What Is the Target? Clinical Mycology and Diagnostics

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1.1 Epidemiology

More than 600 different fungi, yeasts and filamentous fungi, some of them are most commonly known as moulds and dermatophytes, have been reported to infect humans, ranging from common to very serious infections, including those of the mucosa, skin, hair and nails, and other ailments.

Particularly, invasive fungal infections (IFI) are found in patients at risk. Both yeasts and moulds are able to cause superficial, deep and invasive disseminated infections, whereas dermatophytes cause infections of the skin, nails and hair. Dermatophytoses are caused by the agents of the genera *Epidermophyton*, *Microsporum*, *Nannizia* and *Trichophyton*.

Invasive infections encompass mainly immunocompromised patients, e.g. patients with the acquired immunodeficiency syndrome or immunosuppressed patients due to therapy for cancer and organ transplantation or undergoing major surgical procedures. As the patient population at risk continues to expand so also does the spectrum of opportunistic fungal pathogens infecting these patients. Invasive fungal infections may also be serious complications of traumatic injury characterized by fungal angioinvasion and resultant vessel thrombosis and tissue necrosis [1, 2]. In contrast to other settings, posttraumatic IFI occurs through direct inoculation of tissue with spores at the site of injury [3]. Both yeasts and moulds are able to cause superficial, deep and invasive disseminated infections, whereas dermatophytes cause infections of the skin, nails and hair.

1.1.1 Yeasts

Yeasts are fungi with a more or less ball-like shape. Yeasts multiply by budding but may form hyphae or pseudohyphae. Many infections are caused by yeasts with the *Candida* being the most common representative. In the last decades, the expansion of molecular phylogenetics has shown that some genera are polyphyletic, which means that some species are of different genetic origin and therefore unrelated. The genus *Candida* is now associated with at least ten different telemorphic genera including *Clavispora*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces* and *Pichia* [4]. More than 100 *Candida* species are known, whereas the majority of infections are caused by *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* [5]. Other emerging species causing infections have been described. For example, *C. auris* is an emerging multidrug-resistant pathogen that is capable of causing invasive fungal infections, particularly among hospitalized patients with significant medical comorbidities [6].

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Other important genera are *Cryptococcus*, *Malassezia* and *Trichosporon*. Cryptococcal infections occur with a near worldwide distribution in immunosuppressed hosts. This infection is typically caused by *Cryptococcus neoformans*, an encapsulated yeast, and infection is acquired from the environment. *Cryptococcus neoformans* var. *grubii*, *C. neoformans* var. *neoformans* and *C. gattii* are the causes of opportunistic infections which are classified as AIDS-defining illness [7]. Non-*Cryptococcus neoformans* species, including *C. laurentii* and *C. albidus*, have historically been classified as exclusively saprophytic. However, recent studies have increasingly implicated these organisms as the causative agent of opportunistic infections in humans [8].

The lipid-dependent *Malassezia furfur* complex causes pityriasis versicolor, whereas the non-lipophilic *M. pachydermatis* is occasionally responsible for invasive infections in humans. *Trichosporon beigelii* used to be known as the principal human pathogen of the genus *Trichosporon*. Four newly delineated taxa (*T. asahii* and less frequently *T. mucoides*, *T. inkin* and *T. louberi*) are associated with systemic infections in man. *T. mycotoxinivorans* has been described recently as the cause of fatal infections in patients suffering from cystic fibrosis [4].

Saprochaete and *Geotrichum* spp. are rare emerging fungi causing invasive fungal diseases in immunosuppressed patients, mainly in patients with haematological malignancies, but also other non-haematological diseases as underlying disease have been reported [9]. The most important risk factor is profound and prolonged neutropenia [10].

Saccharomyces cerevisiae is a common food organism and can be recovered from mucosal surfaces, gastrointestinal tract and female genital tract of healthy persons. Occasionally, it causes vaginal infections and on very rare occasions invasive infections in immunocompromised and critically ill patients [4].

Rhodotorula species have traditionally been considered as one of common non-virulent environmental inhabitant. They have emerged as an opportunistic pathogen, particularly in immunocompromised hosts, and most infections have been associated with intravenous catheters in these patients. *Rhodotorula* spp. have also been reported to cause localized infections including meningeal, skin, ocular, peritoneal and prosthetic joint infections; however, these are not necessarily linked to the use of central venous catheters or immunosuppression [11].

Pneumocystis jirovecii (formerly known as *P. carinii*) is a unicellular, eukaryotic organism occurring in lungs of many mammals. *P. jirovecii* is a causative agent of *Pneumocystis* pneumonia. Although the incidence of *Pneumocystis* pneumonia (PCP) has decreased since the introduction of combination antiretroviral therapy, it remains an important cause of disease in both HIV-infected and non-HIV-infected immunosuppressed populations. The epidemiology of PCP has shifted over the course of the HIV epidemic both from changes in HIV and PCP treatment and prevention and from changes in critical care medicine. Although less common in non-HIV-infected immunosuppressed patients, PCP is now more frequently seen due to the increasing numbers of organ transplants and development of novel immunotherapies [12].

1.1.2 Filamentous Fungi

Filamentous fungi form colonies of different colours with a more or less woolly surface formed by the filamentous hyphae that may carry conidia (spores) that are disseminated easily via the air (asexual propagation). These fungi are generally perceived as moulds.

Although a wide variety of pathogens are associated with invasive mould diseases, *Aspergillus* spp. are counted among the most common causative organisms. Overall, the genus *Aspergillus* contains about 250 species divided into subgenera, which in turn are subdivided into several sections or species complexes. Of these, 40 species are known to cause diseases in humans. Most invasive infections are caused by members of the *A. fumigatus* species complex, followed by *A. flavus*, *A. terreus* and *A. niger* species complexes [13]. The *Aspergillus fumigatus* species complex remains the most common one in all

pulmonary syndromes, followed by *Aspergillus flavus* which is a common cause of allergic rhinosinusitis, postoperative aspergillosis and fungal keratitis. Lately, increased azole resistance in *A. fumigatus* has become a significant challenge in effective management of aspergillosis. The full extent of the problem is still unknown, but some studies suggest that resistance in *A. fumigatus* may be partially driven by the use of agricultural azoles, which protect grain from fungi [14]. Other species of *Aspergillus* may also be resistant to amphotericin B, including *A. lentulus*, *A. nidulans*, *A. ustus* and *A. versicolor*. Hence, the identification of unknown *Aspergillus* clinical isolates to species level may be important given that different species have variable susceptibilities to multiple antifungal drugs.

Mucormycosis is caused by fungi of the order *Mucorales*. Of fungi in the order *Mucorales*, species belonging to the family *Mucoraceae* are isolated more frequently from patients with mucormycosis than any other family. Among the *Mucoraceae*, *Rhizopus* is by far the most common genus causing infection, with *R. oryzae* (*R. arrhizus*) being the most common one [15, 16]. *Lichtheimia corymbifera*, *Rhizomucor* spp., *Mucor* spp. and *Cunninghamella* spp. are also known to cause jeopardizing infections. *Mucorales* are resistant to voriconazole and caspofungin in vitro and in vivo. The incidence of mucormycosis may be underestimated due to the low performance of diagnostic techniques based on conventional microbiological procedures, such as culture and microscopy. The most useful methods for detecting *Mucorales* are still microscopic examination of tissues and histopathology, which offer moderate sensitivity and specificity. Recent clinical studies have reported that mucormycosis is the cause of >10% of all invasive fungal infections when techniques based on DNA amplification by quantitative used to complement conventional methods [17].

Besides *Mucorales*, the emergence of other opportunistic pathogens, including *Fusarium* spp., *Paecilomyces* spp., *Scedosporium* spp. and the dematiaceous fungi (e.g. *Alternaria* spp.), became evident [5]. *Fusarium* spp., *Alternaria* spp. and *Scedosporium* spp. also account for mould infections among solid organ transplant recipients.

The genus *Fusarium* includes several fungal species complexes. These are ubiquitous soil saprophytes and pathogenic for plants [13]. Only a few species cause infections in humans [18]. Among these are the species complexes *F. solani*, *F. oxysporum*, *F. verticillioides* and *F. fujikuroi* [19]. *Fusarium* spp. have been involved in superficial and deep mycosis and are the leading causes of fungal keratitis in the world [18, 20]. Recently, these fungi have been identified as emerging and multiresistant pathogens causing opportunistic disseminated infections [21, 22].

The genus *Scedosporium* has undergone a taxonomic reclassification. According to the new classification, the most common *Scedosporium* spp. involved in human infections are *S. apiospermum* (telemorphic state, *Pseudallescheria apiosperma*), *S. boydii* (*Pseudallescheria boydii*), *S. aurantiacum* and *S. prolificans* (*Lomentospora prolificans*). Owing to epidemiological reasons, most recent reports divide human infections by these species into mycoses caused by the *S. apiospermum* complex (which includes *S. apiospermum*, *S. boydii* and *S. aurantiacum*) and by *S. prolificans* [13].

Species belonging to the *S. apiospermum* complex are cosmopolitan, being ubiquitously present in the environment, but predominantly in temperate areas. They are commonly isolated from soil, sewage and polluted waters, composts and the manure of horses, dogs, cattle and fowl [23]. *S. prolificans* appears to have a more restricted geographical distribution, being found largely in hot and semiarid soils in southern Europe, Australia and California [24].

Table 1.1 shows the most common yeasts and moulds causing IFI.

1.1.2.1 Relevant Diagnostic Material for Diagnosis of Clinical Mycoses

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.

Table 1.1 Spectrum of opportunistic yeasts and moulds (exemplary, without claiming completeness)

Yeasts		Moulds	
Candida	*C. albicans* *C. glabrata* *C. parapsilosis complex* *C. tropicalis* *C. guilliermondii* *C. auris*	*Aspergillus* species complex	*A. fumigatus* *A. flavus* *A. terreus* *A. niger*
Cryptococcus	*C. neoformans* var. neoformans *C. neoformans* var. grubii *C. gattii*	*Mucorales*	*Rhizopus* spp. *Rhizomucor* spp. *Mucor* spp. *Lichtheimia corymbifera* *Cunninghamella* spp.
Trichosporon	*T. asahii* *T. mucoides* *T. inkin* *T. louberi* *T. mycotoxinivorans*	*Fusarium* species complexes	*F. solani* *F. oxysporum* *F. verticillioides* *F. fujikuroi*
Malassezia	*M. furfur* species complex *M. pachydermatis*	*Scedosporium*	*S. apiospermum* *S. boydii* *S. aurantiacum* *S. prolificans* = *Lomentospora prolificans*
Geotrichum and *Saprochaete*	*G. candidum* *S. capitate* *S. clavata*	*Paecilomyces*	*P. variotii*
Saccharomyces	*S. cerevisiae*	*Scopulariopsis*	*S. brevicaulis*
Rhodotorula	*R. rubra* *R. mucilaginosa* *R. glutinis* *R. minuta*	*Alternaria*	

Superficial samples like swabs, respiratory secretion, sputum or stools are not helpful for the diagnosis of invasive fungal infection as both yeasts and filamentous fungi easily colonize body surfaces.

1.1.2.2 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 or histopathologic evidence of invasion [25]. Because of the limited sensitivity of all these diagnostic procedures, and concerns about specificity of some of them, a combination of various testing strategies is the hallmark of IFI diagnosis [17, 25].

1.1.2.3 Histopathology

Histopathology of excised human tissue samples is the cornerstone for diagnosing and identifying fungal pathogens. Direct examination for the presence of mycelial elements using appropriate staining (e.g. Grocott-Gomori methenamine silver, periodic acid-Schiff, potassium hydroxide-calcofluor white) should be performed on all clinical specimens, including respiratory secretions or any tissue sample [17].

However, 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 [26]. As *Aspergillus* is far more commonly encountered than the other pathogens mentioned, a pathologist often may describe an organism as *Aspergillus* or *Aspergillus*-like based upon morphological features alone. This can hinder diagnosis and may entail inappropriate therapy [27].

1.1.2.4 Microscopy

Direct microscopy is most useful in the diagnosis of superficial and subcutaneous fungal infections and, in those settings, should always be performed together with culture.

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 [28]. Because yeast and moulds can stain variably with the Gram stain, a more specific fungal stain is recommended [29].

Microscopy may help to discern whether an infection is caused by yeast or moulds. The presence of pseudohyphae and optionally blastoconidia indicates the presence of yeast, whereas moulds 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 [30]. However, it is impossible to differentiate between the respective genera of the mentioned fungi. It is important to look for septate and nonseptate hyphae, thus allowing to distinguish between *Aspergillus* sp. and members of the *Mucorales*. Mucoraceous moulds have large ribbon-like, multinucleated hyphal cells with non-parallel walls and infrequent septa. The branching is irregular and sometimes at right angles. Hyphae can appear distorted with swollen cells, or compressed, twisted and folded [30]. Another group of moulds causing tissue invasion with a distinctive appearance is the agents of phaeohyphomycosis, such as *Alternaria* and *Curvularia*. These fungi have melanin in their cell walls and appear as pigmented, septate hyphae [31]. 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.

The most common direct microscopic procedure relies on the use of 10–20% potassium hydroxide (KOH), which degrades the proteinaceous components of specimens while leaving the fungal cell wall intact, thus allowing their visualization [30].

The visibility of fungi within clinical specimens can be further enhanced by the addition of calcofluor white or blankophores. These are fluorophores, which are members of a group of compounds known as fluorescent brighteners or optical brighteners or "whitening agents" and bind to beta 1–3 and beta 1–4 polysaccharides, such as found in cellulose and chitin. When excited with ultraviolet or violet radiation, these substances will fluoresce with an intense blueish/white colour [25]. Optical brightener methods have been shown to be more sensitive than KOH wet mount [31]. 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 [32]. As 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 [33].

Also, lactophenol cotton blue is easy to handle and often used for the detection and identification of fungi. 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 [32].

For detection of *P. jirovecii*, special staining as, for example, direct immunofluorescent staining is required. Sputum induction and BAL are the most commonly used, although non-HIV-infected patients with PCP may require lung biopsy for diagnosis. Standard staining methods include methenamine silver, toluidine blue-O or Giemsa stain. Monoclonal antibodies can be used to detect *Pneumocystis* with a rapid, sensitive and easy-to-perform immunofluorescence assay [12].

1.1.2.5 Culture

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 aetiological agent and may allow susceptibility testing to be performed. Proper collection and transportation of the specimen is essential. Particularly, sterile materials are important for diagnosis of invasive fungal infections. Fungal selective media must be included, and it should be observed that some species take a certain period of time (5–21 days) to grow in culture. Negative culture results do not exclude fungal infection. Identification of the isolate to species level is mandatory [34].

Blood cultures (BC) are the first-line test and currently considered the "gold standard" in the event of any suspected case of systemic mycosis [35]. Several commercial blood culture systems are available. Lysis centrifugation was one of the first systems to detect fungi and became a gold standard [25]. However, the more commonly used automated blood culture systems appear to show the same sensitivity for the majority of invasive fungi [36].

The Bactec System (BD Diagnostic System, Sparks, Md., USA) and the BacT/Alert System (bioMérieux, Marcy l'Etoile, France) are widely used automated systems. The Bactec system proposes a specifically formulated medium for the isolation of fungi, called Mycosis IC/F medium. The recommended incubation period 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 [37, 38]. 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 as this is the case for *Histoplasma capsulatum*.

In 2012, recommendations concerning diagnostic procedures for detection of *Candida* diseases have been published by the ESCMID Fungal Infection Study Group [34]. Concerning candidaemia, 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 of 60 mL blood for adults obtained in a single session within a 30-min period and divided in 10-mL aliquots among three aerobic and three anaerobic bottles. The frequency recommended is daily when candidaemia 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 [39, 40].

Despite the advances in blood culture technology, the recovery of fungi from the blood remains an insensitive marker for invasive fungal infections. Filamentous fungi will be detected to a much lesser extent than yeasts, because most of them do not sporulate in the blood with the exception of *Fusarium* spp. [17, 41]. Concerning *Aspergillus*, only *A. terreus* has been described to be detected by blood cultures.

Cultures of lower respiratory secretions collected by bronchoscopy and bronchoalveolar lavage fluid (BALF) are part of the diagnostic work-up of invasive pulmonary mould infections. However, the yield of BALF culture is notoriously low, usually showing a sensitivity of 20–50% [17]. In addition, positive BALF culture may reflect colonization and not infection, particularly in lung transplant recipients or patients with chronic lung diseases. On the other hand, the ubiquitous nature of airborne conidia and the risk of accidental contamination with moulds may hamper the interpretation of a positive result. It has to be considered that the positive predictive value of culture depends on the prevalence of the infection and thus it is higher among immunocompromised patients [42]. One study suggests that positive BALF culture for *Aspergillus* spp. may be associated with IA in as many as 50% of ICU patients even in the absence of high-risk host conditions [43]. As a consequence, it is recommended that respiratory tract samples positive for *Aspergillus* spp. in the critically ill should always prompt further diagnostic assessment. Attention has to be paid that the absence of hyphal elements or a negative culture does not exclude a fungal infection.

Culture is highly sensitive (98%) in patients with Cryptococcus meningitis [44]. However, in central nervous system, aspergillosis or candidiasis cultures from cerebrospinal fluid (CSF) are less sensitive [45]. All yeasts and moulds 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 obtained specimens. When looking for dermatophytes, all samples are cultured on agar for identification, which takes at least 2 weeks. A negative culture result cannot be confirmed until plates have been incubated for 6 weeks. Treatment of clinically obvious or severe cases should not be delayed for culture results, although treatment may need to be altered according to the dermatophyte grown. 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 [46].

Yeasts are identified by their assimilation pattern and their microscopic morphology and moulds by their macroscopic and microscopic morphology. Commercially available biochemical test systems identify most of the commonly isolated species of yeast accurately, but it has to be kept in mind that no identification or misidentification of more unusual isolates might occur. Due to their slow growth, identification can take several days and in rare occasions even weeks. Certain *Candida* spp. can be identified more rapidly by using chromogenic media.

Chromogenic media have also been shown to allow easier differentiation of *Candida* species in mixed yeast populations than the traditional Sabouraud glucose agar [25].

Identifying filamentous fungi is much more cumbersome. Generally, macroscopic and microscopic morphology is the key to identification. The macroscopic examination of the colonies can reveal important characteristics concerning colour, texture, exudates, pigments, specific structures, growth rate and growth zones, and the texture of the aerial mycelium. The colour 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 [30].

As an alternative to the conventional identification schemes, proteomic profiling by mass spectral analysis has recently emerged as a simple and reliable method to identify yeasts, moulds and dermatophytes [47]. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) is now commonly used in routine laboratories for yeast identification, while the identification of moulds and dermatophytes using this technique is still not as common as for yeasts.

Yeasts including *Candida*, *Pichia* and *Cryptococcus* genera are most easily processed and analysed. Furthermore, closely related yeast species which cannot be discriminated with common biochemical methods such as the *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 [48]. Even *C. auris*, a recently described multiresistant *Candida* species being typically misidentified by commercial API-20C or Vitek-2 systems, is correctly identified by MALDI-TOF [49].

This technique has also been applied directly on positive blood cultures without the need for its prior culturing, and thus reducing the time required for microbiological diagnosis. Results are available in 30 min, suggesting that this approach is a reliable, time-saving tool for routine identification of *Candida* species causing bloodstream infection [25].

The differentiation of moulds like *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and dermatophytes appears to be far more difficult. Reference databases and the database query methods (i.e. comparing and subsequent scoring of the similarity of an unknown spectrum to each database reference spectrum) may directly affect the performance of MALDI-TOF MS for the identification of fungi. While the reference database provided with each commercial MALDI-TOF

MS platform may not be sufficient for routine analyses, some authors noticed that increasing the number of mass spectra obtained from distinct subcultures of strains included in the reference spectrum library (i.e. the number of reference entries) would improve the accuracy of MALDI-TOF MS-based mould identification [50].

Normand et al. developed a free online application which seems to improve the rate of successful identifications [51]. Up to 92.61% of 501 fungal isolates derived from human samples were correctly identified. Only 5% of the identifications were unsatisfactory (i.e. correct at the genus level but not at the species level), and none of the identifications were false at the genus level. These results are better than those usually obtained via phenotypic identification and thus encourage the use of MALDI-TOF in a routine laboratory for mould identification.

1.1.2.6 Surrogate Markers: Biomarkers of Invasive Fungal Infections

Early and reliable diagnosis and rapid initiation of appropriate antifungal therapy has been shown to improve survival significantly. It has been demonstrated that surrogate markers of fungal infections are able to speed up diagnosis and thus further improve treatment and outcomes for patients with IFIs [52].

1.2 Antigen and Antibody Detection

Antibody and antigen detection often provides supplemental information for the diagnosis of invasive fungal infections. Antibody tests are often used in the diagnosis of endemic mycoses, which are often difficult to detect by traditional methods.

1.2.1 Candidiasis

In some cases, antibody tests are a supplemental test in the diagnosis of invasive candidiasis. Interestingly, serum immunoglobulin G (IgG) responses against specific antigens have generally performed better than IgM, suggesting that many patients mount amnestic responses or have ongoing, subclinical tissue invasion [52]. Patients infected with non-*C. albicans* species can be identified by responses against recombinant *C. albicans* antigens [53].

However, it has to be considered that the detection of anti-*Candida* antibodies fails to discriminate between disseminated and superficial infections and may also indicate colonization in uninfected patients. In immunocompromised patients not reliably producing antibodies, diagnosis based on antibody detection is rendered nearly impossible [25, 35]. A number of reports indicate substantial improvement of sensitivity and specificity of invasive candidiasis is when mannan antigen and anti-mannan antibody assays are used in combination. Mikulska et al. [54] reported a combined mannan/anti-mannan sensitivity and specificity for invasive candidiasis diagnosis of 83% and 86%, respectively (compared with separate sensitivities and specificities of 58% and 93% for mannan antigen alone and 59% and 83% for anti-mannan antibodies alone). Thus, detection of serum mannan and anti-mannan antibodies is turning out to be very interesting for earlier diagnosis of invasive candidiasis.

Serial determinations may be necessary. It shows also very high negative predictive value (>85%) and can be used to rule out infection [34].

1.2.2 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 (CrAg) can be detected either by latex agglutination test (LA) or by ELISA. False-positive reactions have been reported in patients with disseminated trichosporonosis, *Capnocytophaga canimorsus* septicaemia, malignancy and positive rheumatoid factor when using the LA. Another assay format is the EIA, the PREMIER Cryptococcal antigen assay (Meridian Diagnostics, Inc.) utilizing 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 [25].

Since 2009, there is also lateral flow assay (LFA) for the detection of the CrAg available [55]. The CrAg LFA is a well-established point-of-care (POC) test and has an excellent test performance, it is easy to use, and test results are available in 10 min. Moreover, the CrAg LFA is temperature stable, and cross-reactions with other fungi are rare. Serum, plasma, urine and CSF specimens can be used and have shown an excellent sensitivity and specificity [56]. Importantly, CrAg LFA is not useful to check treatment response, as the clearance of CrAg is a slow and also independent process that devitalizes the yeast [57, 58]. Therefore, CrAg LFA titres may therefore remain elevated even if therapy is effective [55, 58].

1.2.3 Invasive Aspergillosis (IA)

Aspergillus antibodies are only infrequently detectable in immunocompromised patients but are often helpful in patients with aspergilloma, allergic bronchopulmonary aspergillosis and cystic fibrosis [59].

Significant advances to the field were brought by the introduction of noncultural diagnostic tests in blood and BALF, including galactomannan antigen (GM) testing for invasive aspergillosis and beta-D-glucan (BDG) testing in patients at risk [52]. When noncultural diagnostic tests were introduced, the rate of fungal infections diagnosed pre-mortem (versus postmortem) was shown to increase from 16 to 51% in a large autopsy study [60].

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 the use in serum and BALF [25, 52]. GM testing is currently considered the gold standard when it comes to biomarkers for IA diagnosis as sensitivity and specificity are generally high. Recently, it has been reported that this assay shows a good diagnostic performance when urine and CSF samples are used [52, 61, 62].

However, false-positive and false-negative results of GM have been described in certain patient groups by various authors [25, 42]. False-negative results occur in patients who are receiving antifungal agents other than fluconazole.7 False-positive results occur in patients who are colonized but not infected with *Aspergillus* species. As colonization is undesirable in solid organ transplant or haematology patients at high risk for invasive aspergillosis, results attributed to colonization should not be disregarded but rather should 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 [63].

Patients who have infection with *Fusarium* species, *Paecilomyces* spp., *Histoplasma capsulatum* and *Blastomyces dermatitidis* may also show positive results because these fungi have similar galactomannans in their cell walls. Cross-reactions may occur with non-pathogenic fungi that are closely related to *Aspergillus* spp., such as *Penicillium* spp. False-positive reactions may be due to the presence of GM in blood-derived products, sodium gluconate containing hydration solutions, antibiotics or food products [64–66].

False-positive reactions with piperacillin-tazobactam have been reported in the past, but manufacturing changes have eliminated this problem. Other reported causes of false-positive results include severe mucositis, severe gastrointestinal graft-versus-host disease, blood products collected in certain commercially available infusion bags, multiple myeloma (IgG type) and flavoured ice pops or frozen desserts containing sodium gluconate [67]. However, 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 [25].

1.2.4 Aspergillus-Specific Lateral Flow Device Test (LFD)

In 2012, Thornton et al. developed a new promising LFD for the detection of *Aspergillus* in patients suffering from haematological 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 [68]. Minimal required training, simple handling by using BALF samples without any pretreatment, no need for specially equipped laboratories, rapid availability of test results within 15 min and low costs are the major advantages of the LFD [52]. In case of serum testing, samples need to be pretreated by heating, centrifugation and adding a buffer solution according to the manufacturer's recommendations. Results are read by eye after 15-min incubation time and are interpreted depending on the intensity of the test line as negative (−) or weak (+) to strong (+++) positive. Cross-reactivities are rare with the LFD. It appears that only *Penicillium* spp. cause cross-reactions [55]. In clinical studies, sensitivity and specificity rates were acceptable; in particular in BALF samples, even during antimould prophylaxis/treatment, the overall sensitivity was 56% during antifungals versus 86% without [69]. The combination with other biomarkers is currently the most promising approach to indicate IPA [70–77]. Similar to other fungal diagnostics, sensitivity of the LFD is reduced in the presence of antifungal prophylaxis/treatment. Following extensive appraisal of the prototype LFD, the test has now been formatted for large-scale manufacture and CE marking as an in vitro diagnostic (IVD) device. It shows promising performance in a first clinical study [78].

1.3 1-3-β-D-Glucan (BDG) as a Marker for Invasive Fungal Infection

Whereas GM has the limitation of being able to detect only invasive aspergillosis, BDG as a cell wall component of many pathogenic fungi can be detected in a variety of invasive infections including *Aspergillus* spp., *Candida* spp., *Pneumocystis jirovecii*, *Fusarium* spp., *Trichosporon* spp. and *Saccharomyces* spp. but does not allow differentiation of yeast from mould infections [79]. However, it is absent in mucormycosis and at least according to most authors in cryptococcosis. BDG is a major component of the fungal cell wall. It can be detected by the activation of the coagulation cascade in an amoebocyte lysate of horseshoe crabs (*Limulus polyphemus* or *Tachypleus tridentatus*). Various tests are commercially available. The Fungitell assay (Associates of Cape Cod, Falmouth, MA, USA) has been approved by US FDA and is widely used in Europe, while other assays (Fungitec-G, Seikagaku Corporation; Wako Pure Chemicals Industries Ltd.; Maruha-Nichiro Foods Inc.; Tokyo, Japan) have been commercialized in Asia [17]. The role of serum BDG testing to diagnose IFI has been well documented, but other samples, including BALF and CSF fluid, might work as well [80].

Similar to GM, BDG is included as mycological criterion in the revised definitions of IFI from the EORTC/MSG consensus group [81]. This test is considered to be a useful adjunct, especially for patients with intra-abdominal infections, where the sensitivity of cultures is decreased [81]. Studies in adults suggest that monitoring of BDG might be a useful method to exclude IFI in clinical environment with low to moderate prevalence of IFI. Many potential sources for contamination have been demonstrated and may lead to false-positive results [17]. It has also been reported that dialysis filters made from cellulose significantly increase serum-glucan concentrations and thus may lead to false-positive test results [82]. In addition, patients likely to be colonized with fungi may show false-positive results. Therefore, this test has been recommended for exclusion of fungal infection in case of negative results and can be used in the sense of antifungal stewardship. It is crucial for clinicians to know that the BDG assays should always be interpreted in the context of clinical, radiographic and microbiological findings [35].

A more recent approach is the combined use of BDG and procalcitonin for the differential diagnosis of candidaemia and bacteraemia, which is an important issue in intensive care patients [83].

In children and neonates, the diagnostic role of BDG is unclear. Children have shown higher mean BDG levels than in adults [84]. However, very high levels of BDG exist in neonates and children with proven IFI [85] so that the diagnostic cut-off may be increased to 125 pg/ml in neonates with invasive candidiasis (and not 80 pg/ml as suggested for adults) [86]. Due to a high number of false-positive and false-negative results in paediatric patients with hematologic disorders and HSCT recipients BDG is not considered a reliable efficient diagnostic tool in this population [87].

Concerning cryptococcal meningitis, the role of BDG testing has been debated controversially. Though it was once believed that *C. neoformans* does not contain BDG in its cell wall, detectable levels of BDG in CSF were found to correlate with quantitative fungal cultures, and high CSF BDG levels (>500 pg/mL) and were associated with a three times higher risk of 10-week mortality. Although CSF BDG levels do not have adequate sensitivity or specificity to make this assay the preferred cryptococcal diagnostic test, positive results should warrant further diagnostic testing, especially in high-risk, immunocompromised patients [88]. Nucleic acid amplification tests for direct detection of fungi.

Molecular amplification techniques enable the fast and sensitive detection and identification at a species level by direct detecting and analysing tiny amounts of fungal DNA present in serum and blood without the need of prior cultivation [89]. Multiple in-house PCR assays targeting various genetic sequences (18S rDNA, 28S rDNA, 5.8S rDNA, internal transcribed spacer region, mitochondrial DNA) have been developed for the detection of a broad range of fungi in different specimens such as blood, serum, plasma, BAL, sterile fluids and tissues though only a few of these techniques have been standardized so far. Depending on the primers used, fungal pathogens can be detected generally or more specifically, including rapid identification of particular fungal pathogenic species with suitable primers and assays like real-time PCR [90]. The sensitivity and specificity results of the various techniques are variable, but mostly there is an improved sensitivity observed when compared to classical cultural-based methods [25].

The use of PCR to diagnose medical mycoses has been challenging, however, because fungi have cell walls that impede the efficient lysis of organisms and liberation of DNA, thus leading to false-negative PCR results. On the other hand, some human pathogens are also ubiquitous in the environment and may therefore cause false-positive results [91]. A crucial distinction must be made between identification and detection of fungal pathogens using PCR: identification from culture or biopsies requires specific DNA extraction procedures, since the fungal wall has to be broken to avoid false negatives. By contrast, in serum or plasma, fungal DNA is already free and may be more easily detected. Recent technological advancements such as microarray, multiplex PCR with magnetic resonance and others have mitigated the technical difficulty of performing nucleic amplification in both yeast and mould and as a consequence improved the sensitivity and specificity of PCR-based assays for the identification of human fungal pathogens [92, 93].

Several *Candida*-PCR assays have been developed and evaluated and have shown benefit concerning the enhancement of rapid diagnosis. It has been demonstrated that the use of direct PCR is associated with good sensitivity and specificity for rapid diagnosis when using blood samples [35, 94, 95].

A recently developed and already commercially launched diagnostic test detecting *Candida* bloodstream infections is T2Candida panel [93, 96]. The T2Candida panel in combination with the T2Dx instrument (both T2 Biosystems) forms a fully automated and rapid diagnostic tool for early detection of yeasts. This method is magnetic resonance-based and allows highly sensitive detection directly in complex samples, such as whole blood, and is able to detect five *Candida* spp., namely, *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and *C. glabrata*. The technology allows for the lysis of yeast cells, releasing fungal DNA, then makes copies of the target DNA using PCR and detects the amplified nucleic acids in aqueous solution using magnetic resonance. The platform can use a single blood sample to identify candidaemia within 3 to 5 hours, whereas traditional testing methods can take 6 days or more. This is a magnetic resonance-based diagnostic approach that measures how water molecules react in the presence of magnetic fields

[17, 96]. When particles coated with target-specific-binding agents are added to a sample containing the target, the particles bind and cluster around the target. This clustering changes the microscopic environment of water in the sample, which in turn alters the T2 magnetic resonance signal or the T2 relaxation signal, indicating the presence of the target. This method differs from traditional PCR, where as much as 99% of the fungal DNA target can be lost. T2Candida can detect microbes at a density as low as 1 colony-forming unit (CFU) per ml of whole blood, compared with the 100–1000 CFU/ml typically required for conventional PCR-based methods. Sensitivity of 91.0% and specificity of 98.1% have been reported to be higher than 90% in several studies with PPV 71.6% to 84.2% and NPV ranging from 99.5% to 99.0% [95]. Paediatric patient studies revealed a 100% concordance with blood culture results and T2MR [97].

The speed and sensitivity of T2Candida give it the potential to improve patient care, but the reagents and instrumentation are expensive. A more recent regulatory decision by the FDA gave the superiority claim of T2Candida over blood culture systems. As data is scarce, it is currently under investigation for use in clinical practice [98].

PCR for invasive aspergillosis has been established for whole blood, serum, plasma and other specimens but is very challenging because of the very low amount of DNA in samples [17, 25, 35, 42, 69, 99]. In 2006, the European Aspergillus PCR Initiative (EAPCRI) was launched to seek proposals for a technical consensus. This consensus was possible, thanks to the generalization of real-time quantitative PCR (qPCR), which dramatically reduces the risk of contamination from environmental amplicons and allows quantitative management of the amplification reaction to detect inhibition [100]. Because whole blood is technically more demanding for the extraction steps, serum appears to be a better specimen [101]. However, plasma is now preferred to serum as it shows a better sensitivity [102].

For the time being, the combination of PCR and other biomarkers such as GM or BDG seems to be the most forward strategy. Studies comparing the performance of PCR and fungal biomarkers in serum (GM or BDG) or BAL (GM) have yielded encouraging results, suggesting optimal diagnostic accuracy when combined [17, 103].

A recent meta-analysis showed that the association of GM and PCR tests is highly suggestive of an active infection with a positive predictive value of 88% [104]. However, the combined use of LFD, instead of GM, and qPCR could be a better strategy [99].

Multiplex PCR assays targeting the most clinically relevant *Mucorales* in serum or BAL have also been developed and show promising results for the early diagnosis of mucormycosis but have to be further evaluated and standardized [17].

Panfungal PCR A different method used in molecular diagnostics of fungal infections is the use of a PCR that can detect a wide variety of fungi at once in the same specimen. The technique is fairly simple and is based on the use of primers specifically designed to amplify a region that is conserved among different fungal genera. Nevertheless, limitations should also be considered, such as the facts that panfungal PCR could be less sensitive in case of some fungi, e.g. interference of melanin with the amplification in case of dematiaceous hyphomycetes. Furthermore, presence of a mixed fungal infection or the presence of the microorganism due to colonization or accidental contamination needs to be taken into account when interpreting the results. However, several studies have shown the utility of panfungal PCRs, but still clinical evaluation is needed [91, 105, 106].

When performing panfungal PCR assays, DNA sequence analysis is often required when obtaining the amplification product. For DNA sequence analysis, the results must be compared with those deposited in databases from known organisms in order for an identity to be obtained. Publically available databases for DNA fungal sequence comparisons are available, including those at the National Center for Biotechnology Information (GenBank; www.ncbi.nlm.nih.gov/genbank/), the Centraalbureau voor Schimmelcultures Fungal Biodiversity Center in the Netherlands (CBS-KNAW; www.cbs.knaw.nl), the International Society of Human and Animal Mycology ITS Database (ISHAM; its.mycologylab.org) and the Fusarium-ID database (http://isolate.fusariumdb.org). The use of sequence results can be extremely useful when compared with credible deposits.

Not all fungal deposits within databases, however, have been confirmed to be from accurately identified organisms [107]. This can lead to erroneous results and the misidentification of the cultured specimen. In addition, the choice of the proper target sequence can be critical for the identification of fungi. Although the internal transcribed spacer (ITS) region has been put forth as a universal barcode for the identification of fungi [108], this target cannot always be used alone to discriminate between closely related fungi. Several other DNA targets may be required to identify fungi in the clinical setting, and the choice of targets depends on the suspected genus [109].

The commercially available PCR kit the LightCycler® SeptiFast Test MGRADE, designed to detect the 25 most prevalent microorganisms in blood culture (also comprising 5 *Candida* spp., as well as *Aspergillus fumigatus*) is based on real-time PCR targeting species-specific ITS regions and has been evaluated for a few years now in Europe (Roche Diagnostics GmbH, Mannheim, Germany). The complete test procedure is validated by detection of positive signals generated by an integrated internal control DNA in order to reassure an uninhibited amplification and detection within the test specimen. In case of *Candida* spp. and *A. fumigatus*, the SeptiFast test turned out to be more sensitive than conventional BC and was not affected by the administration of antimicrobial therapy [25, 89, 110].

PCR has been shown to work well in the paediatric population. A potential drawback of PCR testing in these patients is the amount of specimen needed to perform valid testing (about 2 ml), which is markedly more material than that needed for the GM, BDG and LFD tests [80]. Advantages and disadvantages of the various test assays are listed in Table 1.2.

Table 1.2 Current approaches to laboratory diagnosis

Test	Specimen	Advantage	Disadvantage	Recommendation
Histopathology	Tissue	Enables proven diagnosis	Requires biopsy, no identification to genus and species	
Direct microscopy	Any	Low cost	Labour intensive, no identification to genus and species	Better sensitivity when using calcofluor white
Culture	Any	Allows exact identification and susceptibility testing	Slow, dependent on viable organisms	Use of specific media
Galactomannan	Serum, BAL; investigational: CSF, urine	Sensitive, specimens easy to obtain, rapid results	Decreased sensitivity when patient is on antifungals	Useful for monitoring therapeutic response, useful for diagnosing IA when using BAL
Beta-D-glucan	Serum; investigational: BAL, CSF	Sensitive, specimens easy to obtain, rapid results	Lacks specificity, high rate of false-positive results	Especially for exclusion of fungal infections; could be useful as a screening technique when doing serial determinations in haematological patients at high risk
Lateral flow test	Serum, BAL	Sensitive, rapid results Very reliable for detection of cryptococcosis	Performance derived from small studies (IA)	Useful technique in combination with other tests for IA (GM, PCR)
DNA detection	Any	Sensitive, results within several hours	Labour intensive, expensive, only little standardization, may have low	Could be useful as a screening technique when doing serial determinations in haematological

Threshold for patients at high contamination risk

1.4 Antifungal Susceptibility Testing (AST)

Antifungal drug resistance can occur with all drug classes and involves strains with acquired resistance and inherently less susceptible species. In vitro susceptibility testing 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. Thus, it is a useful tool to provide information to clinicians to help to guide therapy [109, 111].

AST may be used for the assessment of the in vitro activity. As elevated antifungal minimum inhibitory concentration (MIC) values represent decreased vitro activity and are associated with poor outcomes and breakthrough infections, this may be used for therapeutic management. Secondly, AST is also used as a means to survey the development of resistance and to predict the therapeutic potential and spectrum of activity of investigational agents. In any case, AST should be clinically useful; thus, it must reliably predict the likelihood of clinical success. There are several factors that also influence outcomes in patients with fungal infections other than antifungal susceptibility. These include (1) the host's immune response, (2) the severity of the underlying disease and other comorbidities, (3) drug interactions and (4) the pharmacokinetics of the agents and concentrations achieved at the site of infection [109].

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 [112, 113] (http://www.eucast.org/ast_of_fungi/). Both of these methods use BMD, although there are some differences in inoculum size and MIC endpoint determination results obtained when testing azoles and echinocandins against *Candida* and azoles against *Aspergillus* species are in close agreement [114]. CLSI also established disk diffusion assays for *Candida* (fluconazole, voriconazole, echinocandins) and *Aspergillus* [115]. Also, interpretative breakpoints have been provided for azoles, caspofungin and micafungin.

Over the past several years, there have been efforts to harmonize the methods and clinical breakpoints (CBP) for antifungal susceptibility testing between these two groups. Some differences do exist, but the results are comparable [116, 117]. One issue with both the CLSI and EUCAST broth microdilution susceptibility testing that has been identified is the problem of interlaboratory variability for caspofungin MICs, with some laboratories reporting low values, whereas others report high values for this echinocandin [118]. This variability seems to be greatest for *C. glabrata* and *C. krusei* and may lead to falsely classifying susceptible isolates as resistant to the echinocandins. Because of this, EUCAST does not recommend susceptibility testing with caspofungin but instead recommends the use of micafungin or anidulafungin MICs as surrogate markers for caspofungin susceptibility or resistance [117]. Studies have clearly demonstrated high concordance rates for anidulafungin and micafungin MICs in detecting mutations within the *FKS* gene that confer echinocandin resistance in multiple *Candida* species [119, 120].

There are also differences in the CBP that define resistance as set by CLSI and EUCAST. Despite the differences in methods, the categorical agreement that is obtained is comparable although some differences have been reported. CBP have not been set for each antifungal agent against each type of fungus. The CLSI has only established breakpoints for fluconazole, voriconazole and the echinocandins against certain *Candida* species, and no breakpoints have been set against moulds or endemic fungi. In contrast, EUCAST has established breakpoints for certain antifungals against yeast and some moulds, including *Aspergillus* species [109].

As these methods are time-consuming and commercially available, test kits for MIC determination are a good alternative. These include gradient diffusions assays, colorimetric assays and automated tests. The antifungal MIC agar-based assay Etest® (bioMérieux) directly quantifies antifungal susceptibility in terms of discrete

MIC values. This method is commonly used for susceptibility testing against various *Candida* species and is also considered a sensitive and reliable method for detecting decreased susceptibility to amphotericin B among *Candida* isolates and *Cryptococcus neoformans* [109]. Several studies have reported very good essential agreement (>90%) between the Etest assay and the CLSI and EUCAST broth microdilution reference methods [121, 122]. A clear benefit of utilizing Etest is 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 as it produces easy to read, sharp zones of inhibition. However, for echinocandins, the paradoxical effect has been 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 [123, 124].

Others have reported less than optimal categorical agreement between the Etest assay and the CLSI broth microdilution method for caspofungin against *C. glabrata* and *C. krusei* based on the revised CLSI echinocandin clinical breakpoints [125–127]. In addition, a recent study reported poor overall agreement between Etest and EUCAST MICs for amphotericin B and posaconazole (75.1%) when used to measure activity against members of the order *Mucorales* and recommended that the Etest assay should not be used when testing these fungi [128].

The YeastOne Sensititre test (Thermo Scientific, Waltham, MA, USA formerly TREK Diagnostic Systems) is a broth microdilution assay format that uses the blue colorimetric dye resazurin (alamarBlue) that is converted to by metabolically active cells to resorufin. Several studies of the YeastOne assay, including multicentre evaluations, have demonstrated excellent reproducibility and very good agreement with the broth microdilution reference methods.

Overall categorical agreement, however, was somewhat lower for caspofungin than micafungin (93.6 vs 99.6%) between the YeastOne assay and the CLSI broth microdilution method, and this was due to the low categorical agreement for caspofungin against *C. glabrata* and *C. krusei* (69.1%) between the two methods [109].

The yeast susceptibility test, Vitek 2 (bioMérieux, France), is a fully automated assay for performing antifungal susceptibility testing. Several studies have reported reproducible and accurate results compared with the CLSI broth microdilution method. One of the limitations of this system for caspofungin is that a correct discrimination between susceptible and intermediate categories for *C. glabrata* isolates is impossible as the lower end of the concentration range is 0.25 μg/mL [109, 122]. In addition, it was reported that 19.4% of caspofungin-resistant *Candida* isolates with known mechanisms of resistance (mutations in *FKS* hotspot regions) were misclassified as susceptible to caspofungin [129].

As azole-resistant *Aspergillus fumigatus* is emerging worldwide, easy test formats are urgently needed. Therefore, a screening method based on an agar-based test has been developed and commercialized (VIP CheckTM, Beneden-Leeuwen, the Netherlands). Multiple colonies are sub-cultured on a four-well plate with a growth control and itraconazole, voriconazole and posaconazole added to the agar. This approach detects with high sensitivity and specificity potential resistance in the isolates in a simplified way, i.e. isolates growing only on the growth-control well excludes resistance [130]. The overall performance of the four-well screening plates was evaluated with respect to the sensitivity and specificity to differentiate between different mutant and WT isolates. The overall sensitivity and specificity for the four-well plate (no growth versus growth) was 99% (range 97%–100%) and 99% (95%–100%), respectively [131]. Sensititre YeastOne can also be used for *Aspergillus*, and some studies have shown that this assay might be useful in detecting resistance to itraconazole and voriconazole [132].

In recent years, progress has been made towards the description of resistance mechanisms at molecular level. There are methods of detection that can be useful for clinical laboratories, but lack of standardization precludes their integration in the routine daily practice. The molecular detection of *Candida* resistance to azoles and to echinocandins and of *Aspergillus* resistance to triazoles can be clinically relevant and could help to design more efficient prevention and control strategies. However, multicentre studies including third-party validation and reproducibility assessment are needed for further acceptance and standardization. New automated and massive sequencing technique could change AST procedures in the upcoming years [45].

Susceptibility testing is indicated to provide the 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 1.4. 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; *Mucorales* 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 1.3 shows the susceptibility pattern of the most common *Candida* spp. In cases where the susceptibility pattern cannot be reliably predicted based on the species identification alone, antifungal susceptibility testing should be performed [111, 133].

Attention has to be paid that for emerging fungal pathogens, such as *Mucorales*, dematiaceous moulds and *Fusarium*, no standardized breakpoints are available as of yet. Species belonging to the order *Mucorales* are more resistant to antifungal agents than *Aspergillus* spp. All species of *Mucorales* are unaffected by voriconazole, and most show moderate resistance in vitro to echinocandins; use of voriconazole as first-line treatment for aspergillosis and use of echinocandins as empirical treatment for febrile neutropenia and disseminated candidiasis have been blamed for the increased incidence of mucormycosis. Amphotericin B and posaconazole show the most potent activity in vitro against the *Mucorales* [13]. Table 1.4 shows the susceptibility pattern of common opportunistic moulds.

In recent years, progress has been made towards the description of resistance mechanisms at molecular level. There are methods of detection that can be useful for clinical laboratories, but lack of standardization precludes their integration in the routine daily practice. The molecular detection of *Candida* resistance to azoles and echinocandins and of *Aspergillus* resistance to triazoles can be clinically relevant and could help to design

Table 1.3 General susceptibility patterns of certain yeasts and moulds

Fungus	AmB	FLU	ITRA	VOR	POS	EC
C. albicans	S	S	S	S	S	S
C. tropicalis	S	S	S	S	S	S
C. parapsilosis	S	S	S	S	S	I
C. glabrata	S	I	I	I	I	S
C. krusei	S	R	S-I-R	S-I-R	S-I-R	S
C. lusitaniae	S to R	S	S	S	S	S
C. guilliermondii	S	R	R	R	R	R
C. auris	X	R				X

AmB amphotericin B, *FLU* fluconazole, *ITRA* itraconazole, *VOR* voriconazole, *POS* posaconazole, *EC* echinocandins, *S* susceptible, *SDD* susceptible dose dependent, *I* intermediate, *R* resistant
"X" denotes that the MICs for the antifungal compound are elevated compared to those for *C. albicans*

more efficient prevention and control strategies [133]. The commercially developed AsperGenius species assay (PathoNostics, Maastricht, the Netherlands) is a multiplex real-time PCR capable of detecting aspergillosis and genetic markers associated with azole resistance [134]. The assay is validated for testing bronchoalveolar lavage (BAL) fluids, replacing the requirement for culture to differentiate susceptible from resistant *A. fumigatus* strains [135, 136]. A novel and highly accurate diagnostic platform has been developed for rapid identification of FKS mutations associated with echinocandin resistance in *C. glabrata* which needs evaluation, and further development to cover the entire FKS mutation spectrum would enhance its appeal as a diagnostic platform [137]. Recommendations for the antifungal susceptibility testing are presented in Table 1.5.

Table 1.4 General susceptibility patterns of selected moulds

Fungus	AmB	ITRA	VOR	POS	ISA	EC
A. fumigatus	S	S	S	S	S	S
A. flavus	S/R	S	S/R	S	S	S
A. terreus	R	S	S	S	S	S
A. lentulus	R	R	R	S/R	S/R	R
Rhizopus spp.	S	S/R	R	S/R	S/R	R
Mucor spp.	S/R	R	R	S/R	S/R	R
Fusarium spp.	S/R	R	S/R	S/R	S/R	R
Scedosporium spp.	S/R	R	S/R	S/R	R	R

AmB amphotericin B, *ITRA* itraconazole, *VOR* voriconazole, *POS* posaconazole, *ISA* isavuconazole, *EC* echinocandins, *S* susceptible, *R* resistant

Table 1.5 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 Consider cross-resistance between fluconazole and all other azoles to be complete for *C. glabrata* In invasive fungal infections In invasive and mucosal infections failing therapy For yeasts and moulds from sterile sites For isolates considered clinically relevant particularly in patients exposed to antifungals
How to test?
Identification to species level For *Candida* spp. perform routine susceptibility testing for fluconazole and according to the local epidemiology include other azoles Selection of susceptibility testing methods: standardized methods • CLSI methods • EUCAST EDef 7.1 – Broth based, M27-A3 – Agar based, M44-A2 Commercial methods • Etest • Sensititre YeastOne • Vitek 2 • Molecular assays Aspergillus—azoles (available) Candida—echinocandins, azoles (in progress) 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

However, multicentre studies including third-party validation and reproducibility assessment are needed for further acceptance and standardization. New automated and massive sequencing technique could change AST procedures in the upcoming years [133].

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