

Contemporary Tools for the Diagnosis and Management of Invasive Mycoses

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Invasive fungal infections have become a major cause of morbidity and mortality over the past 3 decades. Organ transplantation, the use of aggressive chemotherapy, and the availability and widespread use of immunosuppressive treatments for many medical ailments have resulted in large populations of patients who are at risk for fungal disease. Early diagnosis and prompt therapy are instrumental to successful treatment of these infections, but conventional methods for diagnosis of fungal disease are slow and lack sensitivity. Important advances in diagnosing invasive mycoses, particularly in laboratory-based testing, have been realized over the years. Antigen-based assays, new laboratory methods for identification of fungi, and reference guidelines for susceptibility testing have been developed and validated for use in clinical laboratories. We review these technological advances and our understanding of their clinical application and impact.

Historically, the diagnosis of invasive fungal infection (IFI) has been limited to the correlation of clinical signs and symptoms of disease with recovery of the organism from or histopathologic detection of the organism in clinical specimens. As the incidence of fungal disease has increased over the past 30 years, emphasis has been placed on achieving earlier and less invasive means of diagnosis, in hopes of improving the mortality rate associated with these infections. Many advances in fungal diagnostics have thus been realized. This article provides a review of both the conventional diagnostic approaches (i.e., direct microscopy, culture, and histopathologic methods) and the newer non-culture-based methods (i.e., serologic techniques and molecular diagnostics) (table 1).

SPECTRUM OF OPPORTUNISTIC FUNGAL PATHOGENS

The spectrum of possible opportunistic fungal pathogens has expanded beyond well-known fungi such as

Candida albicans, *Cryptococcus neoformans*, and *Aspergillus fumigatus* [1, 2]. New and emerging pathogens include species of *Candida* and *Aspergillus* other than *C. albicans* and *A. fumigatus*; yeasts, including *Trichosporon*, *Rhodotorula*, and *Blastoschizomyces* species; the Zygomycetes; hyaline Hyphomycetes, including *Fusarium*, *Scedosporium*, and *Paecilomyces* species; and a wide variety of dematiaceous fungi (table 2) [1, 2]. It is now clear that there are no truly nonpathogenic fungi and that virtually any fungus can cause a lethal mycosis in a sufficiently immunocompromised host. Although *Candida* species, *Aspergillus* species, and *C. neoformans* account for >80% of all fungal infections in hematopoietic stem cell transplant (HSCT) recipients, solid-organ transplant recipients, and other immunosuppressed patient populations [3], the less common and “emerging” opportunistic fungi pose a significant diagnostic and therapeutic challenge. In most instances, the only means available to diagnose these less common but important mycoses is by isolation in culture and subsequent identification by an experienced mycologist.

CONVENTIONAL METHODS FOR THE LABORATORY DIAGNOSIS OF FUNGAL INFECTIONS

Patients who are at the highest risk for developing opportunistic fungal disease typically have a decreased in-

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Clinical Infectious Diseases 2006;43:S15–27

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1058-4838/2006/4303S1-0003\$15.00

Table 1. Laboratory methods used for the diagnosis of invasive fungal infection.

Conventional microbiologic methods
Direct microscopy (Gram, Giemsa, and KOH/calcofluor stains)
Culture
Identification
Susceptibility testing
Histopathologic methods
Conventional microscopy
Direct immunofluorescence
In situ hybridization
Immunologic and biochemical methods
Histoplasma antigen test
Cryptococcal antigen test
Galactomannan test
(1→3)β-D-glucan test
Molecular methods
Direct detection
Identification

flammatory response, and the infection is often in advanced stages by the time signs and symptoms develop. The challenge in diagnosing fungal infection has, therefore, been 2-fold: first, to identify patients who are at the highest risk for fungal infection so that they can be monitored more closely and, second, to develop laboratory methods that will provide evidence of infection earlier and more reliably than has been possible historically. The newer serologic and molecular diagnostic tests promise to enhance the diagnostic approach to fungal infections; currently, however, conventional microbiologic and histologic methods serve as the cornerstone for the definitive diagnosis of mycoses. Successful diagnosis and treatment of mycotic infections are highly dependent on a team approach that includes clinicians, medical mycologists, and pathologists. Careful stratification of patients according to their risks of acquiring fungal infection will improve the usefulness of both new and established diagnostic approaches.

Direct microscopy. Direct microscopic examination of clinical specimens is a crucial first-line procedure in detecting the presence of fungal elements and is perhaps the most rapid, useful, and cost-effective means of diagnosing fungal infections [4]. Detection of fungal elements microscopically may provide a presumptive diagnosis in <1 h and often serves to guide the laboratory in selecting the most appropriate means by which to culture the clinical material, as well as in interpreting the culture results. Direct microscopy can often provide an etiologic diagnosis of infection caused by *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis* (*posadasii*), *Pneumocystis jirovecii* (*carinii*), or *Penicillium marneffei* (table 3). For other infections, microscopy can usually yield preliminary information that a yeast or mould is present, and, in some instances, the morphologic appearance may provide a presump-

tive diagnosis of the type of infection (e.g., zygomycosis and candidiasis) but not the actual species of the etiologic agent (table 3). The morphologic characteristics of fungi seen on direct microscopic examination include budding yeasts, hyphae, and pseudohyphae. The combination of budding yeast cells and pseudohyphae is characteristic of *Candida* species; however, these structures may also be seen with *Trichosporon* and *Geotrichum* species (table 3).

Direct microscopy, as performed in the microbiology laboratory, most commonly relies on the use of 10%–20% potassium hydroxide containing the fluorophore calcofluor white or the staining of smears or touch preparations with either Gram or Giemsa stain. Calcofluor white binds to the chitin in the fungal cell wall and fluoresces blue-white or green, thus providing a rapid and sensitive means of detecting fungi in clinical material [4, 5]. However, all methods of direct examination are less sensitive than culture, and negative results of direct examination of a clinical specimen never rule out a fungal infection [4].

Histopathologic methods. Visualization of fungal elements in tissue is the cornerstone for the diagnosis of invasive mycoses; however, isolation and identification of the infecting organism are required for a precise diagnosis. For example, the microscopic appearance of the hyphae of hyaline Hyphomycetes (e.g., *Aspergillus*, *Fusarium*, *Acremonium*, *Paecilomyces*, and *Scedosporium* species) in tissue are very similar (table 3), but knowing which specific pathogen is present is important because fungi vary in their antifungal susceptibility profiles.

To detect small numbers of organisms and to clearly define the morphologic features of the infecting organism, special stains, such as Gomori methenamine silver stain and periodic acid–Schiff stain, must be used. The Fontana-Masson stain is used to stain for melanin in the cell wall and may highlight lightly pigmented dematiaceous fungi and aid in differentiating capsule-negative strains of *C. neoformans* (melanin positive) from other yeasts (melanin negative) in tissue. The mucicarmine stain is also used to identify *C. neoformans* in tissue, by staining the polysaccharide capsule of the fungus.

Immunohistologic staining for the identification of fungi in clinical specimens has been attempted, and monoclonal and polyclonal fluorescent-antibody reagents have been developed for differentiating the genera of *Aspergillus*, *Fusarium*, and *Scedosporium* in situ. Unfortunately, the high degree of antigenic relatedness among these and other fungal pathogens, such as *Paecilomyces* species, has resulted in significant cross-reactivity and low specificity of these stains [6]. At this time, there are no commercially available fluorescent-antibody reagents for in situ identification of fungi.

Culture. Fungi have longer generation times than those of most bacteria, which results in slower recovery of the organisms from specimens. Advances in fungal culture capabilities have

Table 2. Spectrum of opportunistic fungal pathogens.

Organic group, examples of specific pathogens
<i>Candida</i> species
<i>C. albicans</i>
<i>C. glabrata</i>
<i>C. guilliermondii</i>
<i>C. kefyr</i>
<i>C. krusei</i>
<i>C. lusitanae</i>
<i>C. parapsilosis</i>
<i>C. rugosa</i>
<i>C. tropicalis</i>
Other yeasts
<i>Blastoschizomyces</i> species
<i>Cryptococcus neoformans</i>
<i>Malassezia</i> species
<i>Rhodotorula</i> species
<i>Saccharomyces</i> species
<i>Trichosporon</i> species
<i>Aspergillus</i> species
<i>A. flavus</i>
<i>A. fumigatus</i>
<i>A. niger</i>
<i>A. terreus</i>
<i>A. ustus</i>
<i>A. versicolor</i>
Zygomycetes
<i>Absidia</i> species
<i>Cunninghamella</i> species
<i>Mucor</i> species
<i>Rhizomucor</i> species
<i>Rhizopus</i> species
<i>Saksenaea</i> species
Other hyaline moulds
<i>Acremonium</i> species
<i>Emmonsia</i> species
<i>Fusarium</i> species
<i>Paecilomyces</i> species
<i>Phialemonium</i> species
<i>Scedosporium</i> species
<i>Scopulariopsis</i> species
<i>Trichoderma</i> species
Dematiaceous moulds
<i>Alternaria</i> species
<i>Bipolaris</i> species
<i>Curvularia</i> species
<i>Cladophialophora</i> species
<i>Dactylaria</i> species
<i>Exophiala</i> species
<i>Phialophora</i> species
<i>Ramichloridium</i> species
<i>Wangiella</i> species

(continued)

Table 2. (Continued.)

Organic group, examples of specific pathogens
Dimorphic fungi
<i>Blastomyces dermatitidis</i>
<i>Coccidioides immitis</i>
<i>Histoplasma capsulatum</i>
<i>Paracoccidioides brasiliensis</i>
<i>Penicillium marneffeii</i>
<i>Sporothrix schenckii</i>
Other, <i>Pneumocystis jirovecii</i> (carinii)

NOTE. This list is not all inclusive.

centered on recovery of fungi from blood. Blood cultures may test negative even when disseminated disease is present; however, detection of fungemia is useful in diagnosing opportunistic infections caused by *Candida* species, *C. neoformans*, *Trichosporon* species, *Malassezia* species, *Fusarium* species, and, occasionally, *Acremonium* species, *Paecilomyces* species, *Scedosporium* species, and *Aspergillus terreus*. The lysis centrifugation method (Isolator; Wampole) and the continuous-monitoring automated blood culture systems are all sensitive methods for the detection of *Candida* species [7]. The development of specialized broth media containing lytic agents, resins, charcoal, or diatomaceous earth, coupled with continuous agitation, has contributed to the improved performance of broth-based systems. However, recovery of *C. neoformans*, *H. capsulatum*, *Malassezia furfur*, and *Fusarium* species may be inferior with broth-based systems compared with the lysis centrifugation method [7]. On the other hand, culture contamination occurs more frequently with the more labor-intensive lysis centrifugation method.

Among the 4 automated, continuous-monitoring blood culture systems that have been developed, the Bactec (Becton-Dickinson) and BacT/Alert (bioMérieux) systems are superior in their capacities for the recovery of yeast from blood. Studies have documented that these systems match the performance of the lysis centrifugation method for the detection of *Candida* species and *C. neoformans* in blood [8, 9]. In addition, the MycoF-lytic culture bottle (Becton-Dickinson) also matches the Isolator system for the recovery of dimorphic fungi [10].

IDENTIFICATION OF FUNGI

Identification of fungi recovered from clinical specimens can be challenging because of their slow growth characteristics and because of the importance placed on morphologic features. Most yeasts are identified on the basis of carbohydrate assimilation and/or fermentation and their macroscopic and microscopic morphologic features after growth on specialized media. Moulds are identified primarily on the basis of their macroscopic and microscopic morphologic features. Newer chro-

Table 3. Characteristic microscopic features of opportunistic and pathogenic fungi in clinical specimens.

Fungi	Microscopic morphologic features in clinical specimens
<i>Candida</i> species	Oval budding yeasts 2–6 μm in diameter. Pseudohyphae and true hyphae may be present.
<i>Cryptococcus neoformans</i>	Spherical budding yeasts of variable size, 2–15 μm in diameter. Capsule may be present or absent. No hyphae or pseudohyphae.
<i>Trichosporon</i> species	Hyaline arthroconidia, blastoconidia, and pseudohyphae, 2–4 by 8 μm in size.
<i>Malassezia</i> species	Small oval budding yeasts. “Bowling pin” appearance with collarette. Both hyphal and yeast forms may be seen in skin scrapings.
<i>Aspergillus</i> species	Hyaline, septate, dichotomously branched hyphae of uniform width (3–6 μm). Conidial heads may be seen in cavitory lesions.
Other hyaline Hyphomycetes ^a	Hyaline, septate, dichotomously branching hyphae. Angioinvasion is common. Adventitious conidiation may be present. May be indistinguishable from <i>Aspergillus</i> species.
Zygomycetes	Broad, thin-walled, pauci-septate hyphae, 6–25 μm wide, with nonparallel sides and random branches.
Dematiaceous fungi	Pigmented (brown, tan, or black) hyphae, 2–6 μm wide. May be branched or unbranched. Often constricted at the point of septation.
<i>Histoplasma capsulatum</i>	Small (2–4 μm in diameter), intracellular, budding yeasts.
<i>Coccidioides immitis/posadasii</i>	Spherical, thick-walled spherules, 20–30 μm in diameter. Mature spherules contain small (2–5 μm in diameter) endospores. Released endospores may be mistaken for yeast. Arthroconidia and hyphae may form in cavitory lesions.
<i>Blastomyces dermatitidis</i>	Large (8–15 μm in diameter), thick-walled budding yeast cells. The junction between mother and daughter cells is typically broad based. Cells may appear multinucleate.
<i>Sporothrix schenckii</i>	Elongated or “cigar-shaped” yeast cells of varying size (rare). Tissue reaction forms asteroid bodies.
<i>Penicillium marneffei</i>	Oval, intracellular yeast cells bisected with a septum (fission yeast).
<i>Pneumocystis jiroveci</i> (carinii)	Cysts are round, collapsed, or crescent shaped. Trophozoites seen on staining with Giemsa or immunofluorescent stains.

^a Includes *Fusarium*, *Scedosporium*, *Acremonium*, and *Paecilomyces* species.

mogenic and molecular methods that couple the presumptive identification of yeasts and moulds with their detection in clinical specimens have been developed to reduce the time to identification.

Chromogenic methods. Media have been formulated to provide the presumptive identification of yeast based on colonial morphologic features. The addition of certain substrates or chromogens to the agar medium allows the direct detection of specific enzymatic activities characteristic of selected species of yeast. CHROMagar *Candida* (CHROMagar) is one such medium that can be used for simultaneous isolation and presumptive identification of *C. albicans*, *Candida krusei*, and *Candida tropicalis*. Use of this medium shortens the time to presumptive identification of the organisms and allows for easier detection of multiple yeast species present in a specimen [11]. CHROMagar may be coupled with the rapid trehalose test [12] for the identification of *Candida glabrata* and has been shown to be useful in the rapid identification and determination of fluconazole susceptibility of *Candida* species directly from positive blood cultures [13]. Other chromogenic media and a rapid colorimetric test based on the detection of L-proline aminopeptidase and β -galactose-aminidase have been developed specifically for the rapid identification of *C. albicans* [14].

***C. albicans* peptide nucleic acid (PNA) fluorescence in situ hybridization (FISH) test.** A new FISH method that uses PNA probes for the identification of *C. albicans* directly from blood

culture bottles that tested positive and in which yeasts were observed by Gram staining has been developed and was approved for use by the US Food and Drug Administration (FDA) in 2004. The *C. albicans* PNA FISH test (AdvanDx) is based on a fluorescein-labeled PNA probe that targets *C. albicans* 26S rRNA. The probe is added to smears made directly from the contents of the blood culture bottle and is hybridized for 90 min. Smears are subsequently examined by fluorescence microscopy. Recent single-center and multicenter studies have documented the excellent sensitivity (99%–100%) and specificity (100%) of this test in the direct identification of *C. albicans* from blood cultures [15, 16]. The FISH results are unaffected by the type of blood culture system or broth formulation (e.g., lytic medium and resin- or charcoal-containing medium) [16]. This approach may provide a time savings of 24–48 h, compared with conventional laboratory methods used for identification. It allows physicians to be notified of the yeast’s identity along with positive blood culture results. Rapid, accurate identification of *C. albicans* should promote optimal antifungal therapy with the most cost-effective agents, resulting in improved outcomes and significant antifungal cost savings for hospitals [17].

Molecular methods for identification. The development of nucleic acid probes has advanced the identification of dimorphic fungi once they are recovered in culture. These tests are available for use in clinical laboratories (AccuProbe; Gen-

Probe) and can be performed in ~2 h. A chemiluminescent-labeled DNA probe specific for target fungal rRNA is incubated with a lysate of the organism. The probe has sensitivity similar to that of the historical and more labor-intensive exoantigen test but demonstrates slightly less specificity, depending on the fungus tested. The probe demonstrates 100% specificity for *H. capsulatum* and *C. immitis*; however, specificity for *B. dermatitidis* is 99.7%, because there is cross-reactivity between that organism and *Paracoccidioides brasiliensis* [18–20].

Amplification-based molecular approaches are being developed to provide more rapid and objective identification of both yeasts and moulds, compared with traditional phenotypic methods [21–24]. Ribosomal targets and internal transcribed spacer regions have shown particular promise for the molecular identification of some fungi. Several recent studies have confirmed the tremendous potential of these approaches as powerful tools in the identification of clinically important yeasts and moulds [21–25]; however, the existing sequence databases are limited with regard to both the quality and the accuracy of their entries [22, 23]. It is anticipated that, with the availability of improved sequencing techniques, broader and more reliable databases, and more readily available kits and software, this technology will be a competitive alternative to the classic mycological identification techniques used for clinically important fungi.

Predictive value of culture detection and identification.

Because fungi are ubiquitous in the environment, their isolation from clinical specimens often represents transient colonization rather than invasive disease. Likewise, contamination of specimens or cultures by environmental organisms, many of which can also serve as etiologic agents of opportunistic mycoses, may confound the interpretation of culture results. Although most isolates of *Candida* species, *C. neoformans*, *H. capsulatum*, and *Fusarium* species obtained from blood cultures are clinically significant, others, such as *Aspergillus* species (not *A. terreus*) and *Penicillium* species (not *P. marneffeii*), are not [7, 26]. Isolation of *Aspergillus* species from cultures of respiratory tract specimens is especially problematic, because this organism is common in the environment, and it can colonize the respiratory tract of any individual without actually causing disease. Direct visualization of the organism in tissue helps to confirm the significance of isolation of *Aspergillus* species from cultures of respiratory tract specimens; however, this is frequently not possible because of the invasive procedure required.

Several investigators have shown that the interpretation of respiratory tract cultures yielding *Aspergillus* species is aided by considering the risk group of the patient [27–29] (table 4). From these studies, it is clear that, for high-risk patients (e.g., allogeneic HSCT recipients, patients with hematologic malignancies, and patients with neutropenia), a positive culture that yields *Aspergillus* species is often associated with invasive dis-

Table 4. Risk of invasive aspergillosis for patients with cultures positive for *Aspergillus* species, by study and risk category.

Risk category	Study, percentage risk (no. of patients with positive culture/total no. of patients)		
	Yu et al. [27]	Horvath et al. [28]	Perfect et al. [29]
High ^a	100 (17/17)	72 (34/47)	57 (117/206)
Intermediate ^b	37 (20/54)	58 (14/24)	15 (228/1510)
Low ^c	0 (0/9)	14 (1/7)	<1 (1/155)

^a Includes allogeneic HSCT recipients, patients with neutropenia, and patients with hematologic cancer.

^b Includes autologous HSCT and solid-organ transplant recipients, patients receiving therapy with corticosteroids, HIV-infected patients, and patients with malnutrition, diabetes, underlying pulmonary disease, or solid-organ cancer.

^c Includes HIV-infected patients, patients with cystic fibrosis or connective tissue disease, and other nonimmunosuppressed patients.

ease. The positive predictive value of a positive culture is lessened for autologous HSCT recipients, solid-organ transplant recipients, and HIV-infected patients [27–29]. The specific identification of the fungus isolated from culture specimens can also help in determining clinical significance; *Aspergillus niger* is rarely a pathogen, whereas *A. terreus* and *Aspergillus flavus* have been shown to be statistically associated with invasive aspergillosis (IA) when isolated from cultures of respiratory tract specimens [29].

IMMUNOLOGIC, BIOCHEMICAL, AND MOLECULAR MARKERS FOR DIRECT DETECTION OF IFI

Rapid, sensitive, specific, noninvasive diagnostic tests for serious fungal infections would allow for more timely and focused application of specific therapeutic measures. As such, tests for detection of antibodies, cell wall components, and fungus-specific nucleic acids have great appeal [30]. Most conventional serologic tests designed to detect specific serum antibodies are ineffective, because many patients who are at risk for fungal disease are not capable of mounting a specific antibody response to infection. Furthermore, determination of the presence of an acute infection typically requires a comparison of the type and quantity of antibody present in acute-phase and convalescent-phase serum samples, an exercise that is not helpful during the acute presentation, when therapeutic interventions are being decided.

Detection of fungal cell wall and cytoplasmic antigens in serum or other body fluids by immunologic or biochemical methods represents the most direct means of providing a serologic diagnosis of IFI. The best examples of this approach are the commercially available tests for the detection of the polysaccharide antigens of *C. neoformans* and *H. capsulatum*. These tests have proven value in the rapid diagnosis of cryptococcal meningitis [31] and disseminated histoplasmosis [32] and will not be discussed further. Of greater importance for

the rapid diagnosis of opportunistic fungal infections are the recently developed galactomannan (GM) immunoassay for the diagnosis of IA [33] and the (1→3)- β -D-glucan (BG) assay for the diagnosis of invasive disease caused by *Aspergillus* and *Candida* species and other opportunistic fungi [34–36].

GM. GM is a cell-wall polysaccharide specific to *Aspergillus* species that is detectable in serum and other body fluids during IA. GM levels, reported as optical density (OD) values, can be measured in body fluids by means of a double-sandwich EIA. In July 2003, the Platelia *Aspergillus* EIA test (Bio-Rad Laboratories) was approved by the US FDA for use in the diagnosis of IA in HSCT recipients and in patients with leukemia. Data presented to the FDA cited a sensitivity of 80.7% and specificity of 89.2% for the diagnosis of IA, based on a multicenter study conducted on serially collected serum samples from 179 HSCT recipients and patients with leukemia, 31% of whom developed proven or probable IA. Parameters used in the study to determine a positive assay result included an OD index value of ≥ 0.5 and two positive assay results for an aliquot of the same serum sample [37].

Despite numerous clinical studies on the use of the GM assay to diagnose and monitor the condition of patients who are at high risk of developing IA, there are many uncertainties with respect to the release of GM from the fungus and site of infection and the variable performance of the test in clinical practice [33]. The variability in assay performance is considered to be multifactorial and includes issues affecting the release of the antigen from the hypha (e.g., fungal strain and stage of growth), leakage of the antigen from the site of infection into the blood, binding of the antigen by blood substances, host factors (e.g., location and extent of fungal disease, antifungal treatment, and age), and methodological factors, such as OD cutoff, definition of a positive “test” (single vs. sequential positive results), and definition of an infected patient [33].

In contrast to the data submitted to the FDA, several studies have used OD values of 1.0 as a positive cutoff and have required that 2 consecutive samples must test positive to declare a positive assay [38]. More recent work, however, has shown that the use of mould-active antifungals can decrease the sensitivity of the test. Although the kinetics of GM in patients receiving mould-active antifungals is not completely understood, it is generally accepted that these agents decrease circulating GM levels and that the use of the lower OD value improves sensitivity [39]. Furthermore, lower thresholds for positivity may increase the lead time to diagnosis without compromising test performance [40]. Irrespective of the OD value used, it is apparent that, in approximately two-thirds of patients, circulating antigen can be detected at a mean of 8 days before diagnosis by other means [33].

Importantly, false-positive GM assay results have been reported for patients receiving piperacillin-tazobactam [41–43]

and amoxicillin-clavulanate [44, 45]. GM is a by-product of the β -lactam antibiotic fermentation process, which likely accounts for the cross-reactivity when these agents are used. It should be understood that GM is a complex sugar also found in other organisms as well as in many food products. These other sources of GM may serve as a source of false-positive assay results [33, 44, 46].

There are several additional unresolved issues regarding the use of GM antigenemia in the diagnosis of IA. Performance characteristics of the GM assay in the solid-organ transplant and pediatric populations need to be established. To date, evaluation in the solid-organ transplant population is limited to 2 prospective studies. One, a study of liver transplant recipients, was limited because of the low incidence of IA [47]. The other, a study of lung transplant recipients, documented high specificity (93%) but low sensitivity (30%), and cases of tracheobronchial aspergillosis went undetected [47]. Evaluations in the pediatric HSCT population have documented a high rate of false-positive results [48].

Measurement of GM in other body fluids, such as bronchoalveolar lavage (BAL) fluid, urine, and cerebrospinal fluid, has also been proposed. Sensitivities of the assay in BAL fluid ranged from 61% to 76%, depending on the OD cutoff value used [49]. Positive and negative predictive values were 100% when BAL testing was combined with high-resolution CT scanning [50]. Appropriate OD cutoff values require validation in larger studies before the testing of specimens other than serum can be recommended. Finally, although animal models support the use of the GM assay for monitoring response to therapy, clinical studies are necessary to determine the kinetics of GM in humans—particularly as they relate to site of infection and use of various antifungal agents—to define the prognostic utility of the assay [40].

BG. BG is a cell-wall constituent of many pathogenic fungi, including *Aspergillus* and *Candida* species, and is detectable in patients’ serum during invasive disease due to these organisms. In addition to patients with IA and candidiasis, BG is also detectable in patients with infections caused by species of *Fusarium*, *Trichosporon*, *Saccharomyces*, and *Acremonium*, which are less common but very important fungal pathogens, especially in immunocompromised hosts [51]. The test does not detect infection with *Cryptococcus* species or *Zygomycetes* because of the low quantities of BG in the cell walls of those fungi [52, 53].

The BG test (Fungitell; Associates of Cape Cod) was approved by the US FDA for the qualitative detection of BG in the serum of patients with symptoms of or medical conditions predisposing to IFI and as an aid in the diagnosis of deep-seated mycoses and fungemia. Detection of BG in serum uses a chromogenic variant of the limulus amoebocyte lysate assay [34]. The assay has been evaluated in a multicenter study of

patients with IFIs and healthy control subjects [35] and for the early diagnosis of IFIs in patients with hematologic malignancies [34, 36]. The latter study included serial monitoring with the assay during at-risk periods. Testing performed on serial serum samples obtained from 283 subjects with acute myeloid leukemia or myelodysplastic syndrome who were receiving antifungal prophylaxis revealed that at least one serum sample was positive for BG a median of 10 days before the clinical diagnosis in 100% of subjects with a proven or probable IFI. This included cases of candidiasis, fusariosis, trichosporonosis, and aspergillosis. The absence of a positive test result had a 100% negative predictive value, and the specificity of the test was 90% for a single positive test result. Specificity increased to 96% when 2 sequential positive results were required for a "true-positive" test result [34].

Although a positive test result for the presence of BG does not identify the infecting fungus, the practical application of this test includes its use as a screening assay (presumptive marker) for invasive fungal infection to allow the earlier initiation of antifungal therapy. Other tests are necessary for the confirmation and identification of the fungal pathogen. In a study designed to assess the benefit of monitoring patients for the presence of both GM and BG, the combination of the 2 tests improved specificity (to 100%) and positive predictive value (to 100%) for the diagnosis of IA, without affecting sensitivity and negative predictive values. Interestingly, the BG assay tended to become positive earlier than the GM assay.

BG is ubiquitous in the environment (e.g., in some types of gauze and laundry starch), and false-positive results may be caused by poor specimen handling, hemodialysis using certain cellulose membranes, exposure to certain types of gauze, and recent receipt of albumin or immunoglobulin products [54–56]. To date, the Fungitell assay has not been evaluated in pediatric or solid-organ transplant populations.

Molecular detection of fungi in clinical specimens. The number of infectious diseases for which diagnosis is achieved by molecular technology is increasing rapidly. To date, however, the majority of molecular assays validated for use in clinical microbiology laboratories are those for the diagnosis of viral diseases. Given the limitations of current fungal diagnostics, the use of PCR as an adjunct to the fungal diagnostic arena is especially promising.

PCR has been studied in greatest detail for the diagnosis of candidemia and IA [30, 49, 57]. Clinical specimens include serum or whole blood, for *Candida* and *Aspergillus* species, and respiratory samples (e.g., sputum or BAL fluid), for aspergillosis [30, 49, 57]. Target sequences vary widely but most often include ribosomal genes (18S rRNA) or internal transcribed spacer regions [25, 30]. Sensitivities range from 78% to 100% for candidiasis and from 33% to 100% in patients with proven IA [30]. Specificities vary, especially with PCR for aspergillosis

where false-positive results may be seen when BAL fluid is tested, because of the transient presence of conidia in the respiratory tract. The use of serum or plasma may be preferable to the use of respiratory tract specimens, because contamination with conidia is much less likely. The combination of quantitative PCR and GM antigen testing of BAL fluid has recently been shown to result in improved sensitivity and specificity, compared with either test alone, in the diagnosis of invasive pulmonary aspergillosis [49]. Likewise, antigen testing and real-time PCR using serum proved to be useful in weekly screening for IA in patients with hematologic disorders [57].

As with bacteria, the combination of broad-range PCR to amplify a product (e.g., 18S rRNA) from all or most common fungi associated with human infection (i.e., "panfungal PCR") is a potentially important strategy [30, 35]. Amplification followed by restriction endonuclease analysis, sequencing, or hybridization to a series of genus- or species-specific probes has been shown to be a viable option in the effort to broaden the diagnosis of fungal infections [24, 25, 58]. Presently, however, despite promising reports, PCR for the diagnosis of IFI has not been widely used in clinical settings. Furthermore, it has not been shown convincingly that PCR can compensate for the limitations of culture in the rapid diagnosis of IFI in the immunocompromised host. Real-time PCR and microarray platforms, coupled with fungal antigen detection, will likely be required to significantly ameliorate this difficult diagnostic problem.

ANTIFUNGAL SUSCEPTIBILITY TESTING

Resistance to antifungal agents is characterized as either intrinsic (i.e., present without prior exposure to the drug) or secondary (i.e., acquired following exposure to the agent). Both intrinsic and secondary resistance are relevant to the treatment of fungal infections. Whereas intrinsic resistance allows for predictable patterns of susceptibility of certain fungi to certain antifungal agents, secondary resistance is less predictable. Susceptibility testing in vitro is therefore playing an increasing role in antifungal drug selection. For example, *C. krusei* is intrinsically resistant to fluconazole, and testing in vitro offers no beneficial information. On the other hand, the susceptibility of *C. glabrata* to fluconazole varies significantly, and in vitro susceptibility results guide the use of fluconazole for the treatment of *C. glabrata* infections, particularly when there has been prior exposure to azoles.

Standardized methods. At present, the state-of-the-art method for antifungal susceptibility testing of yeasts is comparable to that for testing of bacteria [59]. The Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards) Subcommittee on Antifungal Susceptibility Testing has developed and published approved methods for broth dilution testing (CLSI ap-

proved standard M27-A2) [60] and disk diffusion testing (CLSI approved guideline M44-A) [61] of yeasts. These methods are reproducible, accurate, and available for use in clinical laboratories [62–64].

Quality control guidelines and MIC breakpoint criteria have been established for several systemically active antifungal agents, and quality control MIC reference ranges for *Candida* species have been established for microdilution testing of both established agents (e.g., amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole) and newly introduced and investigational agents (e.g., voriconazole, posaconazole, ravuconazole, caspofungin, and anidulafungin) [65, 66]. Quality control limits for disk diffusion testing of fluconazole and voriconazole against *Candida* species have also been published [61, 67, 68]. Interpretive MIC breakpoints have been established for fluconazole, flucytosine, itraconazole, and voriconazole [69–71], and disk diffusion breakpoints have been established for fluconazole and voriconazole [70, 71]. Notably, interpretive breakpoints have not been established for amphotericin B, posaconazole, or the echinocandins, nor have any breakpoints been defined for *C. neoformans*. However, the CLSI has come to a consensus on a standardized method for testing the echinocandins against *Candida* species, and it is expected that interpretive breakpoints for these agents, as well as for posaconazole, will be established in the near future.

Standardized methods have been developed for MIC testing of filamentous fungi (CLSI document M38-A) [72] but require further refinement to establish the in vivo correlation with in vitro data [73, 74]. The M38-A MIC method is applicable to the testing of *Aspergillus*, *Fusarium*, and *Scedosporium* species and the Zygomycetes with the newer azoles (i.e., voriconazole, posaconazole, and ravuconazole); however, it may not be optimal for testing the echinocandins [72, 73, 75]. A recent multicenter study found that, for caspofungin testing with *Aspergillus* species, the minimum effective concentration (i.e., the lowest concentration that results in growth of *Aspergillus* species producing conspicuously aberrant hyphae) offered an end point that gave generally reproducible results (excellent agreement among 14 of 17 laboratories) [75]. The finding of aberrant results in 3 of the 17 laboratories suggests a need for caution and further refinement of the caspofungin (echinocandin) test methodology for *Aspergillus* species [75].

Commercial antifungal susceptibility test systems. Commercial development of microdilution antifungal panels conforming to CLSI M27-A2 guidelines has been gradual [76–78]; however, one product, the Sensititre YeastOne colorimetric plate (Trek Diagnostic Systems), has been cleared by the US FDA for the testing of fluconazole, itraconazole, and flucytosine as a part of patient care. Subsequent to US FDA clearance, the YeastOne system has been used widely in the United States and

elsewhere, with good results in accuracy and reproducibility [79].

The YeastOne system is available in a dry, 96-well panel with the colorimetric growth indicator Alamar Blue and has a shelf life of ~24 months at ambient temperature. After the addition of a broth inoculum and incubation for 24 h, colorimetric MIC results for all test agents are read as the first well showing a color change from red (growth) to blue or purple (growth inhibition) (figure 1). Recently, the new extended-spectrum triazoles (e.g., voriconazole, posaconazole, and ravuconazole) have been added to a panel containing fluconazole in anticipation of these agents gaining FDA approval for the treatment of IFI [78]. The YeastOne colorimetric system provides results comparable to those obtained with the CLSI MIC reference method for 6 systemically active antifungal agents. It is anticipated that the echinocandins will be available on a YeastOne panel in the near future.

The Etest method (AB Biodisk) has been widely used in clinical microbiology as a means of producing accurate, reproducible, and quantitative MIC results by use of an agar diffusion format. This method is based on the establishment of a stable concentration gradient of an antimicrobial agent after diffusion from a plastic strip into an agar medium. When an Etest strip is placed on an agar plate that has been inoculated with a test organism and is incubated for 24–48 h, an ellipse of growth inhibition occurs, and the intersection of the ellipse with the numeric scale on the strip provides an indication of the MIC (figure 2). The Etest has proven to be useful for antifungal testing of both yeasts and moulds. Numerous studies have demonstrated the applicability of the Etest for determining the in

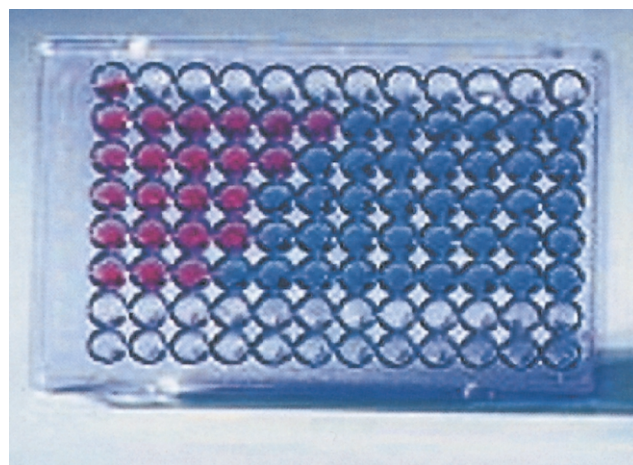


Figure 1. Sensititre YeastOne test panel (Trek Diagnostic Systems) for determination of MICs of antifungal agents against *Candida* species. A different drug is tested in each row of the panel. Red indicates growth, and blue indicates inhibition of growth. The first blue well in each row indicates the dilution representing the MIC for the drug tested in that row. Reprinted with permission from Trek Diagnostic Systems.

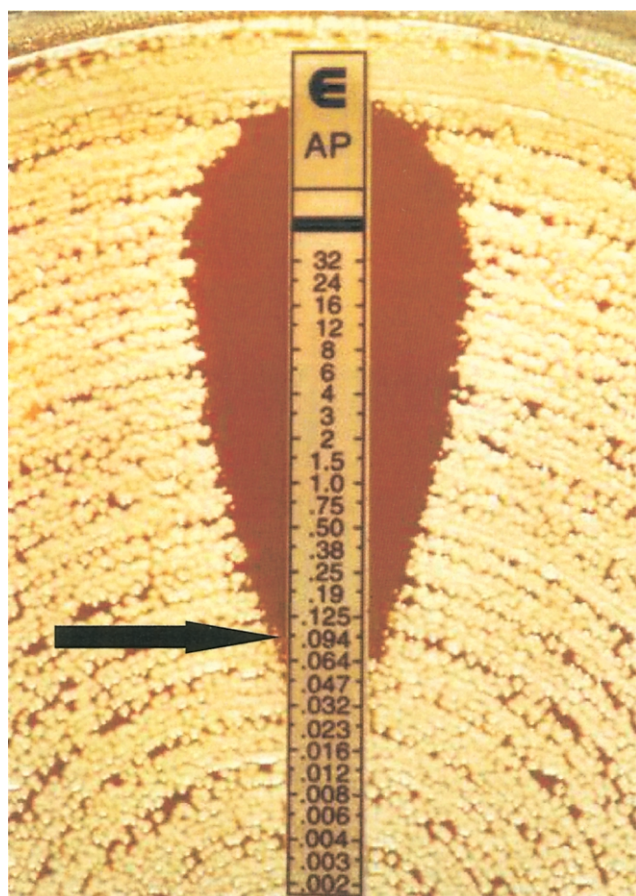


Figure 2. Etest (AB Biodisk) for determination of MICs of amphotericin B to *Candida* species. The MIC is read as the point where the ellipse of the zone of no growth intercepts the test strip (arrow). For amphotericin B, 100% inhibition of growth in the zone is required; for this isolate, the MIC is 0.094 µg/mL. Reprinted with permission from AB Biodisk.

vitro activity of a variety of antifungal agents, including amphotericin B, flucytosine, fluconazole, ketoconazole, itraconazole, voriconazole, posaconazole, and caspofungin [80–88]. Importantly, Etest is the most sensitive and reliable method for detecting decreased susceptibility to amphotericin B among isolates of *Candida* species and *C. neoformans* [88–91].

Interpretation. Similar to antibacterial susceptibility testing, antifungal susceptibility testing can be said to predict the outcome of fungal infections with a degree of accuracy that has been summarized as the “90-60 rule” [59]. According to this rule, infections due to susceptible isolates respond to therapy ~90% of the time, whereas infections due to resistant isolates respond to therapy ~60% of the time. Thus, low MICs are not entirely predictive of clinical success, and higher MICs help to predict which patients are less likely to have a favorable response to a given antifungal agent. The 90-60 rule reflects the fact that the in vitro susceptibility of an infecting organism to the antifungal (or antibacterial) agent is only one of several

factors that may influence the likely success of therapy for an infection.

The interpretive breakpoints for systemically active antifungal agents have been developed by considering data that relate the MICs to known resistance mechanisms, the MIC and disk zone diameter distribution profiles, pharmacokinetic and pharmacodynamic parameters, and the relationship between in vitro activity (i.e., MIC) and clinical outcome, as determined by the available clinical efficacy studies [69, 92]. Currently, interpretive breakpoints exist for 4 systemically active antifungal agents in relation to *Candida* species (table 5). The MIC breakpoints for fluconazole were derived from an extensive database defining the MIC distribution profile coupled with pharmacokinetic/pharmacodynamic parameters indicating that a ratio of 20–25:1 for the area under the serum concentration curve to the MIC was predictive of efficacy and from clinical outcome data from studies of mucosal candidiasis in HIV-infected patients and studies of invasive candidiasis in nonneutropenic patients [69, 92]. Recently, the MIC breakpoints for fluconazole in relation to *Candida* species have been reassessed using available data for 1295 patient-events (692 mucosal infections and 603 invasive infections) from 12 published clinical studies [71]. The clinical success rate was 85% for those episodes in which the fluconazole MIC was ≤ 8 µg/mL (i.e., susceptible), 67% for those episodes in which the MIC was 16–32 µg/mL (i.e., “susceptible dose dependent”), and 42% for those episodes in which the fluconazole MIC was ≥ 64 µg/mL (i.e., resistant) [71].

A similar process was used to establish the MIC breakpoints for voriconazole and *Candida* species [70]. The clinical support for breakpoints of susceptible (≤ 1 µg/mL), susceptible dose dependent (2 µg/mL), and resistant (≥ 4 µg/mL) came from 6 phase 3 primary or salvage/compassionate therapy studies of voriconazole treatment of invasive candidiasis. A statistically significant correlation was observed between MICs and investigator end-of-treatment assessments of outcome [70].

The MIC breakpoints for itraconazole were based on data from the treatment of mucosal candidiasis in patients with AIDS treated with oral itraconazole (capsule and/or solution) for whom serum concentrations of <0.5 µg/mL were common [69]. The category of susceptible dose dependent was applied to those isolates for which the MIC was determined to be 0.25–0.5 µg/mL on the basis of the recognition of improved clinical and microbiological response when serum concentrations were ≥ 0.5 µg/mL. Given the ability to reliably achieve serum concentrations of itraconazole ≥ 1 µg/mL throughout the dosing interval by use of the intravenous formulation, it is interesting to note that 96% of 7299 clinical isolates of *Candida* species were inhibited by ≤ 1 µg/mL itraconazole [93].

MIC breakpoints for flucytosine were derived by considering the MIC distribution profile, the pharmacokinetic/pharmacodynamic properties of the drug, and historical clinical outcomes

Table 5. Antifungal susceptibility testing: interpretive breakpoints with use of Clinical and Laboratory Standards Institute methods.

Antifungal agent ^a	Interpretive breakpoints, $\mu\text{g/mL}$			Comment [reference]
	Susceptible	Susceptible dose dependent	Resistant	
Fluconazole	≤ 8	16–32	≥ 64	Follows 90-60 rule of clinical response ^b [59, 70]
Itraconazole	≤ 0.12	0.25–0.5	≥ 1	Follows 90-60 rule of clinical response ^b [59, 92]; MIC is $\leq 1 \mu\text{g/mL}$ for 96% of <i>Candida</i> species [93]
Voriconazole	≤ 1	2	≥ 4	Follows 90-60 rule of clinical response ^b [59, 70]
Flucytosine	≤ 4	8–16 ^c	≥ 32	Follows 90-60 rule of clinical response ^b [59, 92]
Amphotericin B	≤ 1	...	> 1	Use Etest or antibiotic medium 3
Caspofungin	NA	NA	NA	MIC is $\leq 1 \mu\text{g/mL}$ for 99% of <i>Candida</i> species [96, 98]
Micafungin	NA	NA	NA	MIC is $\leq 1 \mu\text{g/mL}$ for 99% of <i>Candida</i> species [97, 99]

NOTE. The Clinical and Laboratory Standards Institute methods pertain to *Candida* species only. NA, data not available.

^a Only licensed agents are listed.

^b According to the 90-60 rule, infections caused by susceptible isolates respond to appropriate therapy ~90% of the time, whereas infections caused by resistant isolates (or infections treated with inappropriate antimicrobials) respond ~60% of the time.

^c MICs of 8–16 $\mu\text{g/mL}$ for flucytosine are considered to be intermediately susceptible, not susceptible dose dependent.

data [69]. A similar approach was not useful for amphotericin B because of the narrow range of MIC values (0.25–2 $\mu\text{g/mL}$) generated by the CLSI M27-A2 broth-based method. It should be noted that isolates of *Candida* species for which amphotericin B MICs are $>1 \mu\text{g/mL}$ are very uncommon and should be considered as potentially resistant [92].

The optimization of in vitro susceptibility testing of the echinocandins against *Candida* species has been a difficult process [75, 94, 95]; however, collaborative studies conducted by the CLSI subcommittee on antifungals demonstrate [75, 95, 96] that the use of RPMI 1640 broth medium, incubation at 35°C for 24 h, and an MIC end-point criterion of prominent reduction in growth ($\geq 50\%$ inhibition relative to control growth) provides reliable and reproducible MIC results [75]. These testing conditions have allowed investigators to establish a wild-type MIC distribution profile for caspofungin and *Candida* species in which $>99\%$ of clinical isolates are inhibited by $\leq 1 \mu\text{g/mL}$ and the modal MIC is 0.03–0.06 $\mu\text{g/mL}$ [94, 96–99]. MICs for strains of *Candida* species with documented *FKS1* gene mutations [75, 96] and for those strains with resistance that have been reported in the literature [100, 101] are all $>2 \mu\text{g/mL}$ and usually are $>8 \mu\text{g/mL}$. Thus, the present lack of correlation between caspofungin MICs and clinical outcome [102] most likely represents the fact that strains for which MICs are elevated (i.e., $>2 \mu\text{g/mL}$) are very uncommon and that clinical failure is likely due to factors other than the drug-pathogen interaction [59]. The development of breakpoints for caspofungin and other echinocandins is a work in progress but will ultimately take into account all the factors mentioned above for the azole antifungals. However, on the basis of the 2 case reports of acquired resistance to caspofungin, it appears that increasing MICs in serial isolates of *Candida* species may signify the development of clinically important resistance [100, 101].

Antifungal susceptibility testing is now increasingly and ap-

propriately used as a routine adjunct to the treatment of fungal infections [59, 103–105]. Guidelines for the use of antifungal testing and other laboratory studies have been developed [59]. Selective application of antifungal susceptibility testing coupled with broader identification of fungi to the species level should prove to be useful, especially in difficult-to-manage fungal infections [59, 105]. Future efforts will be dedicated to further validating the interpretive breakpoints for established antifungal agents and developing them for newly introduced systemically active agents. In addition, procedures must be optimized for testing non-*Candida* yeasts (e.g., *C. neoformans* and *Trichosporon* species) and moulds [1, 64].

CONCLUSION

The accurate diagnosis of fungal infections by use of conventional mycologic and histopathologic techniques is time consuming and arduous because of the suboptimal sensitivity of these methods. Facilitating an earlier and noninvasive means of diagnosing fungal disease continues to be a major focus for medical microbiologists and clinicians, particularly those who provide care to immunosuppressed patients. Although progress has been made during the past 2 decades—including the standardization of methods for susceptibility testing, the development of more-rapid means of presumptively identifying *C. albicans* in blood cultures with the PNA-FISH assay, and the development of GM- and BG-based antigen assays—much work is left to be done. Future efforts must be directed toward expanding the availability of the new US FDA-approved and CLSI-recommended tests, validating the molecular assays for the diagnosis of fungal disease, and establishing and validating the interpretive breakpoints for all medically important fungi to all licensed antifungal agents.

Acknowledgments

Financial support. This article is supported by an educational grant from Schering-Plough.

Potential conflicts of interest. B.D.A. has received grants and research support from Enzon Pharmaceuticals and Astellas Pharma US; has received consultation fees from Merck, Enzon Pharmaceuticals, Astellas Pharma US, Pfizer, Vicuron Pharmaceuticals, and Schering-Plough; has received honoraria from AB Biodisk; and is a member of the paid speakers' bureau for Astellas Pharma US and Schering-Plough. M.A.P. has received grants and research funding from Schering-Plough, Pfizer, Astellas Pharma US, Vicuron Pharmaceuticals, and Merck; is a consultant for Schering-Plough, Pfizer, Astellas Pharma US, Vicuron Pharmaceuticals, and Merck; has received honoraria from Schering-Plough, Pfizer, Astellas Pharma US, Vicuron Pharmaceuticals, and Merck; and is a member of the paid speakers' bureaus for Schering-Plough, Pfizer, Merck, and Astellas Pharma US.

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