

# Current Status of Nonculture Methods for Diagnosis of Invasive Fungal Infections

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## INTRODUCTION

The frequency of invasive fungal infections has risen dramatically in recent years (209, 216). Early and accurate diagnosis of these infections is important for several reasons, including timely institution of antifungal therapy (164) and to decrease the unnecessary use of toxic antifungal agents. In addition, the availability of accurate and timely diagnoses could reduce the use of empirical antifungal therapy, thereby reducing antifungal selection pressure and the emergence of antifungal resistance. Unfortunately, a major obstacle to the successful treatment of invasive fungal infections is the lack of sensitive and specific methods for the early diagnosis of invasive fungal infections. Standard approaches to the laboratory diagnosis of invasive fungal infections include (i) direct microscopic visualization for the presence of organisms in freshly

obtained body fluids, (ii) histopathologic demonstration of fungi within tissue sections, and (iii) cultivation of the causative fungus and its subsequent identification. However, these approaches often are not sufficiently sensitive and/or specific to diagnose invasive fungal infections, and they sometimes require invasive procedures to obtain the necessary specimens.

This work reviews recent advances of nonculture methods for the diagnosis of invasive aspergillosis, invasive candidiasis, cryptococcosis, blastomycosis, coccidioidomycosis, histoplasmosis, paracoccidioidomycosis, and penicilliosis. Among the nonculture methods we review, detection of a specific host antibody response is attractive because such tests can be performed rapidly and do not require invasive sampling procedures. However, presence of host antibodies does not always correlate with presence of invasive disease, especially in patients whose abilities to produce specific immunoglobulin responses may be impeded by immunosuppressive drugs and/or serious underlying diseases. Detection of macromolecular microbial antigens generally requires a relatively large microbial burden, which may limit assay sensitivity. Nonetheless, several

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examples of successful antigen detection systems have been developed, and some of these are widely used. Other alternatives to standard culture and serologic diagnostic methods include amplification and detection of specific fungal DNA sequences and the detection and quantitation of specific fungal metabolic products.

### DETECTION OF SPECIFIC HOST HUMORAL RESPONSES

Definitive diagnosis of invasive fungal infection is usually based on (i) recovery and identification of a specific etiological agent from clinical specimens or (ii) microscopic demonstration of fungi with distinctive morphological features (e.g., encapsulated *Cryptococcus neoformans* cells in cryptococcosis). However, if neither cultural nor morphological proof of infection is available, other approaches must be used. Detection of specific host antibody responses often provides this supplemental information for the diagnosis of invasive fungal infections. Although serologic testing has been used for many decades to establish presumptive diagnoses of a number of fungal infections (26, 80, 216), antibody tests are seldom used in the diagnosis of invasive candidiasis, invasive aspergillosis, or cryptococcosis (26, 49, 82, 113). Among the reasons for the poor sensitivity and specificity of antibody testing in the case of these diseases are that antibodies are often present in colonized but uninfected patients (216) and that severely immunocompromised patients tend to mount poor specific antibody responses (26, 49, 82, 113). Hence, the diagnosis of these infections by antibody tests will not be discussed here.

Detection of specific host antibody responses, however, is often used in the diagnosis of endemic mycoses, which are often difficult to detect by traditional methods such as culture and staining methods. Conventional serologic tests have limitations, especially when crude mixtures of antigens are used as reagents. These limitations include (i) cross-reactions among different species, (ii) presence of antibodies to common environmental or commensal fungi, (iii) lack of standardization of antigens and methods for detecting quantifying antibodies. This section will review the recent developments as well as the problems associated with serologic testing for fungal infection, with particular attention to the endemic mycoses.

#### Blastomycosis

The diagnosis of blastomycosis is usually made by identifying *Blastomyces dermatitidis* in tissues or exudates by microscopy and/or culture, but serological tests may also provide useful information. Early serologic tests for blastomycosis were generally based on demonstrating antibodies to *B. dermatitidis* A antigens, which were obtained by allowing *Blastomyces* yeast cell walls to undergo autolysis in neutral buffer (79, 91). Although the amount of immunoglobulin G (IgG) antibody to *B. dermatitidis* A antigen correlated with disease activity, antibody could also be detected 1 year or more after successful treatment (14). WI-1 is a 120-kDa protein in the outer cell wall of *B. dermatitidis* that has been purified and used as a target in immunodiagnostic testing (109, 189). Antibodies to WI-1 can be detected earlier than antibody to A antigen, they can persist for longer intervals, and they decline to low or undetectable

levels by 6 months after illness onset in patients with resolution or successful treatment of disease (110, 111).

The main obstacle to using antibody testing to diagnose blastomycosis is cross-reactivity between *B. dermatitidis* and the fungi that cause coccidioidomycosis, histoplasmosis, paracoccidioidomycosis, and nonfungal infections (91, 108). Although a study comparing WI-1 and A antigens demonstrated that WI-1 is more reactive and specific for the binding of serum antibodies to *Blastomyces* (109), the principal site of specific antibody recognition is the same peptide epitope for both WI-1 and A antigens. This is a 25-amino-acid residue tandem repeat and has been cloned from WI-1 and produced in recombinant form in *Escherichia coli* (107). The use of this recombinant antigen that was produced in a nonglycosylated form appears to circumvent problems of antigen cross-reactivity due to post-translational modification.

#### Coccidioidomycosis

Diagnosis of coccidioidomycosis by direct examination of clinical specimens is an insensitive test, mainly because of the small number of *Coccidioides immitis* present in most clinical specimens. Culture, although easily accomplished, should be performed in a biological safety cabinet. Hence, alternative diagnostic procedures may depend on serology.

Serologic diagnosis of coccidioidomycosis is generally based on the detection of antibodies to two *C. immitis* antigens, the tube precipitin (TP) and the complement fixation (CF) antigens. TP antibodies have been associated with primary acute disease and are thought to belong mostly to the IgM class (153). CF antibodies persist during the chronic disseminated phase of the disease and have been described as primarily IgG antibodies (153). However, CF antibodies may not be present in immunocompromised or immunosuppressed patients such as AIDS patients (1, 5, 20, 175), which suggests that this approach may have limited utility in AIDS patients.

The use of either crude or partially purified antigens to detect antibodies to TP and CF antigens contributed to low sensitivity (153) and/or specificity in patients with *C. immitis* infection, histoplasmosis, and blastomycosis (31, 90, 99, 101, 153, 187, 188, 241). Hence, many investigators have been trying to better characterize *C. immitis* antigens in an attempt to develop more-sensitive and -specific tests. Johnson and Pappagianis (94) showed that the CF antigen had chitinase activity, and the amino acid sequence of the recombinant CF protein is partially homologous to two highly conserved domains in the chitinases of several fungi and bacteria. Yang and colleagues (246) cloned a cDNA that encoded the CF antigen of *C. immitis*. Although the recombinant protein was highly sensitive in detecting CF antibody in sera from patients with coccidioidomycosis, it also cross-reacted with sera from patients with histoplasmosis and blastomycosis. Yang et al. (245) reasoned that *Histoplasma capsulatum* or *B. dermatitidis* may also produce a chitinase with a similar conserved domain. If this prediction is correct and the epitopes recognized by CF antibody are distinct from those shared with *H. capsulatum* and *B. dermatitidis*, then the problem of cross-reactivity could be resolved by molecular modification of the CF protein/chitinase gene. Hence, Yang et al. (245) determined whether the epitope(s) that reacted with CF antibody were the same or different from

the epitopes that were shared with *H. capsulatum* and *B. dermatitidis*. The CF antigen was cloned, and unlike the full-length protein and the peptide domain comprised of amino acid residues 20 through 427, a peptide domain comprised of amino acid residues 20 through 310 was specific to anticoccidioidal CF antibody. Moreover, this peptide was recognized by serum antibody in 95% (21 of 22) of patients with active coccidioidomycosis and not by sera from patients with histoplasmosis and blastomycosis (245).

### Histoplasmosis

The standard method for the diagnosis of histoplasmosis remains cultural isolation and identification of *H. capsulatum*. However, culture often requires a 2- to 4-week incubation period before the identification of the fungus is possible, whereas antibody detection methods offer a rapid alternative to microbiological techniques.

Before the 1970s, the most important source of antigens in assessing antibody responses in patients with histoplasmosis was histoplasmin, which was prepared from filtrates of mycelial-phase cultures of *H. capsulatum* grown in synthetic medium (157). Histoplasmin contains *H. capsulatum* species-specific H and M antigens as well as C antigen. Antibodies against H antigen form during active histoplasmosis (24), while antibodies to M antigens may be formed in active and chronic histoplasmosis and are usually the first to arise upon seroconversion (167).

Antigens derived from filtrate of mycelial-phase cultures of *H. capsulatum* also contain components that cross-react with antibodies to other fungal species (100). Therefore, glycosylated and nonglycosylated forms of M antigens were compared, and cross-reactivity with serum from patients with aspergillosis, blastomycosis, coccidioidomycosis, and paracoccidioidomycosis was eliminated when M antigen was treated with periodate (249, 250). Furthermore, a recombinant antigen corresponding to a 60-kDa native *H. capsulatum* antigen was recognized by 100% of sera from histoplasmosis patients and none of the various control sera (37). The H antigen has also been cloned and sequenced and was found to be homologous to extracellular  $\beta$ -glucosidases (45). However, the suitability of recombinant H as a serodiagnostic reagent has not yet been assessed. In addition to the above problems, antibody may be undetectable in patients with human immunodeficiency virus (HIV) infection, most probably due to impaired antibody production (80). Under these circumstances, antigen detection tests described in the next section may be more appropriate tools in the diagnosis of these infections.

### Paracoccidioidomycosis

The definitive diagnosis of paracoccidioidomycosis can be accomplished by direct visualization of *Paracoccidioides brasiliensis* in body fluids or tissues or its isolation and growth in culture. However, the time required to isolate *P. brasiliensis* from clinical specimens represents a hindrance to rapid diagnosis. Serological tests are useful for rapid diagnosis and generally rely on the detection of antibody responses against components of *P. brasiliensis*. However, antibody responses are difficult to detect in AIDS patients with paracoccidioidomycosis.

In contrast, they are useful in patients without AIDS who have paracoccidioidomycosis, especially in cases of disseminated disease (172). Anti-*P. brasiliensis* IgG levels are usually elevated in patients with recently diagnosed paracoccidioidomycosis, and these levels have been shown to be good markers for monitoring patients with the acute or chronic form of the disease and monitoring responses to therapy (12, 23, 32).

Culture filtrates, cytoplasmic extracts, and antigens derived from the cell wall have been used as reagents in serological tests for paracoccidioidomycosis (21, 29, 80, 173, 174). Limitations of these tests include (i) cross-reactivity to other mycotic disorders, mainly histoplasmosis, and (ii) difficulties in standardization of the various tests and reagents (21, 33, 46, 60). Hence, attempts to eliminate these problems include the examination of purified and well-characterized antigens derived from *P. brasiliensis*.

The 43-kDa glycoprotein antigen was the first *P. brasiliensis* recombinant protein to be cloned (198). Although extensively and successfully employed as an antigen in the serological diagnosis of paracoccidioidomycosis (199), this antigen was extracted directly from *P. brasiliensis* cultures, and this may result in variations in antigen lots (30). Furthermore, cross-reactivities were noted with sera from patients with histoplasmosis and lobomycosis (161), and these were predominantly attributed to periodate-sensitive carbohydrate epitopes containing galactosyl residues (2). Other antigenic products included a 58-kDa antigen (60) and a 22- to 25-kDa antigen (61) that were identified by species-specific monoclonal antibodies. The 58-kDa antigen was recognized by immune human sera, but problems associated with purifying this antigen to homogeneity have hindered the extension of this work (60). The 22- to 25-kDa antigen was considered as a potential marker in the immunohistochemical identification of *P. brasiliensis* (61).

A 27-kDa recombinant protein from *P. brasiliensis* has recently been cloned, sequenced, and expressed in *E. coli* (135, 151). Batch production of the recombinant 27-kDa antigenic product is feasible, and large quantities can be produced, thus permitting standardization (152). However, cross-reactions were seen in the cases of serum samples from aspergillosis and histoplasmosis patients, and the 27-kDa antigen is not recognized by serum from all patients with paracoccidioidomycosis (152).

### Penicilliosis

Preliminary diagnosis of penicilliosis is made on the basis of clinical symptoms combined with direct microscopic identification of fission arthroconidia of *Penicillium marneffeii* in clinical specimens. Definitive diagnosis then relies upon the identification or isolation of *P. marneffeii* in clinical specimens. However, cultural methods usually take  $\geq 3$  days. Rapid diagnosis is important because disseminated penicilliosis has a high mortality, and antibody detection permitted the early diagnosis of penicilliosis. Nevertheless, antibody detection may still be required in order to identify individuals with nonspecific symptoms of penicilliosis and patients who have an initial asymptomatic form of the disease.

Patients with penicilliosis develop elevated titers of antibodies against *P. marneffeii* antigens, and antibody response has been reported in immunosuppressed patients with AIDS (215).

However, immunoassays employing either crude antigen preparation or whole fungal cell have limitations. Hence, attempts have been made to circumvent this situation.

A *P. marneffei* gene that encodes a highly antigenic cell wall mannoprotein, Mp1p, has been cloned (35), and antibodies to Mp1p have been demonstrated by immunoprecipitation in *P. marneffei*-inoculated guinea pigs and in patients with penicilliosis (36). Another approach that used the purified recombinant Mp1p protein in an enzyme-linked immunosorbent assay (ELISA)-based antibody test detected 14 of 17 (82%) HIV-infected patients with penicilliosis. Moreover, the specificity of this test appeared to be very good; no false-positive reactions were noted in serum samples from healthy donors, patients with typhoid fever, or patients with tuberculosis (36). Although these studies showed good sensitivity and specificity with the purified recombinant Mp1p protein, prospective studies are required before this approach can be applied to routine usage.

### Summary

Early attempts at antibody detection relied on the use of crude, unfractionated mixtures of antigens, and this resulted in major problems with cross-reactivity. Much of this cross-reactivity was due to nonprotein components (e.g., carbohydrates and phosphorylcholine) that are present in crude antigens prepared directly from yeasts and mycelial stage, fungal cytoplasm, or fungal cell walls. Moreover, tests that rely on crude extracts may be impeded by difficulties in producing reproducible reagents. Hence, most recent efforts have focused on (i) developing more-defined antigens that are derived from the application of recombinant techniques and (ii) using more-advanced assay systems. The use of recombinant antigens prepared in a prokaryotic host can eliminate cross-reactivity due to posttranslational antigen modification. However, problems associated with false positives generated through prior exposure either through skin testing or to the organism itself, and with false negatives resulting from the development of infection in immunocompromised patients, such as those with advanced HIV infection, still exist.

### DETECTION OF FUNGAL ANTIGENS

Another approach to diagnosis of invasive fungal infections is to use immunologic reagents to detect and quantify macromolecular fungal antigens in host body fluids. Ideal antigenic markers for invasive fungal infections should not be present too transiently, and they should be associated with infection rather than colonization. They should be conserved within the fungal species of interest, they should not cross-react with other human and microbial antigens, and they should be present sufficiently early for the starting of antifungal therapy. Moreover, tests for these antigens should be adaptable to formats that can be used in routine clinical laboratories, they should be easy to perform, and they should not be subject to significant interlaboratory variation.

A number of early studies focused on using cell wall components of fungal species as antigenic markers (3, 54, 116, 168, 220, 221). Among these markers are mannan and galactomannans, which have been shown to be useful in the diagnosis of invasive aspergillosis and candidiasis (see Tables 1 and 2).

Early efforts to detect antigenemia were often hampered by the use of insensitive methods with low detection limits (48, 49, 114, 132). Moreover, fungal mannans or galactomannans may be rapidly removed from circulation by the formation of immune complexes and by receptor-mediated endocytosis by Kupffer's cells in the liver (48), thereby limiting the sensitivity of these diagnostic approaches. Hence, attempts have been made toward development of immunoassays with increased specificity and sensitivity for candidiasis and aspergillosis, and there has been much less interest in diagnosis of other systemic fungal infections.

These studies of antigenic markers were not limited to mannoprotein but also included polysaccharide capsular antigens, carbohydrates other than mannoproteins, and soluble proteins. However, some antigen detection assays described in the past employed either poorly standardized reagents or insensitive methodology (26, 48, 49, 80). In general, antigen tests that use polyclonal antibodies raised against crude fungal antigens have significant cross-reactivities with several pathogenic fungi (48, 80). For example, the radioimmunoassay (RIA) based on anti-*H. capsulatum* rabbit polyclonal antibody as capture and detector antibody to detect *H. capsulatum* polysaccharide antigen (HPA) exhibited some degree of cross-reaction with other organisms (71, 80, 228). In addition, assays that use polyclonal antibodies may be subject to variability among different batches of antisera. For instance, detection of cryptococcal capsular polysaccharide antigen by latex agglutination with polyclonal antibodies has proven highly sensitive since its inception in 1960s (11), but may be subject to the type of variation reported for polyclonal serum-based latex bead assays (242). Monoclonal antibody-based immunodiagnostic assays have several advantages over polyclonally based assays, which make the former likely to reduce such variability since they are readily available in unlimited quantities as appropriate and since monoclonal antibodies do not exhibit as much batch-to-batch variability.

This section will review how far the above requirements have been fulfilled and recent development of antigen detection in the diagnosis of invasive candidiasis, invasive aspergillosis, cryptococcosis, and some endemic mycoses.

### Invasive Aspergillosis

Invasive aspergillosis is an increasingly recognized condition in immunocompromised hosts. However, the major problem associated with invasive aspergillosis is the difficulty in diagnosing this infection early enough to be of value in patient management. Cultures of blood or respiratory specimens are seldom positive, especially during the early stages of the disease. The reliable diagnosis of invasive aspergillosis requires invasive procedures to obtain biopsy material for histological and microbiological examination. However, the performance of invasive diagnostic procedures is often precluded by the critical condition of the patients. Antibody detection tests are used as an adjunct to microbiological methods for the diagnosis of invasive aspergillosis, but these tests are often negative because of the fulminant nature of the disease and/or the poor immunological status of the host (26, 49, 113, 114, 115, 216). Therefore, the detection of various antigenic markers of invasive aspergillosis is currently an area of great interest.

TABLE 1. Detection of galactomannan antigen in patients with mycologic and/or histopathologic evidence of invasive aspergillosis

Method	No. positive for antigen/total no. of subjects (%)		Reference
	Patients with proven invasive aspergillosis	Controls <sup>a</sup>	
LA <sup>b</sup> (Pastorex)	2/4 (50.0)		4
LA (Pastorex)	4/13 (30.8)	3/61 (4.9)	130
LA (Pastorex)	18/19 (94.7)	8/90 (8.9)	83
ELISA (Platelia)	9/10 (90.0)		214
LA (Pastorex)	7/10 (70.0)		214
LA (Pastorex)	4/8 (50.0)	5/83 (6.0)	89
ELISA (Platelia)	33/40 (82.5)	1/77 (1.3)	195
LA (Pastorex)	11/40 (27.5)		195
ELISA (Platelia)	3/5 (60.0)	3/17 (17.6)	127
LA (Pastorex)	2/5 (40.0)	1/17 (5.9)	127
ELISA (Platelia)	25/27 (92.6)	2/44 (4.5)	128

<sup>a</sup> Included healthy subjects and/or colonized patients and/or patients with other infections.

<sup>b</sup> LA, latex agglutination.

Many *Aspergillus* cellular products have been studied as markers of invasive aspergillosis and have been reviewed in detail elsewhere (48, 49, 114, 115, 216). Galactomannan, the first antigen detected in body fluids of experimental animals and patients with invasive aspergillosis (3, 54, 116, 168), has been studied extensively and has been shown to correlate with clinical diagnosis and response to antifungal therapy (182, 200, 212, 213). A number of techniques, including enzyme immunoassays (EIAs), RIA, and latex particle agglutination tests have been evaluated for the detection of galactomannan in the body fluids of patients with invasive aspergillosis (194). However, their routine use has been hampered by a low detection limit (5 to 15 ng/ml) (194), resulting in the detection of galactomannan in serum only at advanced stages of the disease, when antifungal therapy is of limited value (50). Most recent efforts to improve the detection of galactomannan have focused on (i) its presence in body fluids of immune complexes that must be dissociated before the antigen of interest is detectable, (ii) use of testing systems capable of detecting the lowest possible amount of galactomannan, and (iii) the superiority of monoclonal antibodies over polyclonal antibodies for immunological detection of galactomannan.

Stynen and colleagues (194) introduced a sandwich ELISA that employs rat monoclonal antibody EB-A2 and is known as Platelia *Aspergillus* (Bio-Rad, Marnes-la-Coquette, France). This test is one of the most sensitive methods currently available to detect galactomannan. Table 1 summarizes studies in which generally available tests were assessed in confirmed cases of invasive aspergillosis (those with mycologic and/or histopathologic evidence of invasive aspergillosis) and in which the total numbers of patients studied were reported. The lower limit of detection of galactomannan for the sandwich ELISA was 0.5 to 1.0 ng per ml of serum, whereas a latex agglutination test which employed the same monoclonal antibody had a threshold of 15 ng/ml (194). Furthermore, the sandwich ELISA became positive earlier than the latex agglutination test and appeared to remain positive after the latex agglutination test had become negative (125, 192, 211).

The Platelia *Aspergillus* assay has a false-positive rate rang-

ing from 1 to 18% with serum samples from patients without *Aspergillus* diseases (18, 127, 194, 195, 196, 214). These occurred especially with samples obtained within 10 days of cytotoxic chemotherapy (196) or 30 days of bone marrow transplantation (195). Other tests and procedures are required to confirm the presence of infection when persistent antigenemia occurs in non-*Aspergillus*-infected samples. The nature of these persistent false-positive reactions remains undetermined. Earlier reports indicated that cyclophosphamide was a potential inducer (81), while others thought of the cross-reactivity with exoantigens from bacteria or yeasts. In addition, a recent study has suggested that heat-resistant galactomannan is not eliminated by the processes of food sterilization and may reach the circulation through damaged intestinal mucosa and cause false-positive results in tests to detect antigenemia (119). Truly false-negative sera, perhaps as a result from limited angioinvasion, low fungal load, high antibody titers, or low level of galactomannan released by the fungus, have been documented but did not exceed 5% (128). In animal models, the prophylactic or preemptive use of amphotericin B may suppress the expression of galactomannan (64), a phenomenon that appears to be due to reduced mycelial growth (182). However, whether other antifungal agents might result in a similar effect remains to be examined.

Despite the drawbacks mentioned above, the development of the Platelia *Aspergillus* assay represents a marked improvement in the serological diagnosis of patients at risk for aspergillosis. Hence, in the case of a positive Platelia *Aspergillus* test result, the collection and testing of serum samples should be continued in order to exclude the possibility of a false-positive result. Furthermore, confirmation of suspected invasive aspergillosis should be obtained by additional radiological and/or microbiological examinations.

### Invasive Candidiasis

Incidence of invasive candidiasis has increased over the past several decades (209, 216). The early diagnosis of invasive candidiasis has been extremely difficult because the clinical signs and symptoms of invasive candidiasis are nonspecific, which leads to a delay in the diagnosis and, consequently, a delay in the institution of appropriate antifungal therapy. Unfortunately, traditional microbiological techniques for diagnosis of invasive candidiasis often fail to detect the disease, as blood cultures are often negative or become positive too late. Moreover, the use of invasive diagnostic techniques for histopathological studies is not permitted by the underlying conditions of critically ill patients. Hence, efforts to develop a rapid diagnostic test have stimulated the development of several serological methods for the diagnosis of *Candida* infection. However, antibody detection in patients with candidiasis is of limited usefulness for two reasons. First, colonization by *Candida* species of the gastrointestinal tract or other sites can elicit antibody responses in uninfected individuals. Second, immunocompromised patients may not mount detectable antibody responses, even when they have deep *Candida* infections.

Unlike the conventional antibody detection tests, the direct detection of *Candida* species antigens has been shown to have potential as an early diagnostic test. The development of an-

tigen detection tests before 1990 has been reviewed extensively elsewhere (48, 49, 169). Table 2 summarizes the results of clinical studies that provided detailed information about (i) confirmed cases of invasive candidiasis, i.e., mycologic and/or histopathologic evidence, and (ii) controls who did not have invasive candidiasis.

The Cand-Tec latex agglutination test (Ramco Laboratories, Houston, Tex.) was used as the first commercially available antigen detection test (7, 118). However, the specificity and sensitivity of the Cand-Tec assay vary among reports (7, 8, 47, 156). Furthermore, false-positive results due to rheumatoid factor have been observed (28). Therefore, it was difficult to confirm the diagnosis of candidiasis by the Cand-Tec assay alone, and the unknown nature and function of the target antigen have impeded its further development.

Other target antigens included a 47-kDa cytoplasmic protein antigen (134) and a 48-kDa antigen (65, 133) of *Candida* species. The 48-kDa antigen, which was subsequently recognized as enolase (65, 133), is a novel marker for the diagnosis of invasive candidiasis (80, 140, 218). Enolase antigenemia was present in patients with candidemia, while the antigen was not present in superficial *Candida* colonization or those who had no evidence of candidiasis (140). Unfortunately, a highly promising test for *Candida* enolase was marketed only briefly before it was withdrawn from the market by its manufacturer (Directigen; Becton Dickinson, Baltimore, Md.).

Another investigational strategy is the detection of circulating  $\beta$ -(1-3)-D-glucan, the cell wall component of *Candida*, and the test is available commercially (Fungitec G-test; Seikagaku Corporation, Tokyo, Japan). High concentrations of  $\beta$ -(1-3)-D-glucan have been detected in rabbits with experimentally induced candidiasis (143) and in patients with invasive candidiasis (144). However, a positive result does not indicate which class of fungi may be causing infection. This is not a major

practical limitation if a broad-spectrum antifungal agent is to be used. However, some potent antifungals are effective against some fungi but not others (e.g., fluconazole or caspofungin), so methods that can identify specific fungal pathogens will be increasingly desirable in the future.

The use of mannan antigenemia (referred to hereafter in this work as "mannanemia") detection for the immunodiagnosis of systemic candidiasis was suggested decades ago by Weiner and Coats-Stephen (221), and it is now one of the most widely studied antigens in patients with candidiasis. A body of literature has been accumulated from various laboratories, suggesting that positive mannan result may correlate with invasive candidiasis (Table 2). Furthermore, studies have also shown a correlation between detectable mannanemia and tissue invasive by *Candida* spp. in patients with candidemia, and mannanemia was less likely to be present in patients with transient or central venous catheter-related candidemia (72). However, the detection of mannanemia in the past has been hampered by the use of insensitive methods that resulted in poor sensitivity and/or specificity (48, 49, 114, 132). Attempts to improve the immunological detection of mannan involved the use of immune complex dissociation by heating sera before performance of the test, the use of a more-sensitive test format, and the use of monoclonal antibodies that react with defined epitopes (85, 92, 170). Several monoclonal antibodies have been characterized and employed in the immunodiagnostic assays. AF1 is one of the monoclonal antibodies that recognized an oligosaccharide shared by a number of mannoproteins from different pathogenic *Candida* species but not *Candida krusei* (44, 72, 73). Another monoclonal antibody, 3H8, as an IgG1, recognized only mannoproteins of high molecular mass present in the *Candida albicans* cell wall but not those of other *Candida* species (131). Other monoclonal antibodies included EB-CA1, for cases in which different species of

TABLE 2. Detection of antigenemia in patients with *Candida* fungemia and/or histopathologic evidence of invasive candidiasis

Antigen detected	Method used	Antibody used <sup>a</sup>	No. positive for antigen/total no. of subjects <sup>b</sup> (%)		Reference
			Patients with proven invasive candidiasis	Controls <sup>c</sup>	
Enolase	Dot immunobinding assay	Polyclonal Ab	28/39 (71.8)	0/40 (0)	140
Enolase	Double-sandwich liposomal immunoassay	Polyclonal Ab	18/24 (75.0)	6/146 (4.1)	218
$\beta$ -Glucan	Limulus test		27/32 (84.4)	5/40 (12.5)	140
Heat-labile antigen	Cand-Tec assay	Polyclonal Ab	30/39 (76.9) <sup>+</sup>	5/40 (12.5) <sup>+</sup>	140
			16/39 (41.0) <sup>++</sup>	2/40 (5.0) <sup>++</sup>	
Heat-labile antigen	Cand-Tec assay	Polyclonal Ab	21/32 (66.0) <sup>+</sup>		118
			14/32 (44.0) <sup>++</sup>		
Heat-labile antigen	Cand-Tec assay	Polyclonal Ab	16/33 (49.0) <sup>+</sup>		156
			2/83 (6.0) <sup>++</sup>		
Mannan	EIA	Polyclonal Ab	6/29 (20.7)	0/14 (0)	118
Mannan	ELISA	Polyclonal Ab	16/19 (84.2)	2/177 (1.1)	151
Mannan	ELISA	Polyclonal Ab	43/58 (74.1)	0/151 (0)	67
Mannan	LA <sup>d</sup> (Pastorex)	MAB	3/12 (25.0)	0/60 (0)	85
Mannan	EIA (ICON)	Polyclonal Ab	13/19 (68.4)	8/95 (8.4)	155
Mannan	Dot immunobinding assay	MAB	10/15 (67.0)	4/57 (7.0)	44
Mannan	LA (Pastorex)	MAB	10/39 (25.6)	0/40 (0)	140
Mannan	EIA	MAB	18/43 (41.8)	3/150 (2)	185
Mannan	LA (Pastorex)	MAB	12/43 (27.9)	0/150 (0)	185

<sup>a</sup> Abbreviations: Ab, antibody; MAB, monoclonal antibody.

<sup>b</sup> Symbols: +, titer of 1:4 or more as the cutoff value for a positive result; ++, titer of 1:8 or more as the cutoff value for a positive result.

<sup>c</sup> Included healthy subjects and/or colonized patients and/or patients with other infections.

<sup>d</sup> LA, latex agglutination.

TABLE 3. Detection of *Histoplasma* antigen by immunoassays

Method	Specimen	No. positive for antigen/total no. of subjects (%)				Reference
		Patients with AIDS and DH <sup>a</sup>	Patients without AIDS with DH	Patients without DH	Controls <sup>b</sup>	
RIA	BAL <sup>c</sup> fluid	19/27 (70.3)	2/4 (50.0)	0/10 (0)	0/122 (0)	226
RIA	Urine	25/27 (92.6)			0/122 (0)	226
RIA	Serum	23/26 (88.5)			0/122 (0)	226
RIA	Urine	75/79 (94.9)	22/27 (81.5)	24/82 (29.3)		229
RIA	Serum	54/63 (85.7)	7/11 (63.6)	6/26 (23.1)		229
RIA	Urine	38/40 (95)	12/16 (75.0)	11/30 (36.6)	1/96 (1.0)	55
EIA	Urine	38/40 (95)	12/16 (75.0)	11/30 (36.7)	1/96 (1.0)	55
ELISA	Serum	8/11 (72.7)	5/8 (62.5)	12/16 (75.0)	8/92 (8.7)	75

<sup>a</sup> DH, disseminated histoplasmosis.

<sup>b</sup> Included healthy subjects and/or colonized patients and/or patients with other infections.

<sup>c</sup> BAL, bronchoalveolar lavage.

the *Candida* genus share both the EB-CA1 epitope distributed on the mannan and mannoproteins of *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *C. krusei* (92). Two assays employing this monoclonal antibody have been marketed as the Pastorex *Candida* latex agglutination test (Bio-Rad) and the Platelia *Candida* Antigen test (a double-sandwich enzyme immunoassay) (Bio-Rad) (185). Although the specificities of these two assays are similar, the EIA is more sensitive than the latex agglutination test (185). Moreover, a recent study showed that repeated testing of serum from high-risk patients with both the Platelia *Candida* Antigen test and two tests for antibodies to *C. albicans* mannan could identify more infected patients than antigen testing alone (248).

Despite the attempt to improve the immunodiagnostic detection of mannan, most assays, like the Pastorex latex agglutination test, still lack sensitivity due to the rapid clearance of the antigen from patients' sera, especially when only a single serum sample is tested at the time of clinically suspected infection. For example, in the study by Sendid et al. (185), mannanemia was detected in 40% of patients from whom multiple serum samples were available and in 11% of patients from whom only one sample was available. Thus, repeat serum sampling is required in order to improve the sensitivity for detection of the *Candida* mannan antigen.

Currently, antigen detection tests are useful for the diagnosis of invasive candidiasis; however, all assays have certain limitations reflected by either sensitivity or specificity or both. Perhaps a combination of two assays may increase the accuracy of diagnosis of invasive candidiasis. Furthermore, repeated serum sampling may also improve the reliability of antigen detection tests for the diagnosis of candidiasis.

### Cryptococcosis

Culture of *C. neoformans* from body fluids is the traditional and definitive means of microbiologic diagnosis of cryptococcosis, but this method may require several days to detect and identify the organisms. Direct visualization of *C. neoformans* in body fluids by India ink preparation is rapid, but this method is relatively insensitive. Hence, a rapid and reliable test is required.

The detection of cryptococcal capsular polysaccharide antigen is one of the most valuable rapid serodiagnostic tests for fungi performed on a routine basis. The detection of crypto-

coccal antigen by latex agglutination based on latex particles coated with antibody raised against cryptococcal capsule has gained widespread appeal because of its ease of use and greater sensitivity compared to other conventional immunodiagnostic methods. Detection of cryptococcal capsular antigen by polyclonal IgG-based latex agglutination tests is also widely available. However, the type of variation reported for polyclonal serum-based latex bead assays may compromise routine usage (242). Furthermore, extra testing with latex control reagents or extra procedures is needed in order to identify potential false-positive results caused by interfering substances such as rheumatoid factor. Moreover, false-positive reactions have also been reported in patients with disseminated trichosporonosis, *Capnocytophaga canimorsus* septicemia, and malignancy (48, 216). False-negative reactions are occasionally caused by a prozone-like effect and can be corrected by dilution of the specimen or by pronase treatment (48).

A mouse monoclonal IgM-based latex agglutination assay, Murex Cryptococcus Test (Murex Diagnostics, Norcross, Ga.), effectively eliminates false-positive reactions with rheumatoid factor (59, 106). However, the Murex cryptococcus test has lower sensitivity than the rabbit polyclonal IgG-based cryptococcal antigen latex agglutination system (93). Another test format is an EIA, the PREMIER Cryptococcal antigen assay (Meridian Diagnostics, Inc.), utilizing a polyclonal capture system and a monoclonal detection system. The PREMIER EIA was as sensitive and specific as the latex agglutination system for the detection of capsular polysaccharide in serum and cerebrospinal fluid (CSF) (69, 203). The main advantages of PREMIER EIA over latex agglutination assay are that the PREMIER EIA (i) does not react with rheumatoid factor, (ii) can be run with a fairly large number of samples, and (iii) gives fewer false-positive reactions.

### Histoplasmosis

HPA can be detected in the blood and urine (Table 3), particularly in patients with disseminated histoplasmosis and in patients with severe pulmonary manifestations (77, 224, 228), as well as in the CSFs and bronchoalveolar lavage fluids of patients with disseminated histoplasmosis (223, 225, 226).

Detection of HPA by RIA in a reference laboratory is an established method for the diagnosis of histoplasmosis and monitoring the response to treatment (55, 227, 228, 251). How-

ever, the limitations of using RIA include (i) the requirement of radioactivity, which may not be adapted into a kit form easily, and (ii) the use of polyclonal antisera, which has shown interassay variability (224) as well as cross-reactivity with other dimorphic fungi such as *B. dermatitidis* (71, 222, 228, 251), *P. brasiliensis* (55, 222), and *P. marneffei* (222). Thus, a more specific detection system through the application of monoclonal antibody is likely to reduce the cross-reactivity with other dimorphic fungi and interassay variation (75, 77). Hence, Gomez et al. (75) raised a monoclonal antibody that recognized a species-specific epitope on a 69- to 70-kDa antigen of histoplasmosis by inhibition ELISA. The sensitivity of the inhibition ELISA using this monoclonal antibody was 71%, and the specificity was 98% with normal human sera from areas of endemicity (75). Moreover, it offered the advantages of monitoring clinical outcome and antigenemia level (77).

### Paracoccidioidomycosis

It has been known for some time that antigen, in the form of immune complexes, is present in patients with paracoccidioidomycosis (6, 38). Antigen detection tests may be more effective than antibody tests for diagnosing paracoccidioidomycosis, particularly in immunocompromised individuals and in those who have previously been exposed to *P. brasiliensis* (80). However, the detection of antigenemia in paracoccidioidomycosis patients also has some difficulties because (i) insensitive reagents and methodology were used and (ii) cross-reactions occurred in patients with histoplasmosis, aspergillosis, and cryptococcosis (66, 70, 181).

Recently, Gomez et al. (78) have developed an inhibition ELISA for the detection of circulating antigen with a monoclonal antibody P1B directed against an 87-kDa determinant of *P. brasiliensis* (78). Despite the high sensitivity (80.4%) and the usefulness in the follow-up of paracoccidioidomycosis patients (76), cross-reactions were observed with sera from patients with other mycoses, mainly aspergillosis and histoplasmosis (78). In addition, the inhibition ELISA did not prove helpful in the search for the 87-kDa antigen in urine (78). Other developments included the detection of protein components such as the 43-kDa glycoprotein (139, 160) and 70-kDa antigenic protein (30). The 43- and 70-kDa antigens can be detected separately or concurrently in urine samples by immunoblotting using rabbit antiserum raised against culture filtrate (183). The immunoblot method detected the two antigens separately or concurrently in specimens from 91.7% of 12 patients and did not detect them in specimens from patients with other diseases, from healthy individuals, or from other controls (183). The 43-kDa antigen remained present in urine samples during the treatment period; diminished reactivity occurred during clinical recovery, and increased reactivity occurred in samples during relapses (183). The detection of these antigens in urine appears to be a promising method for the diagnosis of paracoccidioidomycosis, monitoring the effects of treatment, and reducing the incidence of relapsing infection. However, the test format may be too cumbersome for use in the routine laboratory.

### Penicilliosis

Studies of antigen detection for the diagnosis of penicilliosis is still in the infancy period because *P. marneffei* infections have only become significant in the last 15 years. Nevertheless, Kaufman and coworkers (102) have recently developed immunodiffusion and latex agglutination tests using rabbit antiserum raised against a culture filtrate of fission arthroconidia of *P. marneffei* for diagnosis of penicilliosis. The immunodiffusion and latex agglutination tests detected circulating *P. marneffei* antigen in serum of patients with penicilliosis with sensitivities of 58.8 and 76.5%, respectively. Recently, a better sensitivity result has been reported by using a polyclonal antibody-based ELISA; 100% of 33 patients with penicilliosis were found to have antigen in their urine (53). However, false-positive results were found in undiluted urine samples obtained from healthy volunteers and patients with cryptococcosis, melioidosis, and other bacterial infections. Although progressive dilution of urine samples increased the specificity of the ELISA to 100%, the sensitivity of the test was reduced (53). Perhaps the incorporation of specific monoclonal antibodies may improve both the sensitivity and specificity of penicilliosis antigen detection.

Another antigenic target method that has been explored recently is the detection of specific mannoprotein for the diagnosis of penicilliosis. Cao et al. (35) cloned an MP1 gene encoding an antigenic cell wall mannoprotein (Mp1p) of *P. marneffei* (35) and developed an Mp1p antigen-based ELISA. The assay was specific for *P. marneffei* and detected the organism in 17 (65%) of 26 AIDS patients with penicilliosis (34). Six of the nine patients who were antigen test negative, including both immunocompetent penicilliosis patients, were antibody positive, suggesting that the Mp1p antigen may be removed more effectively in hosts with an intact immune system (34). Thus, the antigen test would be more useful for patients who have more of a compromised immune system while the antibody test may be more sensitive for patients who are immunocompetent or who have a better humoral immune system. Nonetheless, the Mp1p antigen-based ELISA offers certain advantages: (i) it is quantitative, which may be of value in the monitoring of antifungal therapy and may as well be important as a prognostic indicator because it may reflect both the fungal load and the host's ability to clear the fungal antigen, and (ii) the preparation of the antigen can be reproducible since it depends on the purification of a specific recombinant protein (34).

### Summary

Many researchers have shown that detecting fungal antigens is useful in the diagnosis of invasive fungal infections. However, not all antigen assays for invasive fungal infections provide sufficient sensitivity and/or specificity for routine use. The problems in the past included (i) the formation of immune complexes that are rapidly removed from circulation, causing the transient presence of antigen and thereby resulting in the insufficient sensitivity for detection of disease; (ii) the use of polyclonal antibody, resulting in the limited quantities of antiserum and variability from batch to batch, and (iii) the use of insensitive immunological methods, in which the detection of the lowest threshold is not possible. Moreover, some tests do



not indicate which fungal species is responsible for infection. To date, only the cryptococcal polysaccharide capsular antigen test is widely used in the routine laboratory worldwide, while the *Aspergillus* antigen detection test is available in Europe.

Detection of galactomannan by the Platelia *Aspergillus* assay for the diagnosis of invasive aspergillosis is promising; detection of the antigen has been shown to correlate with clinical diagnosis. However, positive results should always be confirmed to exclude false positives and, wherever possible, should be substantiated by additional clinical and/or other microbiological examinations. Antigen detection for the diagnosis of invasive candidiasis is more investigational. Although showing potential role as an early diagnostic test, negative results for *Candida* antigen are common in many patients with invasive infection due to the transient character of antigen circulation. Furthermore, detection of *Candida* antigens remains difficult as no sensitive or specific assays are available. RIA for the detection of HPA is an established method for diagnosing histoplasmosis and monitoring the response to treatment. However, the test may not be suitable for general laboratory usage. Hence, an inhibition ELISA has been developed and has comparable sensitivity and specificity to those of the RIA for the detection of antigen. It may be a reasonable alternative to the established RIA and offers a potential for wider availability of histoplasma antigen testing and development as a kit. Detection of *P. brasiliensis* circulating antigens in body fluids offers potential advantages over antibody detection, both for the initial diagnosis of paracoccidioidomycosis and the follow-up. Among different tests developed to detect circulating paracoccidioidal antigen, the inhibition ELISA is promising, but more studies are required to ascertain its usefulness and feasibility in antigen detection in the routine clinical laboratory. Studies of antigen detection for the diagnosis of penicilliosis are still in the infancy period, and this area of research remains largely unexplored.

In conclusion, our review of the results obtained in published studies indicates that several criteria should be met before an antigen test is ready for widespread use: (i) immune complexes must be dissociated before the detection of specific antigen such as galactomannan and mannan; (ii) reagents should be standardized and prepared in a reproducible manner (the use of monoclonal antibodies or polyvalent epitope-specific antibodies is likely to meet this criterion); (iii) a sensitive immunological method that can detect the lowest threshold should be used; (iv) methodology should be performed easily, should not be subject to inter- and intralaboratory variation, and should carry no risk to laboratory personnel; and (v) prospective clinical study is required. Such considerations must remain paramount in the future development of antigenic detection schemes.

#### DETECTION OF SPECIFIC NUCLEIC ACIDS

The increasing incidence of fungal infections in immunocompromised patients has focused attention on the rapid and accurate diagnosis of invasive fungal infections using molecular biological techniques. Nucleic acid hybridization and amplification methods are fundamental to molecular diagnosis. These methods have the potential to provide both high detection rates and identification of specific fungal pathogens, as the

latter becomes increasingly important with the widespread use of antifungal therapy and the problem of antifungal resistance. The use of molecular diagnostic tools to detect fungal specific nucleic acid sequences has recently been reviewed (171, 211, 216), and many researchers have reported the usefulness of DNA-based methods for the diagnosis of invasive fungal infections. However, most of the studies were performed in limited numbers of patients, and no large prospective clinical trials have yet been reported. Furthermore, several questions need to be addressed before the DNA-based method can be adapted to daily clinical routine; these include questions of (i) which DNA targets are best for commercial kits used in routine diagnostic laboratories, (ii) what are the optimal methods for extracting fungal DNA from clinical specimens obtained from various sites, and (iii) which detection methods are best for routine use.

#### Sample Preparation

Specimen preparation can have a significant impact on the sensitivity and reproducibility of a molecular diagnostic test. In general, the sample preparation method should release intracellular DNA from the fungal cell wall and/or thick capsule; it must concentrate DNA targets that may be present in very small amounts; and it must eliminate protein debris, contaminants, potential inhibitors, and other extraneous materials without degrading the target DNA. At present, there are many protocols for sample preparation, but no universal method for optimally extracting, purifying, and concentrating fungal DNA from clinical specimens is available. Nonetheless, fungal DNA has been extracted and purified from different clinical samples, including whole blood (22), serum and plasma (97, 103, 123, 243), bronchoalveolar lavage fluid (17, 138, 166, 202), and CSF (163). However, the efficiencies of the molecular diagnostic methods applied to different types of clinical samples might not be equivalent, as DNAs present in different clinical samples are probably different in origin. For example, one study showed that PCR was more often positive when serum was used for testing than when whole blood was used for testing of specimens from patients with invasive candidiasis (13).

#### Target Selection

Targets that have been used in molecular diagnostic tests for fungal infections include single and multicopy nuclear and mitochondrial genes (Tables 4, 5, and 6) and RNA. In general, molecular diagnostic methods targeting multicopy genes have better detection thresholds than those targeting single-copy genes (Table 7). Among multicopy genes, mitochondrial DNA has been used in the PCR-based detection of *C. albicans* (142) and *Aspergillus* species (17, 95). However, the variability of mitochondrial DNA among different strains may be a limiting factor. Others have targeted the multicopy ribosomal genes in order to maximize sensitivity and specificity. The ribosomal genes contain conserved sequences that are common to all fungi and also variable domains and highly variable internal transcribed spacer (ITS) regions. The conserved sequences can be used to screen for fungal infection, while the variable sequences can be exploited for species identification. Indeed, favorable results targeting this ribosomal gene as a tool for

TABLE 4. Nucleic acid detection in patients with *Candida* fungemia and/or histopathologic evidence of invasive candidiasis

Gene	Method <sup>a</sup>	Annealing temp (°C)	No. of cycles	Detection method	Sample	No. with positive reaction/total no. of subjects (%)		Reference
						Patients with proven invasive candidiasis	Controls	
Actin	sPCR	55	30	Hybridization with radiolabeled probe	Serum	11/14 (78.6)	0/29 (0)	97
Chitin synthase	sPCR	54	30	Southern blotting	Blood	15/16 (93.4)	0/34 (0)	96
P450 <sup>b</sup>	nPCR	55, 45	35, 35	Ethidium bromide staining	Serum	16/18 (88.9)	0/6 (0)	42
ITS	sPCR	55	40	EIA	Serum	28/28 (100.0)	3/31 (9.7)	27
18S rRNA	sPCR	62	35	Southern blotting	Blood	8/8 (100.0)	3/100 (3.0)	56
P450	sPCR	59	40	REA <sup>c</sup>	Blood	13/14 (92.9)	18/58 (31.0)	146

<sup>a</sup> Abbreviations: sPCR, standard PCR; nPCR, nested PCR.

<sup>b</sup> First and second values for annealing temperature and number of cycles were used in the first and second PCR, respectively.

<sup>c</sup> REA, restriction enzyme analysis.

fungal detection and identification have been obtained (Tables 4 and 5).

### Amplification and Detection Methods

Nucleic acid hybridization and amplification methods are fundamental to molecular diagnosis. Hybridization techniques employ a DNA probe to determine whether a particular organism is present. The probe is a single strand of DNA synthesized such that it corresponds with a recognized sequence in the DNA or RNA of the suspected infectious agent. In situ hybridization assays has been used effectively to localize the DNA and RNA of infectious agents in routinely processed tissues, and no DNA extraction is required (171). This technique has been reported for the identification of *Candida* spp. (120, 121), and *Aspergillus* spp. (145, 154). Although the entire procedure is rapid and easy to perform, the sensitivity of the assay is often lower than those of other molecular biological

assays, especially those including nucleic acid amplification (39, 87, 171).

PCR is the most frequently used amplification procedure because it can readily adapt to many applications. However, when a single-copy gene is used as the target for PCR, special amplification steps such as nested PCR are often necessary to achieve the necessary degree of sensitivity. For example, the sensitivity with nested PCR was 1,000 times higher than that of the single PCR for the detection of fungal infections (25, 42, 240). The PCR-generated product is usually analyzed by ethidium bromide-stained gel electrophoresis. Although gel electrophoresis is simple and inexpensive, it is much less sensitive than Southern blotting (63, 141, 159, 202).

Detection of PCR products by ethidium bromide staining and by Southern blotting is time-consuming, and the interpretation of results may be subjective. Hence, PCR-EIA—a three-part method which included PCR amplification, hybridization with the complementary labeled probe, and detection of reac-

TABLE 5. Nucleic acid detection in patients with histology and/or mycologic proven invasive aspergillosis

Gene	Method <sup>a</sup>	Annealing temp (°C)	No. of cycles	Detection method(s)	Sample <sup>b</sup>	No. with positive reaction/total no. of subjects (%)		Reference
						Patients with proven invasive aspergillosis	Controls <sup>c</sup>	
Alkaline protease	sPCR	63	42	EtBr <sup>d</sup> staining-Southern blotting	BAL fluid	4/4 (100.0)	6/46 (13.0)	202
26S ITS	sPCR	56	32	EtBr staining-Southern blotting	BAL fluid or other respiratory specimen	3/3 (100.0)	4/17 (23.5)	193
18S rRNA	sPCR	62	40	EtBr staining-Southern blotting	BAL fluid	4/4 (100.0)	0/14 (0)	138
5S rRNA	ISH				Tissue	12/12 (100.0)	0/18 (0)	145
Mitochondrial	cPCR	60	40	EtBr staining-hybridization	BAL fluid	3/3 (100.0)	12/49 (24.5)	17
18S rRNA	nPCR	50/65	30/30	EtBr staining-Southern blotting	Serum	14/20 (70.0)	0/20 (0)	243
Mitochondrial	cPCR	55	45	ELISA	BAL fluid	2/6 (33.3)	0/19 (0)	16
Mitochondrial	sPCR	58	40	ELISA	BAL fluid	3/3 (100.0)	0/57 (0)	95
18S rRNA	nPCR	50/65	30/30	EtBr staining	Serum	4/4 (100.0)		244
18S rRNA	sPCR	62	35	ELISA	Blood	7/7 (100.0)	0/10 (0)	126
18S rRNA	nPCR	57/60	40/30	EtBr staining	Serum	4/4 (100.0)		104
18S rRNA	nPCR	65/65	23/35	EtBr staining	Blood	6/6 (100.0)	1/188 (0.53)	186
18S rRNA	nPCR	65/65	23/35	EtBr staining	BAL fluid	6/6 (100.0)	4/188 (2.1)	186
Large rRNA	nPCR	58/58	31/31	EtBr staining	Serum	6/6 (100.0)	4/19 (21.1)	230

<sup>a</sup> Abbreviations: sPCR, standard PCR; cPCR, competitive PCR; nPCR, nested PCR; ISH, in situ hybridization.

<sup>b</sup> BAL, bronchoalveolar lavage.

<sup>c</sup> Included healthy subjects and/or colonized patients and/or patients with other infections.

<sup>d</sup> EtBr, ethidium bromide.

TABLE 6. Investigational molecular markers for detection of cryptococcosis and other endemic mycoses

Organism	Method used <sup>a</sup>	Detection method(s) <sup>b</sup>	Amplification target	Amplicon size(s) (bp)	Annealing temp (°C)	Total no. of cycles	Reference
<i>B. dermatitidis</i>	sPCR	Species-specific probe	28S rDNA		50	50	184
	sPCR	EtBr staining FCES	ITS	315	55	30	207
<i>C. immitis</i>	sPCR	Species-specific probe	28S rDNA		50	50	184
<i>C. neoformans</i>	nPCR	EtBr staining	rDNA	183	66	31	84
	sPCR	Species-specific probe	28S rDNA		50	50	184
	sPCR	SSCP analysis	18S rRNA	197	55	31	217
	sPCR	Southern blotting	18S rDNA	343	62	50	159
	nPCR	Southern blotting	URA5 gene	345 and 236	63	36	201
	nPCR <sup>c</sup>	EtBr staining	ITS	415	55, 55	20, 30	165
	sPCR	EtBr staining + FCES	ITS	315	55	30	207
	sPCR	Species-specific probe	28S rDNA		50	50	184
<i>H. capsulatum</i>	sPCR	EtBr staining + FCES	ITS	299	55	30	207
	sPCR	Species-specific probe	28S rDNA		50	50	184
<i>P. marneffei</i>	sPCR	EtBr staining + FCES	ITS	280–283	55	30	207
<i>Paracoccidioides</i> species	sPCR	Species-specific probe	28S rRNA	220			184

<sup>a</sup> Abbreviations: sPCR, standard PCR; nPCR, nested PCR.

<sup>b</sup> Abbreviations: EtBr, ethidium bromide; FCES, fluorescent capillary electrophoresis system; SSCP, single-strand conformational polymorphism.

<sup>c</sup> First and second values for annealing temperature and number of cycles were used in the first and second PCR, respectively.

tion products in an EIA that provides either a colorimetric or fluorescence readout—was developed (57, 63, 68, 95, 126). The sensitivity of PCR-EIA to detect candidemia and aspergillosis was higher than that by ethidium bromide staining (68). In addition, the PCR-EIA format provided further amplification without losing the species-specific binding associated with Southern blotting, multiple samples can be assayed in parallel, and semiquantitation of DNA is possible (27, 68).

The TaqMan PCR (Perkin-Elmer Corp., Applied Biosystems, Foster City, Calif.) is another approach that combines PCR, probe hybridization, and signal generation in one step (15, 171). The TaqMan probe consists of a reporter dye with a fluorescein derivative at the 5' end, a 3' quencher dye, and a 3' blocking phosphate group. The fluorescence emission of the reporter dye is suppressed in the intact probe by Förster-type energy transfer. During PCR, the probe is cleaved by the 5' nuclease activity of *Taq* polymerase only when it is hybridized to a complementary target. When probe-specific PCR probe has been generated, an increase in reporter dye fluorescence, resulting from the cleavage between the reporter and quencher, occurs. The amount of reporter dye released is pro-

portional to the amount of DNA amplified by PCR. The TaqMan fluorescence assay enables samples to be analyzed as soon as 5 to 10 min after PCR is complete, and no postamplification manipulation, which might reduce a significant source of laboratory contamination, is required (15). In addition, it was shown to be 10-fold more sensitive than detection by ethidium bromide-stained agarose gel (171). Although studies have shown that the TaqMan assay could detect isolates of *Candida* species and *Aspergillus fumigatus* (15, 171), further studies are required to confirm its usefulness in the clinical setting.

Recently, a quantitative PCR assay with the LightCycler (Roche Diagnostics, Mannheim, Germany) amplification and detection system was described (125). This technology combines rapid thermocycling with glass capillaries with online fluorescence detection of the PCR amplicon; cycling is achieved by alternating heated air and air of ambient temperature. The detection system is based on fluorescence resonance energy transfer with two different specific oligonucleotides: hybridization probe 1 is labeled with fluorescein, while the second hybridization probe is labeled with the fluorophore LightCycler Red 640. Both probes can hybridize in a head-to-

TABLE 7. Lower threshold of nucleic acid detection methods for diagnosis of invasive candidiasis

Target DNA <sup>a</sup>	Method used <sup>b</sup>	Detection technique <sup>c</sup>	Detection limit	Reference
Actin	sPCR	Hybridization with radiolabeled probe	0 cells/0.1 ml	97
HSP	sPCR	EtBr staining	100 CFU/ml	43
Mitochondrial DNA	sPCR	Southern blotting	3 cells/0.1 ml	141
rRNA	sPCR	REA	15 cells/100 µl	88
Chitin synthase	sPCR	Southern blotting	10 CFU/0.1 ml	96
P450	nPCR	EtBr staining	1 pg of DNA	42
P450	sPCR	Southern blotting	10–20 cells/0.1 ml	25
5S rRNA + NTS	sPCR	EtBr staining	15 CFU/ml	86
5.8S rRNA + ITS	sPCR	EIA	2 cells/0.2 ml	68
18S rRNA	sPCR	Southern blotting	10–15 CFU/0.1 ml	210
18S rRNA	sPCR	EthBr-Southern blotting	10–100 cells/ml	158
18S rRNA	ISH		2–3 cells/0.5 ml	120
18S rRNA	sPCR	Southern blotting	1 CFU/ml	56
18S rRNA	sPCR	ELISA	5 CFU/ml	126

<sup>a</sup> Abbreviations: NTS, nontranscribed spacer; HSP, heat shock protein.

<sup>b</sup> Abbreviations: sPCR, standard PCR; nPCR, nested PCR; ISH, in situ hybridization.

<sup>c</sup> Abbreviations: EtBr, ethidium bromide; REA, restriction enzyme analysis.

tail arrangement, bringing the two fluorescent dyes into close proximity. A transfer of energy between the two probes results in emission of red fluorescent light, which is measured by photohybrids, and the level of fluorescence is proportional to the amount of DNA generated during the PCR process. This technology is promising because (i) specific detection of *C. albicans* and *A. fumigatus* has been achieved, (ii) the fungal load in clinical samples can be determined, (iii) amplification and postamplification analyses are performed in closed glass capillaries, thus minimizing the risk of carryover contamination; and (iii) the whole amplification and detection process requires only 45 min (125).

Lastly, a method for amplifying *Aspergillus* RNA in blood samples that does not require temperature cycling has recently been described. This method was more sensitive than PCR for detecting *Aspergillus* 18S ribosomal sequences, but it has not yet been assessed in patients with proven presence or absence of invasive aspergillosis (124).

### Species Identification

Most molecular diagnostic methods are able to screen patients in the initial stages of fungal infection, but not all protocols can identify the source of the DNA to the genus or species level. For example, 18 different species of fungus were detected by a PCR method employing a universal primer that amplified a highly conserved region in the 18S ribosomal DNA (rDNA) (158), but this method cannot differentiate among these species. However, the subsequent probing of the amplicons with species-specific probes discriminated among individual fungal pathogens to species level (56, 126, 184). Although these methods appear promising in the field of diagnostics, the use of species-specific probes is not always an efficient approach in mycology, given the large number of potentially pathogenic fungi. Others have reported the use of restriction fragment length polymorphism by enzyme digestion of PCR product. This approach was able to classify five broad groups of fungi: *Candida* spp., *Cryptococcus*, *Aspergillus* and clinically related septate molds, zygomycetes, and dimorphic fungi (88). Another approach is the use of single-strand conformational polymorphism to delineate the difference between fungal species and/or genera (217). This technique included PCR amplification of a conserved region of the 18S rRNA with further separation of genus and species on the basis of exploiting small but phylogenetically important base pair differences among medically important fungi (217). Minor sequence variations in highly conserved DNA segment will cause subtle changes in conformation that forms in short-strand DNA changes after they are denatured. This conformationally different fragment can then be separated electrophoretically under nondenaturing conditions.

### Diagnostic Considerations

The detection and identification of fungal pathogens by DNA-based methods can yield results sooner than cultivation (57, 84, 159). Furthermore, in experimentally infected animals and patients, the sensitivity can be higher than those of cultures and serologic tests for diagnosing invasive fungal infections (27, 42, 56, 95, 146, 159, 210, 240). DNA results have

correlated with clinical improvement and effect of treatment, and these have been demonstrated in patients with invasive aspergillosis (56, 126, 244), invasive candidiasis (56), and cryptococcal meningitis (165). Although the disappearance of fungal DNA from the blood of patients correlated with successful therapy, no experimental data are available to explain these aspects of therapy. Further evaluation is necessary for the DNA detection in monitoring of invasive fungal infections with animal models.

Molecular diagnostic methods may not distinguish individuals who are colonized from those who are infected (17, 138, 148, 202). For example, false-positive results of 31% and ranging from 8 to 38% have been reported in the diagnosis of candidiasis (147) and aspergillosis (17, 138, 193, 202). The most likely explanations of false-positive results are the colonization of the patients' airway by conidia and/or hyphae and the contamination of sample and/or reaction buffers by environmental fungi (122). Adoption of rigorous working practices and appropriate decontamination procedures may help to control the risk of contamination. In addition, avoiding specimens that are more susceptible to aerocontamination (e.g., bronchoalveolar lavage fluid) may reduce the chance of false positivity (104, 243). False-negative results were reported by Kan (97) in 21% of patients with positive blood cultures for *Candida* species. This could be due to the low sensitivity of a method designed to detect a single-copy gene.

### Summary

DNA-based diagnostic tests have the potential to reduce the time for laboratory identification of pathogens that are slow growing or difficult to culture. Highly sensitive and specific detection of fungal pathogens was demonstrated in a limited number of patients, but no prospective comparison of different sensitive methods for the detection of fungal DNA is available. Simplification and/or standardization methods for DNA extraction and DNA detection will facilitate introduction of molecular technology to routine clinical mycology laboratories. Other technical problems that need to be solved include the risk of contamination and the inability to distinguish colonized individuals from infected patients. Nonetheless, the approach of using primers that target multicopy genes is likely to provide the high degrees of sensitivity that are needed for initial diagnosis of serious fungal infection. It is not yet clear whether methods that target DNA sequences that are shared by many different fungal species or those that are specific to one or a few pathogens will be most useful in clinical practice.

### DETECTION OF DISTINCTIVE FUNGAL METABOLITES

An entirely different approach to diagnosing an infectious disease is to demonstrate a distinctive microbial metabolic product in the body fluids of an infected host. The feasibility of this approach is illustrated by the demonstration of several fungal metabolites in the body fluids or tissues of infected or colonized hosts. Examples include (i) ethanol in CSF samples from humans with *C. neoformans* meningitis (208), (ii) aflatoxin B1 in urine specimens from frogs in which *Aspergillus flavus* mycelial mats had been implanted intraperitoneally (19), and (iii) oxalic acid crystals in the lung of a human with an

*Aspergillus* fungus ball (112). Although these studies established that fungal metabolic products can accumulate and be detected in host body fluids or tissues, none of these fungal products is a useful diagnostic marker.

#### D-Arabinitol in *Candida* Infections

The first microbial metabolite to be used as a practical diagnostic marker was the five-carbon acyclic polyol D-arabinitol (40). In 1979 and 1980, three groups independently found that (i) *C. albicans* produced large amounts of D-arabinitol in culture, (ii) animals and/or humans with invasive *Candida* infections had higher serum arabinitol concentrations than did uninfected or colonized controls, and (iii) humans with abnormal renal function had higher serum arabinitol concentrations than did those with normal renal function, whether or not invasive *Candida* infection was present (105, 180, 231).

Although these results were promising, they did not establish the usefulness of arabinitol as a diagnostic marker for candidiasis, because it was not yet known (i) how frequently clinical isolates of the medically important *Candida* species produce substantial amounts of arabinitol, (ii) how renal dysfunction influences serum arabinitol concentrations, (iii) whether the excess arabinitol observed in serum is derived from microbial or host metabolism, and (iv) whether colonization by D-arabinitol-producing *Candida* species could also cause increased serum arabinitol levels. Therefore, Bernard et al. (9) showed that all strains of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *Candida pseudotropicalis* tested produced large amounts of D-arabinitol in culture, whereas no strain of *C. glabrata*, *C. krusei*, or *C. neoformans* tested produced D-arabinitol. Next, Wong et al. (233) showed that D-arabinitol is excreted into the urine quantitatively at a rate equal to the creatinine clearance rate. Since urinary creatinine excretion is usually constant, these results imply that the ratios of arabinitol concentration to creatinine concentration in serum or urine (i) are proportional to the rate at which arabinitol appears in the body from any source and (ii) can be used to correct for differences in renal function. In addition, Wong et al. (234) showed that (i) the serum and urinary arabinitol/creatinine ratios in rats with invasive *C. albicans* infection rose directly in proportion to total arabinitol appearance and to *C. albicans* colony counts in the kidneys and (ii) the amounts of excess arabinitol demonstrated in the infected rats were similar to the amounts produced when the same *C. albicans* strain was cultured in vitro. Lastly, Wong et al. (237) showed that animals that were heavily colonized with *C. albicans* had no more arabinitol in their serum or urine than did uncolonized controls. Taken together, these results implied that the excess arabinitol observed in the body fluids in invasive candidiasis was produced by *C. albicans* and that arabinitol is a quantitative diagnostic marker for invasive candidiasis.

Gold et al. (74) measured serum arabinitol concentrations and serum arabinitol/creatinine ratios in 25 cancer patients with histologically proven invasive candidiasis and in 88 uninfected controls with a variety of underlying neoplastic diseases and/or renal failure. The serum arabinitol/creatinine ratios in the uninfected controls with normal renal function were indistinguishable from the values obtained for the uninfected controls with abnormal renal function, thereby verifying that ara-

binitol/creatinine ratios can be used to correct for differences in renal function. Moreover, among the 25 infected patients, 16 (64%) had serum arabinitol/creatinine ratios at least 2 standard deviations above the mean value for the controls, compared to 12 (48%) with at least one positive blood culture.

Since *Candida* produces D-arabinitol (9, 105) and since known mammalian metabolic pathways produce only L-arabinitol (206), one way in which the diagnostic sensitivity and specificity of body fluid arabinitol measurements might be improved is to differentiate fungal D-arabinitol from host L-arabinitol. The results obtained with several different enantioselective analytical methods supported the hypothesis that measuring D-arabinitol selectively yielded better results than measuring total arabinitol nonselectively (10, 178, 179, 236, 239).

Unfortunately, the enantioselective methods used in these early studies were cumbersome and time-consuming, or they required costly instrumentation that is seldom available in hospital laboratories. Therefore, two more practical approaches have been explored. The first is to quantify D-arabinitol enzymatically, using NAD-dependent D-arabinitol dehydrogenase (ArDH) from *Enterobacter aerogenes* (190–192), *C. tropicalis* (197), or *C. albicans* (247). A major advantage of the methods that use *E. aerogenes* ArDH is that test kits are available commercially in Japan (Nacalai Tesque, Kyoto, Japan). However, mannitol is an alternative substrate for *E. aerogenes* ArDH and is often present in normal human serum (177). A colorimetric assay that uses *C. tropicalis* ArDH and a clinical chemistry autoanalyzer to quantify D-arabinitol in serum is highly specific for D-arabinitol (162, 197), and this assay has recently been improved by (i) using fluorometric detection to increase its speed and sensitivity and (ii) substituting recombinant *C. albicans* ArDH for native *C. tropicalis* ArDH, which insures the availability of large amounts of the key reagent from a non-pathogenic source (247).

A second approach is to use gas chromatography (GC) with a chiral stationary phase to determine the ratio of D-arabinitol to L-arabinitol (D-/L-arabinitol ratio) in serum and/or urine. Although this method requires analytical instrumentation that is not available in hospital laboratories (combined GC-mass spectrometry [GC-MS]), it is not necessary to determine the absolute concentrations of D- and L-arabinitol in order to determine the D-/L-arabinitol ratios. Therefore, serum and urine samples dried on filter papers can be sent to a central laboratory with the necessary instrumentation and expertise (176). One problem with this approach is that any factor that influences serum and/or urine L-arabinitol concentrations must also influence the D-/L-arabinitol ratios, and there have been no detailed studies of the means by which L-arabinitol is distributed, metabolized, or excreted in animals or humans.

Table 8 summarizes the results of several clinical studies that have evaluated D-arabinitol as a diagnostic marker for candidiasis. The early studies in which serum arabinitol concentrations were determined by GC and GC-MS (105, 180), a study in which serum arabinitol/creatinine ratios were determined by GC (74), studies that used enzymatic assays to determine serum D-arabinitol/creatinine ratios (67, 204, 205, 219) and studies that used GC-MS to determine the serum or urine D-/L-arabinitol ratios (41, 117, 179) all support the usefulness of D-arabinitol, both as an initial diagnostic marker and for mon-

TABLE 8. Detection of arabinitol in body fluids of patients with *Candidia* fungemic and/or histopathologic evidence of invasive candidiasis

Detection method(s)	Specimen	Expressed results	Cutoff value	No. positive for arabinitol/total no. of cases (%)		Reference
				Patients	Controls <sup>a</sup>	
GLC	Serum	Arabinitol	1 µg/ml	15/20 (75)	3/93 (3.2)	105
GC-MS	Serum	Arabinitol	1.2 µg/ml (8 µM)	9/11 (82)	0/6 (0)	180
GLC	Serum	Arabinitol/Cr	1.51 µg/ml/mg/dl	16/25 (64)	3/88 (3.4)	74
GC-MS	Serum	D-Arabinitol/L-arabinitol	2.2	10/12 (83)		117
Enzymatic fluorometric assay	Serum	D-Arabinitol/Cr	1.5 µmol/mg	29/58 (50)	10/151 (6.6)	67
Enzymatic fluorometric assay	Urine	D-Arabinitol/Cr	1.4 µmol/mg	9/11 (82)	4/43 (9.3)	205
Enzymatic chromogenic assay	Serum	D-Arabinitol/Cr	4.0 µmol/liter/mg/dl	31/42 (74) <sup>d</sup>	28/206 (14)	219
				25/30 (83) <sup>e</sup>		
GC-MS	Urine	D-Arabinitol/L-arabinitol	4.0	15/17 (88)	4/94 (4.3)	117
GC	Urine	D-Arabinitol/L-arabinitol	4.6	10/10 (100)	4/67 (6.0)	41

<sup>a</sup> Included healthy subjects and/or colonized patients and/or patients with other infections.

<sup>b</sup> GLC, gas-liquid chromatography.

<sup>c</sup> Cr, creatinine.

<sup>d</sup> Fungemia.

<sup>e</sup> Persistent fungemia.

itoring responses to therapy. However, other investigators have reported elevated serum arabinitol levels in conditions other than candidiasis or renal failure (52, 98), or they did not find high serum arabinitol levels in animals or humans with candidiasis (51, 52, 58, 136). Some of these results can be explained by analytical artifacts (235), others could not be reproduced when they were examined in greater detail (232, 237), and some unfavorable results remain unexplained. Nevertheless, it is clear from the largest studies in which well-validated analytical methods were used that D-arabinitol is a useful diagnostic marker for invasive candidiasis. Indeed, the results obtained in the large study by Walsh et al. (219) are among the best reported to date in any large prospective evaluation of a non-culture diagnostic method for invasive candidiasis.

### Mannitol in *Aspergillus* and *Cryptococcus* Infections

The six-carbon acyclic polyol mannitol is produced in large amounts by many different fungi (149, 150, 206). Moreover, healthy humans have low serum mannitol concentrations (177), and mannitol is eliminated by urinary excretion at a rate equal to the glomerular filtration rate. Since these properties all contribute to the usefulness of D-arabinitol as a diagnostic marker, mannitol has also been examined as a potential diagnostic marker for mannitol-producing pathogenic fungi. Initial studies established that multiple clinical isolates of *A. fumigatus*, *A. flavus*, and *Aspergillus niger* (B. Wong, unpublished observations) and of *C. neoformans* (240) all produced large amounts of mannitol in culture. Moreover, two groups have shown that animals with experimental *A. fumigatus* infections had higher levels of mannitol in body fluid and/or tissue than did uninfected controls (64, 238), and one group has shown that animals with experimental cryptococcal meningitis had higher levels of mannitol in CSF than did uninfected controls (240).

To our knowledge, there have been no published studies that have evaluated mannitol as a diagnostic marker for aspergillosis in humans. In unpublished studies, we found that a few patients with biopsy- or autopsy-proven invasive aspergillosis had markedly elevated serum mannitol concentrations and

mannitol/creatinine ratios, but most did not. Moreover, unexpectedly high serum mannitol levels were also observed in a small proportion of healthy controls (B. Wong, unpublished observations). Also, Megson et al. (137) measured CSF mannitol concentrations in 21 AIDS patients with cryptococcal meningitis. Although many of these patients had substantially higher CSF mannitol concentrations than the normal values reported by others (177), this study did not include any uninfected controls, and there was no correlation between the concentrations of mannitol and cryptococcal polysaccharide antigen in CSF. Thus, despite the fact that animal studies have shown that two medically important fungi produce large amounts of mannitol in infected mammalian tissues, available data do not support the usefulness of mannitol as a diagnostic marker for aspergillosis or cryptococcosis.

### Summary

The development of D-arabinitol as a diagnostic marker for invasive candidiasis demonstrates the feasibility of this general approach and some key properties of a useful diagnostic marker. If a microbial metabolic product is to be used as a diagnostic marker, it should be produced in large amounts by the microbial species of interest both in culture and in infected mammalian tissues, it should be present in trace amounts at most in the body fluids of uninfected controls, and the means by which the compound is eliminated from the body should permit rates of microbial production to be deduced from concentrations in body fluid. If these criteria are met, a sensitive and accurate method for detecting and quantifying the metabolite of interest is required, and the necessary methodology should ideally be available in hospital laboratories. It has been known since the late 1980s that D-arabinitol is a useful diagnostic marker for invasive candidiasis, but the unavailability of practical methods for detecting and quantifying small amounts of D-arabinitol in body fluids limited the availability of this diagnostic approach. Therefore, the recent development of the filter paper method for determining D-/L-arabinitol ratios and an autoanalyzer method in which a recombinant microbial enzyme is the key reagent may expand the availability of this

valuable diagnostic approach and may also encourage investigation of the diagnostic value of other distinctive metabolites that might serve as markers for other infections that are difficult or impossible to diagnose using standard methods.

### CONCLUSIONS

The incidence of invasive fungal infections has increased dramatically in recent years. Early and specific diagnosis is vital, and the decision to treat a patient with invasive fungal infections is based mainly on clinical and mycological information. However, the traditional methods used in routine practice for the diagnosis of invasive fungal infections may be insensitive and somewhat nonspecific. Based on a greater understanding of the pathogenesis of fungal infection and virulence determinants of fungal pathogens, new approaches to diagnose invasive fungal infections are being developed.

Clearly, the diagnosis of fungal infections has moved forward in the past few years, and some of these newly developed methods are likely to accomplish earlier detection of the infections. However, unfavorable results, in terms of sensitivity and specificity, have been reported. Moreover, the results available from most of the individual tests reviewed here are not adequate by themselves to guide decisions about when to initiate antifungal therapy, which antifungal drug or drugs to use, or when therapy is to be stopped. In addition, these newly developed methods may be too cumbersome for routine usage. Hence, further fine-tuning of these methods is needed to simplify their use and to improve their sensitivity and/or specificity so that they will be valuable in guiding clinical treatment decisions.

Despite that fact that traditional methods may be insensitive and/or nonspecific, they are not entirely useless. To date, they are the best methods that are available in the routine clinical laboratory. Hence, it is likely that maximum information will be available by combining both traditional culture and morphological methods with one or more of the newer diagnostic modalities reviewed here. Unfortunately, few studies have examined this multimodality approach prospectively and rigorously. Prospective studies of combined modalities, culture and morphology, and nonculture methods should clarify the role of these tests in the clinical study setting.

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