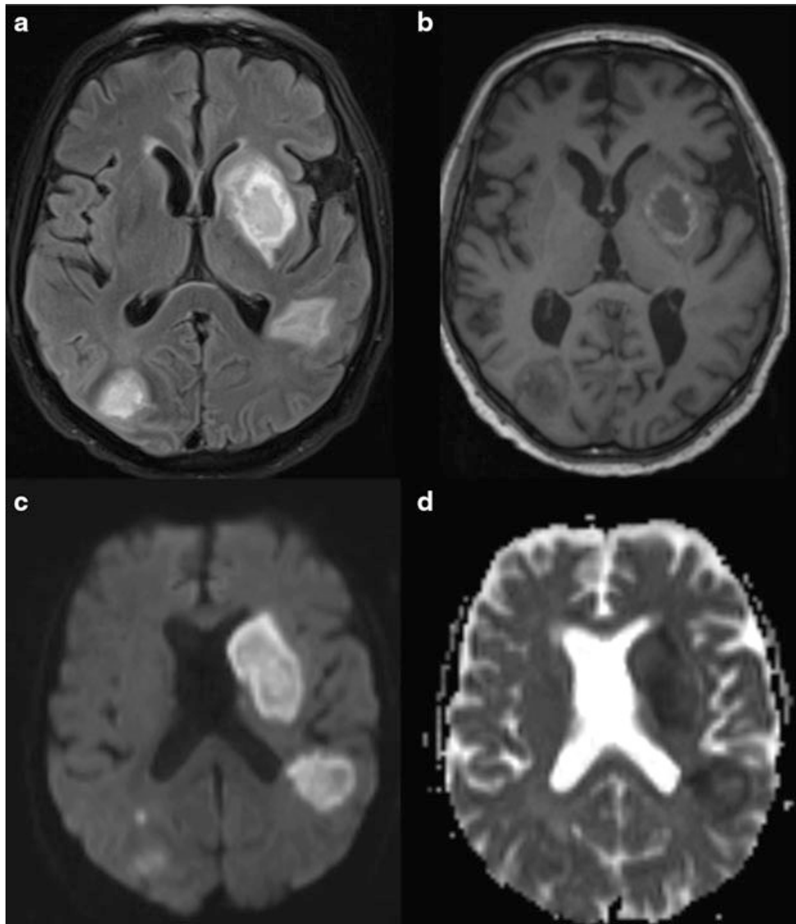

Fig. 13.2. Invasive aspergillosis of the brain in a 70-year-old male patient with lung carcinoma and chemotherapy. (**a**) Axial T2 tirm MR image shows bihemispheric hyperintense lesions. (**b**) Unenhanced axial T1 weighted MR image shows bihemispheric hypointense lesions with a rim of high signal intensity representing blood products. (**c**) Diffusion-weighted images shows high signal intensity in all lesions. (**d**) Apparent diffusion coefficient (ADC) map shows low signal intensity in the center of the lesions, a finding indicative of restricted diffusion

infections caused by *Fusarium* spp. and *Scedosporium* spp. are associated with the recovery of the pathogen in blood cultures in approximately 50% of all cases (Arendrup et al. 2012).

In case of suspected meningitis, 3–5 ml of **cerebrospinal fluid** (CSF) should be collected in sterile, screw-capped tubes. Immediate transport of all collected fluid to the laboratory at room temperature is mandatory (CLSI 2012). CSF is best for detection of *Cryptococcus* meningitis (Denning et al. 2003), but is less sensitive for the detection of central nervous system aspergillosis or candidiasis (Arendrup et al. 2012).

Tissue biopsies are of high diagnostic value. However, for some fungi (e.g. Mucorales), homogenization of the biopsy material reduces the culture yield and is it preferable to inoculate culture plates with slices of minimally manipulated tissue (Arendrup et al. 2012).

The confirmation of many fungal infections is based on samples obtained from lower respiratory tract specimens. Sputum specimens are acceptable, but increasing the number of sputum samples examined increases the sensitivity of detection, with three samples providing optimum yield in invasive aspergillosis.

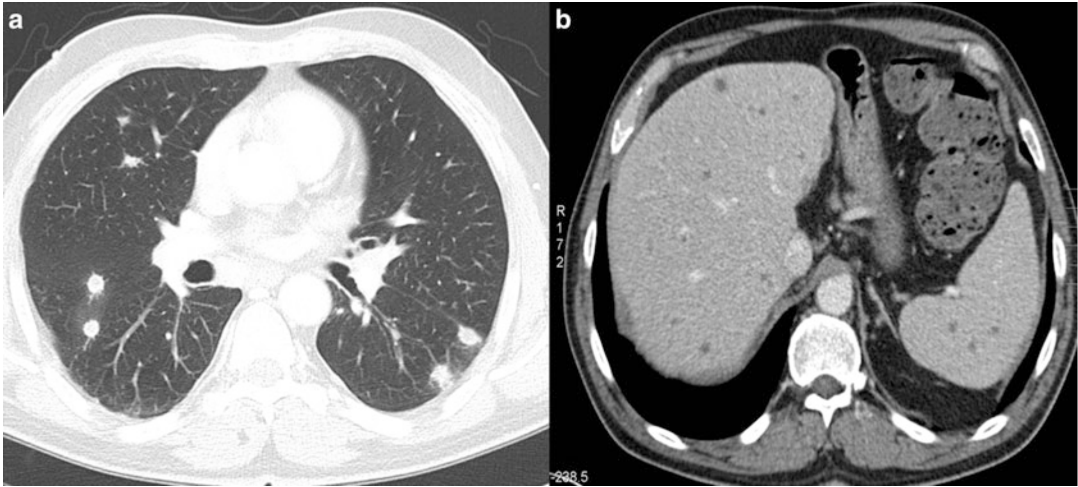

Fig. 13.3. Systemic candidiasis in a 40-year-old male patient with chronic myeloic leukemia. (**a**) Axial CT scan shows multiple bilateral intrapulmonary nodules smaller than 1 cm. (**b**) Axial contrast-enhanced CT shows countless hypoattenuating lesions in the liver and the spleen representing *Candida* microabscesses

Bronchoalveolar lavage (BAL) fluid provides a more representative sample from the lower respiratory tract and allows CT scan abnormalities to be directly sampled. The presence of *Candida* spp. in BAL fluid, similarly, does not correlate with invasive lung infection and should be interpreted as of low significance (Meersseman et al. 2009; Arendrup et al. 2012).

B. Currently Available Diagnostic Methods

Currently available laboratory methods for diagnosing invasive fungal infections include microscopic detection, isolation of the fungus, serologic detection of antibodies and antigen, and histopathologic evidence of invasion (Alexander 2002). For definite diagnosis of proven invasive fungal infections, histological and cultural evidence from biopsies, resection material, or other specimens obtained from normally sterile body sites is required (De Pauw et al. 2008). However, it is not always possible to fulfill these criteria and a combination of different methods is necessary to detect and identify the fungal organism causing the disease.

1. Histopathology

Histopathology is the cornerstone for diagnosis and identification of fungal pathogens. However, as with culture methods, it may not be possible to obtain a tissue sample from critically ill patients. In addition, identifying the specific pathogen based solely on morphological characteristics can be difficult or impossible because several different organisms may have similar histopathological characteristics, e.g., *Fusarium* spp. and other filamentous fungi are indistinguishable from *Aspergillus* in tissue biopsies (Alexander and Pfaller 2006). Because *Aspergillus* is far more commonly encountered than the other pathogens mentioned, a pathologist often may describe an organism as *Aspergillus* or *Aspergillus*-like on the basis of morphological features alone. This can hinder diagnosis and may entail inappropriate therapy (Chandrasekar 2010). Molecular tools can aid in the identification of fungal pathogens from histopathology samples (see Sect. 6).

2. Microscopy

Direct microscopy is most useful in the diagnosis of superficial and subcutaneous fungal

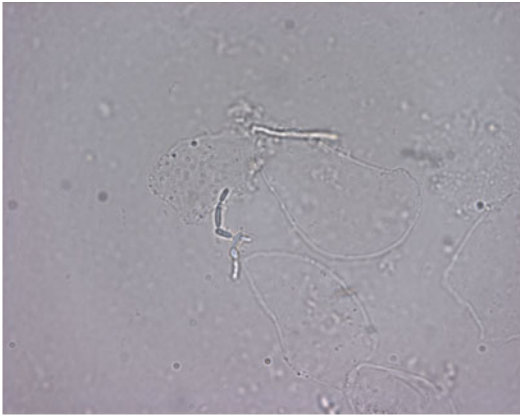

Fig. 13.4 KOH preparation demonstrating pseudohyphae of *Candida* spp.

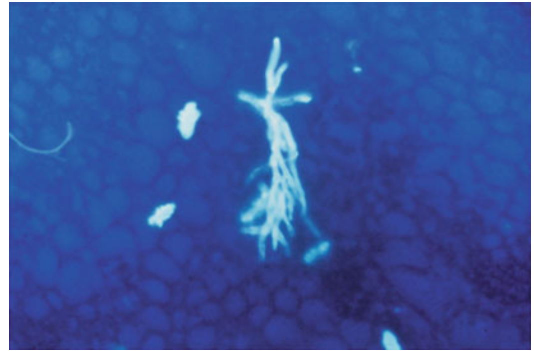

Fig. 13.5 Microscopy with an optical brightener showing dichotomously branched hyphae indicative of *Aspergillus*

infections. Recognition of fungal elements can provide a reliable and rapid indication of the mycosis involved. Various methods can be used: unstained wet-mount preparations can be examined by light-field, dark-field, or phase contrast illumination (Richardson and Warnock 2003).

The most common direct microscopic procedure relies on the use of 10–20% potassium hydroxide (KOH; Fig. 13.4), which degrades the proteinaceous components of specimens while leaving the fungal cell wall intact, thus allowing their visualization (Lease and Alexander 2011).

The visibility of fungi within clinical specimens can be further enhanced by the addition of calcofluor white or blankophores (Willinger 2006). These are fluorophores, which are members of a group of compounds known as fluorescent brighteners, optical brighteners, or "whitening agents" and bind to β-1-3 and β-1-4 polysaccharides, such as found in cellulose and chitin. When excited with ultraviolet or violet radiation, these substances will fluoresce with an intense bluish/white color (Harrington and Hageage 2003). The high intensity of the elicited fluorescence allows rapid and reliable microscopic screening (Fig. 13.5). Optical brightener methods have been shown to be more sensitive than KOH wet mount.

This has also been shown in a study of respiratory samples (mostly BAL) from transplant recipients and neutropenic patients, indicating a sensitivity of 88% and a specificity of 99% for the detection of *Aspergillus*-like elements by blankophor in comparison with a 76% sensitivity for culture (Andreas et al. 2000; Vyzantiadis et al. 2012). The procedure is also suitable for disclosing fungi in Gram-stained microscopical mounts. Filamentous fungi like aspergilli, which stain poorly by the Gram procedure, may be unveiled on Gram-stained microscopic mounts after removal of immersion oil by subsequent blankophor staining (Ruchel and Schaffrinski 1999). In addition, optical brightener methods have been shown to be more sensitive than KOH wet mount in a number of specimen types (Chander et al. 1993). Furthermore, calcofluor or blankophor can be combined with 10–20% KOH, although it is better to pre-soften the tissue with KOH before adding the fluorescent stain (Vyzantiadis et al. 2012).

Because optical brighteners provide a rapid and sensitive method for the detection of most fungi, their use is encouraged for respiratory samples, pus, tissue samples, and fluids from sterile sites when a fluorescence microscope is available.

Similarly, lactophenol cotton blue is easy to handle and often used for the detection and identification of fungi (Fig. 13.6). Other stains are frequently used in direct microscopy, such as the India ink wet mount, which is useful for visualization of encapsulated fungi, particularly *Cryptococcus neoformans*. Although a negative direct examination cannot rule out fungal disease, visualization of fungal elements in specimens can often secure initial information helpful in the selection of empirical antifungal therapy (Lease and Alexander 2011).

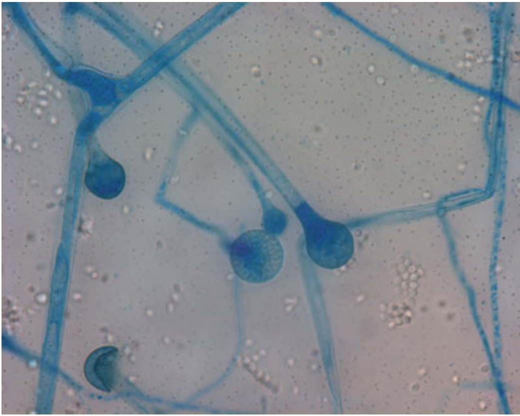

Fig. 13.6. Staining of *Lichtheimia corymbifera* using lactophenol cotton blue

Because yeast and molds can stain variably with the Gram stain, a more specific fungal stain is recommended (CLSI 2012). Microscopy may help to discern whether an infection is caused by yeast or molds (Table 13.1). The presence of pseudohyphae and optionally blastoconidia indicates the presence of yeast. Molds are most commonly seen as hyaline hyphomycetes, generally characterized by parallel cell walls, septation (cross-wall formation in hyphae), lack of pigmentation, and progressive dichotomous branching as in *Aspergillus*, *Fusarium*, or *Scedosporium* species (Vyzantiadis et al. 2012). *Aspergillus* septate hyphae have a diameter of 3–6 μm and each branch is approximately equal in width to the originating stem. Branching at 45° angles is characteristic. Because many other filamentous fungi present with a similar appearance in clinical samples on direct microscopy, one can usually only say that the infection is due to one of the agents of **hyalohyphomycosis**, although the balance of probability is that most infections will be due to *Aspergillus* (Patterson 2003).

In addition, it is important to look for septate and non-septate hyphae, thus allowing to distinguish between *Aspergillus* spp. and members of the Mucorales. **Mucoraceous molds** have large (3–25 μm in diameter, average 12 μm) ribbon-like, multinucleated hyphal cells with nonparallel walls and infrequent septa. The branching is not dichotomous, irregular, and sometimes at right angles. Hyphae can appear distorted with swollen cells, or compressed, twisted, and folded (Vyzantiadis et al. 2012). Another group of molds causing tissue invasion with a distinctive appearance are the agents of phaeohyphomycosis, such as *Alternaria* and *Curvularia*. These fungi have melanin in their cell walls and appear as pigmented, septate hyphae. These are the agents of phaeohyphomycosis (Revankar 2007). The detection of fungal hyphae and/or arthrospores in skin, nail, or hair samples may indicate the presence of dermatophytes but give no special hint as to the species involved.

3. Antigen and Antibody Detection

Except for **cryptococcosis**, where antigen testing can reliably prove infection, serological tests mainly provide supplemental information for the diagnosis of invasive fungal infections. Antibody tests are often used in the diagnosis of endemic mycoses, which may be difficult to detect by traditional methods. In some cases, antibody tests are a supplemental test in the diagnosis of invasive candidosis, but have two major drawbacks: detection of anti-*Candida* antibodies fails to discriminate between disseminated and superficial infections, and antibodies are often present in colonized but uninfected patients. In order to diagnose invasive candidosis, a rise in antibody titers has to be observed in serial serum samples. However, because immunocompromised patients do not reliably produce antibodies it is rendered nearly impossible to diagnose invasive candidosis in these patients solely by antibody detection (Willinger 2006). *Aspergillus* antibodies are only infrequently detectable in immunocompromised patients, but are often helpful in patients with aspergilloma or allergic bronchopulmonary aspergillosis and in patients with cystic fibrosis (Kappe and Rimek 2010). Another approach to the diagnosis of fungal infections is the detection of antigens in blood or other body fluids, whether it is used as a single test or in combination with antibody detection.

Table 13.1. Characteristic morphology of fungi in clinical specimens by direct microscopic examination (selection of certain fungi)

Fungal group and representatives	Microscopic morphology in clinical specimens
Yeasts	
Candida spp. (except *C. glabrata*)	Single budding of moderately variable size 2.5–7.5 µm in size Pseudohyphae and true hyphae may be present in certain species
C. glabrata	Blastoconidia of uniform size (2.5–4 µm) No production of hyphae or pseudohyphae
Cryptococcus neoformans	Spherical budding yeasts of variable size 2–15 µm in diameter Capsule may be present or absent No hyphae or pseudohyphae
Pneumocystis jirovecii	Intracystic bodies that resemble parentheses facing one another are pathognomonic 4–6 µm in size Seen on histological stains Cysts often found in clusters No budding Best visualized in histological sections using special stains Trophozoites seen on staining with giemsa or immunofluorescent stains
Hyaline hyphomycetes	
Aspergillus *Acremonium* *Fusarium*	Hyaline, septate, dichotomously branching hyphae Uniform width (3–6 µm) *Aspergillus* may produce conidial heads in specimens from cavitary pulmonary lesions
Paecilomyces *Scedosporium* *Scopulariopsis* *Trichoderma*	Conidiation may be visualized on histopathological examination of some of the non-*Aspergillus* hyphomycetes
Mucorales	
Cunninghamella *Lichtheimia* (formerly *Absidia*)	Broad, thin-walled, non-septated hyphae 6–25 µm wide
Mucor *Rhizomucor* *Rhizopus* *Saksenaea*	Nonparallel sides and random branches
Dematiaceous fungi	
Alternaria *Bipolaris* *Curvularia* *Cladophialophora*	Pigmented (brown, tan, or black) Septate hyphae 2–6 µm wide
Dimorphic fungi	
Blastomyces dermatitidis	Large, spherical, thick-walled, budding yeast cells 8–15 (max. 40) µm diameter Typically broad-based junction between mother and daughter cells Cells may appear multinucleate
Coccidioides immitis	Spherical, thick-walled spherules Vary in size (20–30 µm) Mature spherules contain endospores Released endospores may be mistaken for yeast Hyphae and arthroconidia may be found in cavitary lesions
Histoplasma capsulatum	Small budding yeasts 2–5 µm in size
Paracoccidoides brasiliensis	Large, multiple-budding yeasts Variable size 2–30 µm in diameter Large cells are surrounded by smaller buds around the periphery (“mariner’s wheel appearance”)

(a) Invasive Candidosis

Mannan is a *Candida* cell wall constituent and can be detected in the serum of patients with invasive candidosis (IC), but it is quickly degraded and cleared from the bloodstream. Furthermore, antimannan antibodies are found in patients either colonized or infected by *Candida* spp. However, it has been shown in patients developing IC that mannan and antimannan antibody titers complement each other (Peman and Zaragoza 2012). In fact, when these tests are used together, their sensitivity and specificity for diagnosing IC improves significantly (Marchetti et al. 2012). In practice, the two more commonly used tests are Platelia Candida Ag® and Platelia Candida Ab® assays (Bio-Rad Laboratories, Marnes-la-Coquette, France). It has been shown that detection of both antigen and antibody is superior to evaluation of each one separately as it presents a sensitivity of 83% and a specificity of 86% in IC; consequently, its use might be helpful in these patients (Mikulska et al. 2010). Nevertheless, the results of mannan antigen and antibody detection varies depending on the *Candida* species implicated; the tests perform best for *C. albicans*, *C. glabrata*, and *C. tropicalis* and worst for *C. parapsilosis* and *C. krusei*. Another important benefit of this combined antigen–antibody detection is the ability to make an earlier diagnosis than with blood cultures, in some cases. Serial determinations may be necessary. These assays can help to detect the infection early because they can be positive 6 days, on average, prior to blood cultures. The method also shows very high negative predictive value (>85%) and can be used to rule out infection (Cuenca-Estrella et al. 2012). However convincing this may be, many experts feel that these assays do not have sufficient sensitivity or specificity to influence their clinical decision-making (Kullberg et al. 2011).

Another option is the Cand-Tec latex agglutination test (Ramco Laboratories, Houston, TX, USA), which detects an antigen, not specifically defined. Though the sensitivity and specificity of this test vary among reports (Yeo and Wong 2002), it is still widely used. However, false-positive reactions due to the rheumatoid factor and renal insufficiency (Ruchel 1993) have been observed and it is generally recommended to use better-defined assays.

An indirect immunofluorescence assay to detect *Candida albicans* germ tube-specific antibodies (CAGTA) is commercially available as *C. albicans* IFA IgG® (Vircell Laboratories, Granada, Spain) and has shown an overall sensitivity of 77–89% and a specificity of 91–100% thus being useful in the diagnosis of IC in critically and noncritically ill patients (Quindos et al. 2004). In addition, Peman et al. (2011) found a high rate of positive results when using this test. The presence of this biomarker was the only protective factor independently associated with mortality, and positivity was not modified by either colonization or previous antifungal treatment. However, there are no recommendations to use this biomarker yet.

(b) Cryptococcosis

The detection of cryptococcal capsular polysaccharide is one of the most valuable rapid serodiagnostic tests for fungi performed on a routine basis. The cryptococcal antigen can be detected either by the latex agglutination test or by enzyme-linked immunosorbent assay (ELISA). The test has gained widespread appeal and is suited to prove disseminated cryptococcosis (De Pauw et al. 2008). False-positive reactions have been reported in patients with disseminated trichosporonosis, *Capnocytophaga canimorsus* septicemia, malignancy, and positive rheumatoid factor. Another assay format is the Premier Cryptococcal Antigen enzyme immunoassay (Meridian Diagnostics, Inc., Cincinnati, OH, USA) that utilizes a polyclonal capture system and a monoclonal detection system. The Premier EIA was reported to be as sensitive as the latex agglutination system for the detection of capsular polysaccharide in serum and cerebrospinal fluid. In addition, it does not react with rheumatoid factor and gives fewer false-positive results (Yeo and Wong 2002).

(c) Invasive Aspergillosis

The most commonly used, commercially available antigen test for *Aspergillus* detection is the double-sandwich ELISA test Platelia Aspergillus® (Bio-Rad Laboratories, Marnes, France), which is validated for use in serum and bronchoalveolar lavage (BAL) fluid and

Table 13.2. Characteristics of the galactomannan antigenemia test

Parameter	Description
Specimen	Serum, BAL
Criteria for positivity	Two consecutive serum specimens with GMI ≥0.5 Always repeat the test before implementing therapy for IA
Population	Prolonged neutropenia, allogeneic SCT, ICU patients, COPD patients

GMI galactomannan index, *SCT* stem cell transplantation

Table 13.3. Causes for false-positivity or cross-reactivity in the galactomannan antigenemia test

Patient groups with false-positive results	False-positivity caused by galactomannan contamination	Cross-reactivity caused by similar cell wall galactomannan
Infants with intestinal colonization by *Bifidobacterium* spp.	Piperacillin-tazobactam Amoxicillin-clavulanate	*Penicillium* spp. *Alternaria* spp.
Chronic graft- versus-host disease	Other beta-lactam antibiotics Plasmalyte (sodium gluconate)	*Paecilomyces* spp. *Cryptococcus* spp.
People following diets rich in soy protein and other foods	Other intravenous hydration or nutrition fluids containing sodium gluconate Echinocandins Chronic graft- versus-host disease Diets rich in soy protein and other foods	*Histoplasma capsulatum* *Geotrichum* *Neosartoria* *Lichtheimia ramosa*

has been evaluated in oncohematologic patients and in receptors of hematopoietic precursors with prolonged neutropenia (Table 13.2). This test has also been evaluated in non-hematological ICU patients and patients suffering from chronic obstructive pulmonary disease (COPD) (Meersseman et al. 2008; Guinea et al. 2010; Hage et al. 2011; He et al. 2011; He et al. 2012). The Platelia Aspergillus® detects **galactomannan** (GM), which represents a heteropolysaccharide cell wall component of the *Aspergillus* cell wall and is released by *Aspergillus* species during hyphal growth. To optimize its diagnostic value in patients at risk of infection, it is recommended to perform two GM determinations a week, mainly in oncohematologic patients and in hematopoietic stem cell transplant recipients with prolonged neutropenia (Peman and Zaragoza 2012). Circulating GM may be detected at a median of 5–8 days before clinical signs and symptoms of IA become evident. Furthermore, its concentration corresponds to the fungal tissue burden, and may therefore be used to monitor the patient's response to antifungal treatment (Maertens et al. 2009).

Pfeiffer et al. (2006) presented a meta-analysis of the diagnostic value of GM detection with the Platelia Aspergillus® assay, showing that for proven and probable cases of IA, the pooled (adult and pediatric) sensitivity was only 61%, whereas the overall specificity was 93%. In general, the negative predictive value (NPV) and the specificity are excellent (>95%), suggesting that the assay can be used to rule out the diagnosis of IA. However, false positive and negative results of GM have been described in certain patient groups (Table 13.3) by various authors (Wheat and Walsh 2008; Hage et al. 2011; Peman and Zaragoza 2012). Solely testing for antigenemia does not replace other tests for IA. To maximize sensitivity, testing should precede empiric antifungal therapy and positive results should be confirmed on a new specimen (Wheat 2003). Generally lower sensitivity rates have been obtained from pediatric patients than adult patients in studies evaluating the utility of GM antigenemia test in early diagnosis

of IA (Oz and Kiraz 2011). When using this test it has to be considered that results may be falsely positive or negative and have to be correlated with clinical and laboratory findings.

Antigen has been detected in body fluids other than sera. The detection of antigen in BAL fluid was described in various studies, indicating the presence of pulmonary aspergillosis. A meta-analysis evaluating the accuracy of BAL galactomannan determined the sensitivity to be 90% and specificity 94% (Guo et al. 2010). A positive result increased the probability of having IA by about sixfold, whereas a negative result decreased the probability to 1%. Whereas the sensitivity remained unchanged, at about 85%, the specificity increased from 89% to 94% by using a cut-off of 1.0 instead of 0.5. BAL was judged to be more useful than serum, by comparison with results of another meta-analysis (Pfeiffer et al. 2006).

Hage et al. (2011) state when using BAL, positive results caused by airway colonization may complicate the use of the test for diagnosis. As shown, results may be false-positive in patients colonized with *Aspergillus*, *Penicillium*, and *Paecilomyces* (Clancy et al. 2007; Husain et al. 2007). When combined results of those studies were analyzed, sensitivity was unchanged but specificity increased using a cut-off of 1.0 (Muller et al. 2002). Results in colonized specimens were at least 1.0 in 8 out of 12 cases (Husain et al. 2008). However, BAL galactomannan was negative in hematology patients who were colonized with *Aspergillus* species (Bergeron et al. 2010). As colonization is undesirable in solid organ transplant or hematology patients at high risk of IA, results attributed to colonization should not be disregarded, but rather prompt additional investigation to exclude invasive disease or to assess the effectiveness of antifungal prophylaxis or therapy, and follow-up evaluation for subsequent invasive disease (Hage et al. 2011). All in all, GM detection in BAL fluid seems to be useful in establishing or excluding the diagnosis of IA in ICU patients (Hage et al. 2011).

In addition, the test might allow the detection of GM in other specimens such as cerebrospinal fluid, urine, and homogenates/extracts of tissue specimens. Few data exist on the performance of the galactomannan assay for homogenized tissue specimens, nevertheless the data are promising (Lackner and Lass-Flörl 2013).

Monitoring for antigen clearance or rebound may provide useful information for assessing the effectiveness of therapy. Declining levels in patients responding to therapy and rising concentrations in those with fatal outcomes have been observed by several authors (Bretagne et al. 1997; Maertens et al. 2001). Thus, failure of antigenemia to decline may suggest treatment failure and support consideration of modifying the therapy.

Studies on patients who have undergone solid organ transplantation report sensitivity ranging from 67% to 100% and specificity from 40% to 98% (Clancy et al. 2007; Husain et al. 2007; Pasqualotto et al. 2010). Detection of antigen in BAL was more sensitive than detection of antigen in serum or isolation of *Aspergillus* from the BAL or lung biopsy, as highlighted in an evaluation of proven cases in hematology patients (Maertens et al. 2010). In that study, the sensitivity was 100% for BAL antigen, 55% for serum antigen, 62% for culture, and 74% for microscopy. In organ transplant recipients, sensitivity was 82% for antigen detection compared to 73% for culture and/or microscopy (Husain et al. 2008).

Recently Thornton et al. (2012) described a new promising diagnostic method for the detection of *Aspergillus* in patients suffering from hematological malignancies. The technology is based on the detection of *Aspergillus*-specific JF5 by MabJF5 monoclonal antibodies. The JF5 is an extracellular glycoprotein that is exclusively secreted during active growth of the fungus and represents a surrogate marker of *Aspergillus* infection. Evaluating exclusively the activity of *Aspergillus* rather than the antigen presents a major advantage compared with the GM test and, at the same time, limits the risk of cross-reactivity or a false-positive result due to contaminated materials because the JF5 test only detects germinating conidia that represent a potential infectious risk. The technique has been incorporated into an immuno-chromatographic lateral-flow device ('point of care' diagnostic tool), which is easy to use. The test aims to detect invasive pulmonary aspergillosis using blood or/and serum samples from patients in only 15 min. However, further studies are needed to prove the test's performance in a clinical setting (Lackner and Lass-Flörl 2013).

4. (1,3)-β-D-Glucan as a Marker for Invasive Fungal Infection

(1,3)-β-D-Glucan (BDG) is a component of the cell walls of many fungal organisms; its presence can be detected in sera when observing activation of the factor G of the horseshoe crab coagulation cascade (Bellanger et al. 2011). The presence of BDG in serum has been shown to be a reliable marker of invasive fungal infection (Karageorgopoulos et al. 2011), and it has recently been added to the EORTC/MSG guidelines as a biological criterion for invasive fungal diseases other than zygomycosis and cryptococcosis (De Pauw et al. 2008). It has also been proposed as a noninvasive marker for pneumocystosis (Bellanger et al. 2011). However, one has to be aware that this test does not detect cryptococcosis and fungal colonization or superficial infections.

The contribution of BDG to IFI screening has mainly been investigated for patients with hematological malignancies. More recently, studies have reported the contribution of the BDG assay for detecting invasive candidiasis in patients in surgical intensive care units (Mohr et al. 2011; Ostrosky-Zeichner et al. 2011).

Karageorgopoulos et al. (2011) recently presented a meta-analysis including 2979 patients (594 with proven or probable IFIs) showing a pooled sensitivity of 76.8% and a specificity of 85.3%. However, marked statistical heterogeneity was noted. BDG has good diagnostic accuracy for distinguishing proven or probable IFIs from no IFIs and can be useful in clinical practice, if implemented in the proper setting and interpreted after consideration of its limitations. In addition, the detection of BDG precedes the appearance of symptoms, radiologic signs, and the empirical antifungal treatment in some patients (Peman and Zaragoza 2012).

5. Culturing Techniques

Culture remains one of the key methods for diagnosing fungal infection. Though often slow, sometimes insensitive, and sometimes confusing with respect to contamination, culture may yield the specific etiological agent and may allow susceptibility testing to be performed. Proper collection and transportation of the specimen is essential. In particular, sterile materials are important for diagnosis of IFI.

Blood cultures (BC) are the first-line test and currently considered the "gold standard" in the event of any suspected case of systemic mycosis (Ostrosky-Zeichner 2012). Several commercial blood culture systems are available.

Lysis centrifugation has been considered the "gold standard" for recovering pathogenic yeasts, as well as thermally dimorphic fungi from blood (Willinger 2006). In particular, dimorphic molds can be most reliably and rapidly recovered in the lysis centrifugation system. Lysis centrifugation involves incorporation of a tube containing an anticoagulant and a lytic agent and exploits the possibility that white blood cell lysis releases viable fungal cells. However, it is time consuming and prone to contamination (Berenguer et al. 1993). When older BC systems are used as comparators, lysis centrifugation procedures show higher efficacy, whereas current automated systems have improved the sensitivity of blood cultures for detecting microorganisms (Cuenca-Estrella et al. 2012). In comparison to current automated blood culture systems, in recent studies lysis centrifugation does not appear more sensitive for the majority of invasive fungi (Morrell et al. 1996; Arendrup et al. 2010).

Of the automated systems, there are two that are widely used: the Bactec System (BD Diagnostic System, Sparks, MD) and the BacT/Alert System (bioMérieux, Marcy l'Etoile, France). Studies have documented that these systems match the performance of lysis centrifugation methods for the detection of yeast. The Bactec system proposes a specifically formulated medium for the isolation of fungi, called Mycosis IC/F medium. This medium is a brain–heart broth enriched with sucrose, chloramphenicol, and tobramycin to inhibit bacterial growth, and a lysis agent, saponin. The incubation period recommended by the manufacturers for Bactec Mycosis IC/F and BacT/Alert FA vials is 14 and 5 days, respectively. In various studies, the vast majority of the *Candida* species were detected in 5 days (Meyer et al. 2004; Ericson et al. 2012). The main reason for 14 days of incubation for Bactec Mycosis IC/F vials is to detect the growth of filamentous fungi, which may take longer.

In 2012, recommendations concerning diagnostic procedures for detection of *Candida* diseases were published by the ESCMID Fungal Infection Study Group (Cuenca-Estrella et al. 2012). Concerning candidemia, the number of BC 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 for obtaining the BC is one right after the other from different sites, and venipuncture remains the technique of choice. A BC set comprises 60 ml blood for adults obtained in a single session within a 30-min period and divided into 10-ml aliquots among three aerobic and three anaerobic bottles. The frequency recommended is daily when candidemia is suspected, and the incubation period must be at least 5 days.

When these recommendations have been followed, the sensitivity of BC to detect *Candida* is 50–75% although lower sensitivity rates in neutropenic patients and those undergoing antifungal treatment have been reported (Arendrup et al. 2008, 2011). **It should be noted that fungal pathology in direct Gram stain from positive BC flasks can provide a reasonably reliable basis for discrimination between *C. albicans* and non-*albicans* species** (Harrington et al. 2007).

Despite the advances in blood culture technology, the recovery of fungi from blood remains an insensitive marker for IFI. Filamentous fungi will be detected to a much lesser extent than yeasts. For IA, BCs represent a limited diagnostic tool because *Aspergillus fungaemia* is rarely encountered, even in the setting of disseminated disease (Duthie and Denning 1995; Denning 1998; Girmenia et al. 2001). Mainly, *A. terreus* has been described as being detected by blood cultures, other *Aspergillus* species very rarely. However, recently it has been described (Rosa et al. 2011) that BACTEC automated systems may allow detection of *A. fumigatus*, *A. flavus*, and *A. terreus*. In this study, BACTEC Plus Aerobic/F vials seemed to be advantageous for *Aspergillus* detection in blood samples collected from patients under antifungal treatment.

Culture is highly sensitive (98%) in patients with *Cryptococcus* meningitis (Denning et al. 2003). However, in central nervous system aspergillosis or candidiasis, cerebrospinal fluid (CSF) cultures are less sensitive (Arendrup et al. 2012). For *Candida* meningitis, 80% of CSF cultures have been described as positive (Sanchez-Portocarrero et al. 2000). All yeasts and molds obtained from sterile sites, including blood and continuous ambulatory peritoneal dialysis (CAPD) fluids, and intravenous-line tips should be identified to species level. This is also valid for bronchoscopically gained specimens. Treatment of clinically obvious or severe cases should not be delayed for culture results, although treatment may need to be altered according to the species. The presence or absence of fungal elements on microscopy is not always predictive of positive culture results, and if a clinician is faced with unexpectedly negative results, investigations should be repeated while alternative diagnoses are considered (Moriarty et al. 2012).

However, the interpretation of results obtained from a culture of the respiratory tract specimens is difficult because of the possible transient colonization by yeasts, on the one hand, and the ubiquitous nature of airborne conidia and the risk of accidental contamination with molds, on the other hand. It has been observed that the cultivation of *Aspergillus* spp. from normally sterile sites and the presence of *Aspergillus* in respiratory samples from immunocompromised children at risk of IA are highly indicative of infection (Muller et al. 2002). Detection of *Aspergillus* species in BAL fluid is highly suspicious for invasive disease in febrile granulocytopenic patients with new pulmonal infiltrates. However, the absence of hyphal elements or a negative culture does not exclude the diagnosis.

6. Identification of Cultured Fungi

Yeasts are identified by their **assimilation pattern** and their **microscopic morphology**, and molds by their **macroscopic and microscopic morphology**. Due to the slow growth of some species/isolates this can take days to weeks. As for *Candida* spp., the use of chromogenic media and test kits allows rapid identification and can accelerate the otherwise slow identification of yeasts. Identification of the most com-

mon yeasts can be performed immediately when visible growth is observed (Fig. 13.7). Chromagar Candida (Becton Dickinson, Franklin Lakes, NJ, USA) and Candida ID Agar (bioMérieux, Marcy l'Etoile, France) have been shown to allow easier differentiation of *Candida* species in mixed yeast populations than the traditional Sabouraud Glucose agar. Moreover, more rapid identification of *Candida* species can be achieved on these media than with conventional test kits. These media reduce the need for subculture and further biochemical tests and considerably simplify and shorten the identification procedure, mainly for *C. albicans*, *C. tropicalis*, and *C. krusei* (Willinger and Manafi 1999; Fricker-Hidalgo et al. 2001; Willinger et al. 2001). However, *C. glabrata* the second most common *Candida* species cannot be identified reliably on chromogenic media.

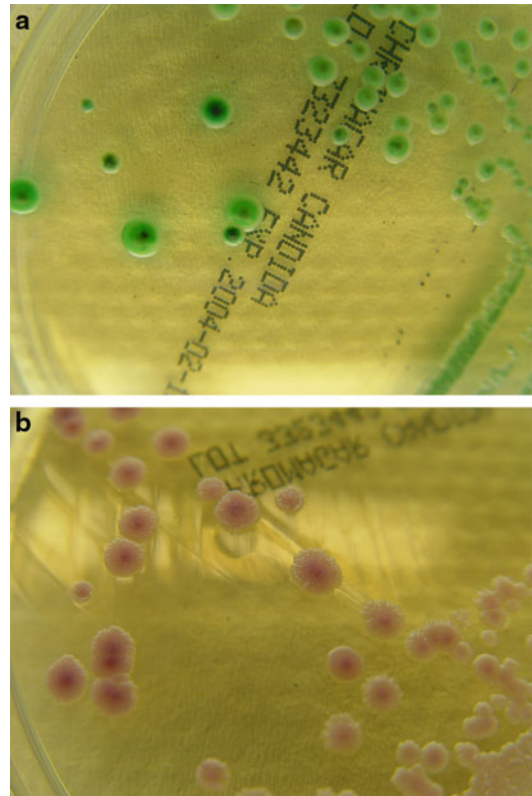

Fig. 13.7. Pigmentation of various *Candida* species on ChromagarCandida® : (**a**) *C. albicans* and (**b**) *C. parapsilosis*)

Identifying filamentous fungi can be much more cumbersome. Generally, macroscopic and microscopic morphology is the key to identification (de Hoog et al. 2009). Macroscopic examination of the colonies can reveal important characteristics concerning color, texture, exudates, pigments, specific structures, growth rate and growth zones, and aerial (the hyphae projecting above the medium surface) and submerged or vegetative mycelium (the portion that penetrates the medium in order to secure the colony and absorb nutrients). The color of the reverse of the colony must be recorded along with any pigment that diffuses into the medium. In addition, microscopic elements have to be evaluated for identification. The production of characteristic conidia and hyphae enables the identification of a large number of molds, at least to the genus level (Vyzantiadis et al. 2012). Standard references including identification algorithms are available (de Hoog et al. 2009) but, despite this, morphology-based identification of molds requires a high degree of experience.

Commercially available biochemical test systems identify most of the commonly isolated species of yeast accurately but may result in no identification or misidentification of more unusual isolates (Freydiere et al. 2001). Furthermore, samples for these tests must be incubated for 1–3 days before results are obtained. Identification of molds may also be cumbersome and tedious because it is mainly based on morphology. As an alternative to the conventional identification schemes, proteomic profiling by mass spectral analysis was recently evaluated for use in species differentiation of a variety of microorganisms. Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF) is emerging as a rapid and accurate tool for identifying pathogens, not only for bacteria but also for molds and yeast species. This technology generates characteristic mass spectral fingerprints that are unique signatures for each microorganism and are thus ideal for an accurate microbial identification at the genus and species levels and have the potential to be used for strain typing and identification (Stevenson et al. 2010; Croxatto et al. 2012). Furthermore, molecular tools can aid in the identification of cultured fungi (see below)

7. Mass-Spectrometry-Based Identification of Fungal Pathogens

This technique probably presents the major paradigm change of the past decades in the way in which species are determined in microbiology because it allows the identification of microorganisms within a few minutes. Currently, there are four commercial systems available: the MALDI Biotyper (Bruker Daltonics, Bremen, Germany), the AXIMA@SARAMIS database (AnagnosTec, Potsdam, Germany and Shimadzu, Duisburg, Germany), and the Andromas (Andromas, Paris, France) and VITEK MS systems (bioMérieux, Marcy l'Etoile, France). Among fungi, ascomycetous and basidiomycetous yeasts including *Candida*, *Pichia*, and *Cryptococcus* genera are most easily processed and analyzed (Bader 2013).

Recent studies showed that far more than 90% of clinical *Candida* isolates from different species could be correctly identified by MALDI-TOF MS (Marklein et al. 2009; van Veen et al. 2010). Furthermore, closely related yeast species that cannot be discriminated with common biochemical methods, such as *Candida ortho-/meta-/parapsilosis*, *Candida glabrata/bracarensis/nivariensis*, *Candida albicans/dubliniensis*, *Candida haemulonii* group I and II complexes, or the phenotypically similar species *Candida palmioleophila*, *Candida famata*, and *Candida guilliermondii*, can be resolved without difficulty by MALDI-TOF MS (Bader 2013).

This technique has also been applied directly on positive blood cultures without the need for prior culturing and has thus reducing the time required for microbiological diagnosis. The reliability of this technique has not only been evaluated for bacteria but also for yeasts directly from blood culture bottles. Spanu et al. (2012) demonstrated that the identification results were concordant with those of the conventional culture-based method for 95.9% of *Candida albicans* and 86.5% of non-*albicans Candida* species. Results were available in 30 min, suggesting that this approach is a reliable, time-saving tool for routine identification of *Candida* species causing bloodstream infection.

Far less data are available for the differentiation of molds like *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., and dermatophytes (Wieser et al. 2012). As MALDI-TOF MS identification can only be performed from cultured fungi, the various growth forms of molds, such as mycelium and conidia, complicate the analysis due to differences in protein composition. Also, in strongly pigmented isolates such as those from *Fusarium* spp. or *A. niger*, the conidial melanin pigment inhibits the ionization of the analyte (Dong et al. 2009; Buskirk et al. 2011). This can be overcome by growth in liquid cultures suppressing pigment formation or by pre-analytical washing steps. However, adjustments and optimizations are needed to enhance the performance of MALDI-TOF MS-based identification systems for routine diagnostics of molds (Bader 2013).

MALDI-TOF MS also has the potential to be a useful and rapid identification method for dermatophytes. The identification of dermatophytes is currently based on morphological criteria, is time consuming, and is hindered by intraspecies morphological variability and the atypical morphology of some clinical isolates. A more rapid method seems to be important for improving and shortening the period of identification. In recent studies using different systems, a high level of identification was obtained for the most important clinical fungal dermatophytes species, *Trichophyton rubrum*, *T. interdigitale*, *T. tonsurans*, and *Arthroderma benhamiae*, thus demonstrating that MALDI-TOF MS might also represent a fast and very specific method for species identification of dermatophytes (Erhard et al. 2008; de Respinis et al. 2012; Nenoff et al. 2013).

C. Antifungal Susceptibility Testing

In vitro antifungal susceptibility testing (AST) is often used to select agents with likely activity for a given infection, but perhaps its most important use is in identifying agents that will not work, i.e., to detect resistance. Currently, there are **two independent standards** for broth microdilution (BMD) susceptibility testing of

Candida and filamentous fungi: the Clinical and Laboratory Standards Institute (CLSI) methods and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods (Lass-Flörl et al. 2010; Pfaller 2012). Both of these methods use BMD, although there are some differences in inoculum size and minimum inhibitory concentration (MIC) endpoint determination, results obtained when testing azoles and echinocandins against *Candida* and azoles against *Aspergillus* species are in close agreement (Pfaller et al. 2011).

Data gathered by these standardized tests are useful (in conjunction with other forms of data) for calculating clinical breakpoints and epidemiologic cut-off values (ECOFFs). Clinical breakpoints should be selected to optimize detection of non-wild-type strains of pathogens and should be species-specific and not divide wild-type distributions of important target species. ECOFFs are the most sensitive means of identifying strains with acquired resistance mechanisms (Pfaller 2012). The wild-type distribution for a species is defined as the distribution of the MIC for isolates that exhibit no acquired or mutational resistance to the drug in question. This also means that a non-wild-type isolate possesses an acquired or mutational resistance mechanism. The upper limit of the wild-type population is defined as the ECOFF. Breakpoints should never divide the wild-type population, as this will lead to a random susceptibility classification (Rodriguez-Tudela et al. 2010). Both reference test methods offer breakpoints and interpretative data for *Candida* spp. and *Aspergillus*. The CLSI breakpoints for fluconazole, voriconazole, and the echinocandins have recently been revised for *Candida* species (Pfaller and Diekema 2012). Although the CLSI documents only provide guidelines for *Candida* spp., some investigators have applied the CLSI breakpoints to *Cryptococcus* spp. and correlations have been demonstrated between higher MICs and treatment failures (Jenkins and Schuetz 2012). For *Aspergillus* species, breakpoints do not yet exist, but susceptibility results can be interpreted as wild type or higher than wild type using ECOFFs (Arendrup et al. 2012).

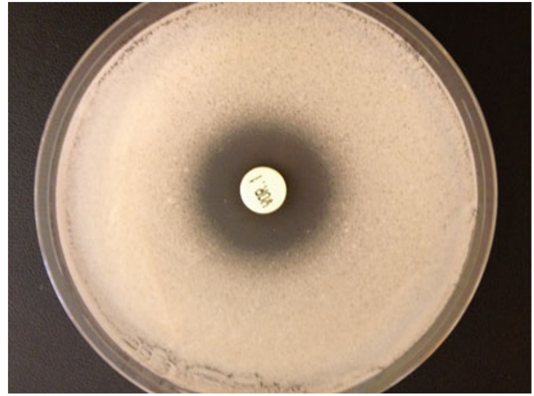

Fig. 13.8. Disk diffusion test (RPMI Agar)

Because the BMD testing method is difficult to perform in daily practice in clinical microbiology laboratories, other testing approaches have been investigated. Disk diffusion antifungal susceptibility testing is a simple and cost-effective method for both yeasts and molds (Fig. 13.8). The CLSI has developed agar-based, disk diffusion testing for yeasts (CLSI 2004, 2009) and molds (CLSI 2010a, b). Disk diffusion testing has been standardized for fluconazole and voriconazole, as well as for echinocandins versus *Candida* species. Also, interpretative breakpoints have been provided for azoles, caspofungin, and micafungin.

There are also commercially available test kits for MIC determination that exhibit potential advantages in terms of ease of use, flexibility, standardization, and rapidity of results. **Etest** (bioMérieux, Marcy l' Etoile, France) is very often used and directly quantifies antifungal susceptibility in terms of discrete MIC values (Fig. 13.9). RPMI-based agars are recommended for use (Pfaller et al. 2000). Also, it is recommended that Mueller-Hinton agar is supplemented with 2% glucose and 0.5 μg/ml methylene blue, which appears to enhance the formation of inhibition ellipses with clear edges and less growth within the ellipse (Pfaller et al. 2004). The method is reliable and reproducible, is suitable for yeast and molds, and has been shown to correlate well with the CLSI methodology (Szekely et al. 1999). A clear benefit of utilizing Etest is in assessing the susceptibility to amphotericin B, as this method gives much broader MIC ranges than BMD. Etest is also highly suitable for determining the activity of echinocandins against yeasts because it produces easy-to-read, sharp zones of inhibition. However, for echinocandins the so-called paradoxical effect has been

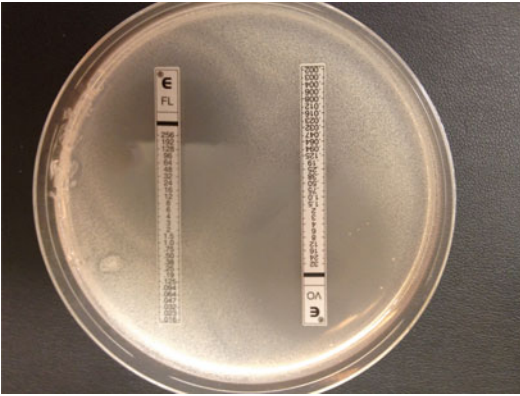

Fig. 13.9. *Candida albicans* and Etest (fluconazole, voriconazole), RPMI-medium

observed for *Candida* and *Aspergillus* in vitro. The paradoxical effect refers to an attenuation of echinocandin activity at higher concentrations despite an inhibitory effect at lower drug levels. It appears to be species-related and varies with the echinocandin. The effect has been noted most often for caspofungin and is not related to FKS1 mutations or upregulation of echinocandin sensitivity of the glucan synthase complex in the presence of drug. The clinical relevance of this in vitro effect is uncertain (Wiederhold 2009; Chen et al. 2011).Another test system is the **VITEK 2**, a fully automated commercial system (bioMérieux, Marcy l'Etoile, France) intended for antifungal susceptibility testing of yeasts. The system reliably detects resistance among *Candida* spp. and demonstrates excellent quantitative, qualitative, and categorical agreement with the reference method in various studies (Pfaller et al. 2004, 2007; Posteraro et al. 2009). However, correct discrimination between fluconazole-susceptible and intermediate *C. glabrata* isolates is not possible because the revised species-specific susceptibility breakpoint is not included in the VITEK 2 detection range (MIC of $\leq$0.250 to $\geq$4 mg/l). Astvad et al. (2013) demonstrated that the VITEK 2 allowed correct categorization of all wild-type isolates as susceptible for caspofungin. However, despite an acceptable categorical agreement, it failed to reliably classify isolates harboring *fks* hot spot mutations as intermediate or resistant, which was in part due to the fact that the detection range did not span the susceptibility breakpoint for *C. glabrata*. Furthermore, EUCAST breakpoints for Caspofungin have not been established, thus the VITEK 2 cannot be used with echinocandins as an alternative for the EUCAST reference method. **Sensititre YeastOne** (Trek Diagnostic Systems, Sun Prairie, WI, USA) is a colorimetric antifungal susceptibility testing MIC plate that exhibits high agreement with the CLSI BMD method (Pfaller et al. 2008).

Susceptibility testing is indicated to provide a basis for selection of appropriate antifungal treatment in individual patient cases and for epidemiological reasons in order to continuously follow susceptibility patterns and thereby detect any emergence of resistance at an early stage. Recommendations for AST are displayed in Table 13.4. However, for individual patient care the isolate should be identified to species level to predict the susceptibility pattern. Important examples of fungi that have low susceptibility to antifungal agents include: *C. krusei*, which is intrinsically resistant to fluconazole and less susceptible to amphotericin B than other *Candida* spp.; *Aspergillus* spp., *Scedosporium apiospermum*, *Trichosporon* spp., and *Scopulariopsis* spp., which are resistant to amphotericin B; zygomycetes, which are resistant to all licensed azoles; and *C. glabrata*, which is frequently less susceptible to fluconazole than other *Candida* spp. For better illustration, Table 13.5 shows the susceptibility pattern of the most common *Candida* spp. In cases where the susceptibility pattern cannot be reliably predicted on the basis of species identification alone, antifungal susceptibility testing should be performed (Arendrup et al. 2012).

VI. Molecular Tools in the Diagnosis of Fungal Pathogens

Molecular species identification has become increasingly important in medical mycology. In the past, this method was mainly applied to fungal isolates that did not form fruiting bodies in culture or to rare isolates that could not be identified by classical phenotype-based methods. However, there is a constantly increasing demand for fast diagnosis in the clinical setting and molecular tools allow rapid species (or at least genus) identification from minute amounts of culture without the need for prolonged subculture to induce fruiting body formation. Only recently have these advantages have been challenged by MS-based identification (see above). Furthermore, molecular

Table 13.4. Antifungal susceptibility testing: when and how to test

When to test?
- Routine antifungal testing of fluconazole and an echinocandin against *C. glabrata* from deep sites
- In invasive fungal infections
- In invasive and mucosal infections failing therapy
- For *Candida* species from sterile sites
- For isolates considered clinically relevant particularly in patients exposed to antifungals

How to test?
- Identify the isolate to species level
- Perform routine susceptibility testing for fluconazole and, according to the local epidemiology, include other azoles
- No testing of isolates with a high rate of intrinsic resistance
  - *C. lusitaniae* and amphotericin
  - *C. krusei* and fluconazole, flucytosine
  - *C. guilliermondii* and echinocandins
  - *A. terreus* and amphotericin B

As described by Lass-Flörl et al. (2010), Arendrup et al. (2012), Pfaller and Diekema (2012) – modified

Table 13.5. General susceptibility patterns of *Candida* species

Candida spp.	AmB	Flucytosin	FLU	ITRA	VOR	POS	EC
C. albicans	S	S	S	S	S	S	S
C. tropicalis	S	S	S	S	S	S	S
C. parapsilosis	S	S	S	S	S	S	S to R
C. glabrata	S to I	S	S-DD to R	S-DD to R	S-DD to R	S-DD to R	S
C. krusei	S to I	I to R	R	S-DD to R	S	S	S
C. lusitaniae	S to R	S	S	S	S	S	S

As described by Pappas et al. (2009) – modified
AmB amphotericin B, *FLU* fluconazole, *ITRA* itraconazole, *VOR* voriconazole, *POS* posaconazol, *EC* echinocandins, *S* susceptible, *SDD* susceptible dose-dependent, *I* intermediate, *R* resistant

species identification (as with MS-based species identification) requires a far lesser expertise of the personnel and – if reliable databases are used for species identification – is likely to generate more reliable results than phenotype-based identification. **Consequently, modern medical mycology has incorporated molecular tools for standard diagnostic procedures.** It is likely that these tools will continue to find their niches in medical mycology even in view of the growing applicability of MS-based species identification. The reason for this is that even with new, as-yet unrecognized fungal pathogens that are not available within identification databases, molecular data can assign an unidentified pathogen to a group of fungi much better than MS patterns.

A. Identification of Cultured Fungal Pathogens

Several technologies have been used in the past for species identification of fungal pathogens. These include restriction-fragment-length polymorphism, random amplified polymorphic DNA, and hybridization-based tools including microarrays and others (Balajee et al. 2007). However, with **sequence-based** tools having become affordable and technically accessible for many medical mycology laboratories, these tools have certainly evolved to be of major importance in the medical mycology laboratory. These techniques can also identify rare species as long as the database is sufficiently large and can suggest related species in the case of novel fungal pathogens (Kurzai et al. 2003).

DNA extraction and PCR amplification from cultured fungi are normally unproblematic and several protocols for extraction of fungal DNA have been described in the literature. A simple protocol, which generally results in sufficient amounts and quality of DNA, is included in the *Atlas of Clinical Fungi* (de Hoog et al. 2009). Some available protocols have much lower yields, mostly due to suboptimal procedures for breaking the fungal cell wall (e.g., protocols based on repeated freezing/thawing), but due to sufficient amounts of DNA in the culture material they can still be useful for extracting fungal DNA. Conventional PCR amplification with 25–35 cycles is sufficient to amplify the target region for sequencing. Several **target regions** have been described in older literature, including parts of the ribosomal gene clusters like the D1/D2 domain of the large subunit (LSU). Sequencing of the LSU in the British reference laboratory between 2004 and 2006 allowed an identification of all clinical isolates that could not be identified by conventional methods (Linton et al. 2007). The most useful target regions for species identification in general are the **internal transcribed spacer regions** (ITS1/ITS2) (Guarro et al. 1999; Balajee et al. 2007).

These regions are intercalated between the more conserved sequences encoding for the ribosomal RNAs (rRNAs), which can be used as a starting point for PCR amplification using "universal" primers, and accumulate mutations with higher frequency than the "functional" rRNAs. The typical fungal ribosomal gene cluster encodes for the 18S rRNA followed by the first internal spacer (ITS1), the 5,8S rRNA, ITS2, and the 28S rRNA gene, which is similar to the arrangements found in other eukaryotic cells (Iwen et al. 2002).

It has been shown that ITS sequencing is regularly sufficient to identify unknown fungal isolates (Rakeman et al. 2005). However, it must be kept in mind that the variability of the ITS regions may differ between fungal species. In fact, ITS sequencing has been used for subspecies strain typing in some species (e.g., for *Pneumocystis jirovecii*), whereas other species have been found to have highly conserved ITS sequences (Iwen et al. 2002). Disadvantages of ribosomal target sequences include a lack of discriminative power in some clinical situations (e.g., discrimination of *Aspergillus* species complexes) (Balajee and Marr 2006) or the presence of heterologous copies in some genera (e.g., some *Fusarium* spp.) that may interfere with amplification and/or sequencing (O'Donnell and Cigelnik 1997). In these cases, other target sequences like elongation factor 1 (EF-1), the RNA-polymerase subunit RPB2, rodlet protein RodA, or β-tubulin can be useful (O'Donnell et al. 1998; Balajee et al. 2007). It has been stated that the success of a sequencing strategy in a clinical microbiology laboratory lies in: (i) the choice of locus, (ii) the amenability of the region to PCR amplification and sequencing, (iii) the reliability of interpretation of the results, and (iv) the availability of a sequence database for comparison (Balajee et al. 2007). The last step of sequence-based species identification relies on sequence comparisons using BLAST tools (most commonly BLAST for comparison of nucleotide sequences) and is strongly dependent on the quality of the database. In most cases, GenBank, a non-curated open-source database is used for initial BLAST analysis. Several studies have clearly demonstrated, that GenBank includes wrong and wrongly assigned sequences, low quality sequences, and other traps that may interfere with a correct species identification (de Hoog and Horre 2002; Nilsson et al. 2006).

The study by Nilsson et al. (2006) suggested that many entries are to some degree incorrect (20%!) and lack reasonable and up-to-date annotation. This problem is further aggravated by constant changes in fungal taxonomy, which are normally not reflected by the database, and the fungal specificity of several names reflecting an identical organism (names for teleomorph, anamorph, and synanamorph). Because sequences deposited in GenBank cannot easily be corrected – it requires action by the initial depositor – erroneous database entries do accumulate over time.

Thus, although GenBank is undoubtedly a highly important tool, a rigorous evaluation of all molecular species identification results is mandatory and a reference laboratory should always be involved in cases of remaining doubt. Evaluation can be achieved by selectively

looking for homology with type strain-derived sequences from known sources and/or authors. In addition, the presence of a number of homologous sequences from the same species but from different sources is usually an encouraging sign. Finally, all molecular results should be carefully evaluated together with the morphological characteristics of the respective isolate and current taxonomic resources. In fact, other web-based repositories can be used for this purpose, including for example the homepage of the Centraalbureau voor Schimmelcultures (CBS), DoctorFungus, or MycoBank (Crous et al. 2004; Robert et al. 2005). When these limitations are kept in mind, species identification based on ITS sequencing is usually reliable with homologies >98%, although there is no clear cut-off for unequivocal species identification (Nilsson et al. 2008). Some commercial databases have become available but are so far not used broadly (Balajee et al. 2007).

B. Molecular Tools for Diagnosing Invasive Aspergillosis

Although a vast variety of PCR protocols for detecting *Aspergillus* species in specimens now exist, no standard protocol has been defined and included in the internationally accepted criteria for the diagnosis of IA (EORTC/MSG) (Löffler and Kurzai 2011). Reasons for this are the missing evaluation of the clinical usefulness and the lack of standardization. Because *Aspergillus* spores are ubiquitously present, it is a great challenge to avoid false-positive results due to contamination, especially in respiratory samples. Therefore, the focus has changed to blood or serum samples with lower risk of contamination. However, recently, the analysis of respiratory samples has been reconsidered for PCR diagnostics of IA.

In general, **fungal DNA concentration in blood or serum samples is very low**. Therefore, a sample volume of at least 3 ml needs to be used for DNA isolation to gain a sufficient amount of *Aspergillus* DNA for PCR analysis, as recommended by the European Aspergillus PCR Initiative (EAPCRI). Due to its inhibitory effect on DNA-degrading enzymes, EDTA should be the anticoagulant of choice. Also, EDTA does not interfere with PCR as other anticoagulants may do. Choosing the right DNA isolation protocol has great impact on the diagnostic quality of *Aspergillus* PCR (White et al. 2010; White et al. 2011). Critical steps in DNA isolation that influence the efficacy of DNA extraction are the lysis of erythrocytes and leucocytes, fungal cell wall disruption, and the elution volume. Omitting lysis of either blood cells or fungal cell wall is associated with a decreased analytic sensitivity. As recommended by EAPCRI, fungal cell wall disruption following blood cell lysis should not be performed with protocols based on enzymatic digestion because they are time consuming, costly, and bear the risk of fungal DNA-contaminated enzyme preparations. For subsequent DNA purification and DNA extraction, commercially available kits can be used (Loeffler et al. 2002). Controls are essential to detect possible fungal DNA contamination of buffers, which has been reported (Loeffler et al. 1999). The optimum elution volume is 60 µl. Neither an increase above 100 µl nor an arbitrary reduction of the elution volume is useful because they lead to a significant decrease in detection rate and in DNA concentration, respectively (White et al. 2010).

The impact of fungal DNA amplification on the diagnostic quality is not as high as the previously described DNA extraction. To ensure enhanced sensitivity of the PCR, a target sequence with multiple genomic copies, high cycle numbers, and amplification with real-time PCR should be preferred. The target sequences most commonly used are within the ribosomal gene clusters 5,8S rRNA, 18S rRNA, and 26 rRNA, with the internal spacers ITS-1 and ITS-2 in between, as mentioned earlier. The genome of *A. fumigatus* contains 38–91 copies of this gene cluster, although this may vary strongly between different strains (Herrera et al. 2009). Mitochondrial DNA sequences also exist in high copy numbers and can also be used (Costa et al. 2002). Combining both target sequences could gain additional sensitivity (Millon et al. 2011).

Several commercial assay are available. MycAssay™ Aspergillus from Myconostica (Manchester, UK) detects *Aspergillus* DNA from lower respiratory tract specimens and serum by real-time PCR. An appropriate fungal DNA extraction and purification kit

(MycXtra®) is offered as well. Another diagnostic assay based on real-time PCR analysis is the SeptiFast kit offered by Roche Diagnostics (Mannheim, Germany). Although originally designed for sepsis diagnostics, it was the first PCR system on the market to detect *A. fumigatus* (Steinmann et al. 2009). SeptiFast not only detects *A. fumigatus*, but also five different *Candida* species. It appears that, for *Candida* spp. and *A. fumigatus*, the SeptiFast methodology is more sensitive than conventional BC (Westh et al. 2009). ViraCor-IBT Laboratories (Lee's Summit, MO, USA) provides a laboratory service for detection of *Aspergillus* spp. that is also based on real-time PCR. Molzym (Bremen, Germany) offers a variety of kits depending on the type of specimen (e.g., SepsiTest for whole blood samples) to detect more than 345 different pathogens including *C. albicans, C. tropicalis, C. parapsilosis, C. krusei, C. glabrata, Cryptococcus neoformans*, and *A. fumigatus* by DNA sequencing. In the case of SepsiTest, broad range primers hybridize within conserved sequences on rRNA genes. SIRS Lab (Jena, Germany) created the DNA detection system VYOO that allows multiplex PCR and detection of 34 bacteria and 7 fungal pathogens (*C. albicans, C. dubliniensis, C. tropicalis, C. parapsilosis, C. krusei, C. glabrata*, and *A. fumigatus*) as well as 5 antibiotic resistance genes in one step by hybridization of microbial DNA onto microarrays. Prior to DNA detection, pathogen DNA is selectively purified using a novel protocol designed by SIRS LAB (Bruns et al. 2009; Sachse et al. 2009). Pathogen DNA can be enriched by affinity chromatography due to selective binding of a specific protein to non-methylated CpG dinucleotide motifs mainly found in microbes. However, this method is not feasible for samples from neutropenic patients (Springer et al. 2011).

C. Other Molecular Tools for Diagnosing Fungal Infection from Clinical Specimens

A vast variety of PCR protocols ranging from specific protocols for selected pathogens to pan-fungal PCRs have been described in the literature and we have restricted ourselves here to describing protocols for diagnosis of IA because these are clearly the best-evaluated molecular tools in medical mycology. However, with the changing epidemiology of fungal infections and the constant emergence of rare pathogens, new studies on molecular tools for aiding diagnosis of invasive mycoses are certainly warranted. The example of *Aspergillus* PCR can serve as a **role-model** and will most likely open the way to standardization and defined quality control, which are urgently required for diagnostic tools in the twenty-first century.

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