Diagnostic Strategies for Invasive Fungal Infections in Patients With Hematologic Malignancies and Hematopoietic Stem Cell Transplant Recipients

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Abstract
Invasive fungal infections (IFIs) frequently occur and are associated with high morbidity and mortality in patients with hematologic malignancies (HMs) and hematopoietic stem cell transplant (HSCT) recipients. Early diagnosis of IFI in these patients facilitates prompt institution of therapy and leads to improved clinical outcomes. This article reviews widely used methodologies for diagnosing IFIs in patients with HM and HSCT recipients. Advantages and limitations of radiologic studies; microbiologic and histopathologic techniques; fungal biomarker assays, including those for galactomannan antigen and β-(1-3)-D-glucan; and molecular assays that are available to establish an early diagnosis of clinically relevant invasive fungal infections are discussed. Recommendations are provided regarding effective use of these methodologies in clinical practice. (JNCCN 2013;11:941–949)

Because of prolonged neutropenia and severe immunosuppression, patients with hematologic malignancies (HMs) and hematopoietic stem cell transplant (HSCT) recipients are at high risk for invasive fungal infections (IFIs), which are associated with substantial mortality in patients with HMs and are the leading cause of infectious mortality in HSCT recipients. Early and accurate diagnosis of IFI can guide the most optimal treatment and lead to improved clinical outcomes.

Candida spp and Aspergillus spp are the most common fungal pathogens in HMs and HSCT recipients and are the subject of this article. Candida is a commensal organism, constituting part of the normal flora of the gastrointestinal tract, residing on oral and gut mucosal surfaces and skin. The most common portal of entry is through breaches in mucosa or through the skin. Locally invasive mucosal infections and fungemia are the most common Candida syndromes observed in this patient population. Less commonly, hepatosplenic candidiasis may occur. In contrast, Aspergillus is an airborne organism, and the usual portal of entry is the nasal passages and respiratory tract. Pneumonia is the most common Aspergillus clinical syndrome, with sinusitis, orbital cellulitis, and disseminated infection less commonly seen.

Candida infections of the mucosa occur early and throughout the course of neutropenia and/or immunosuppressive therapy. Candida fungemia is rarely the cause of first neutropenic fever, but may be a cause of persistent or recurrent neutropenic fevers; it is not responsive to empiric antibiotics and is typically seen late during the first or second week of neutropenia or later. In contrast, invasive aspergillosis (IA) typically develops beyond 2 weeks of neutropenia in patients with acute leukemia, particularly those with antecedent cytopenias or iron overload. For HSCT recipients, the highest risk for IA occurs within 100 days after HSCT, particularly in patients with graft-versus-host disease (GVHD) requiring administration of high doses of systemic steroids. Widespread use of azole prophylaxis has led to a substantial decline in the incidence of invasive candidiasis. IA and other invasive mold infections have now emerged as the major type of IFI in patients with HMs and HSCT recipients.
Diagnostic Approaches
Early diagnosis of IFI is often challenging and requires careful consideration of clinical, radiologic, and laboratory parameters. The EORTC/Invasive Fungal Infections Cooperative Group and the Mycoses Study Group defined 3 categories of IFI: proven, probable, and possible, which are based on diagnostic certainty. Proven IFI requires microbiologic and/or histopathologic confirmation. However, microbiologic and histopathologic techniques have suboptimal sensitivity and specificity, are time-consuming, and frequently require invasive approaches to obtain tissue for diagnosis. To minimize these shortcomings, current diagnostic algorithms of IFIs rely more on radiology and antigen and molecular testing of clinical samples. In many cases, these newer methodologies can be performed with less-invasive means, yet allow timely and accurate diagnosis of IFI, often without the need for more invasive procedures to obtain tissue samples for microbiologic and/or histopathologic confirmation.

Radiologic Studies
Radiology does not have a substantive role in the diagnosis of most Candida syndromes, except for hepatosplenic candidiasis. Characteristic nodular hypodensities, especially with a bull’s eye appearance, in the liver and/or spleen should lead to a strong suspicion for hepatosplenic candidiasis. Histopathologically, these lesions are composed principally of inflammatory cells and are not abscesses. Accordingly, the syndrome is often silent (and the lesions not apparent) during deep neutropenia, and may first manifest at the time of neutrophil recovery.

In contrast, radiologic imaging is important for diagnosing IA. Although chest radiography (CXR) is often used because of its ease of performance, its diagnostic accuracy is suboptimal in patients with neutropenia. Therefore, CT of the chest is preferred, because CXR can be normal in up to 10% of patients with pulmonary IA, whereas only 3% of chest CT scans are falsely negative. High-resolution CT scans have a sensitivity of greater than 85%, a high negative predictive value of greater than 85%, and an average time gain of 5 days compared with CXR, and the information provided often leads to changes in clinical management. The pulmonary abnormalities caused by IA are generally nodular. In most patients with IA, lung nodules are at least 1 cm in diameter, multiple, bilateral, and often peripherally located. More-specific CT imaging signs of IA include a “halo sign,” wherein the central nodular area of fungal invasion is surrounded by a ground glass–appearing hemorrhage or a crescent sign. The halo sign is seen early in the course of IA, and develops in nearly two-thirds of patients. Initiation of anti-mold therapy at the time of a halo sign is associated with improved survival. Consolidation, caviess, and air crescent signs are also seen later. In appropriate clinical settings and with supporting microbiology, radiologic abnormalities, such as a dense, well-circumscribed nodule; an air crescent sign; or a cavity, are used as components of the diagnostic criteria for IA. Recent studies note that other types of infiltrates are also frequently seen with IA, including localized air space consolidation and tree-in-bud and ground glass opacities in an airway-invasive form of pulmonary aspergillosis (as contrasted with the more classic angioinvasive form). The radiologic abnormalities of pulmonary mucormycosis are similar to those of IA; however, the presence of concomitant sinusitis, multiple (>10) nodules, pleural effusion, and a history of prior voriconazole prophylaxis are more frequently present in mucormycosis than IA.

Serial CT scans can also be useful to monitor the response to antifungal therapy. It can take several weeks or even months for radiologic pulmonary abnormalities related to IFI to fully resolve, and in patients with neutropenia they typically worsen during the first 7 to 10 days of therapy, but subsequently improve with continued antifungal treatment. Clinicians should also be aware that parenchymal infiltrates and clinical symptoms may appear or get worse during the recovery from severe neutropenia or withdrawal of immunosuppressive therapy, even in the case of microbiological response, because of enhancement of a neutrophil-driven inflammatory response at the sites of tissue damage by fungal pathogens (also known as immune reconstitution syndrome). Discrepancies in radiologic and clinical responses can be also seen if superimposed infection by viral, bacterial, or another mold pathogen is present. In these situations, monitoring with serial serologic markers is helpful in identifying microbiologically responding patients with worsening clinical symptoms. Besides pulmonary abnormalities,
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Besides pulmonary IA, in patients with pulmonary IA, 39% of cases and BAL does not increase the yield. Invasive fungal infections, or liver or spleen abscesses in appropriate clinical context should alert the clinician to the possible diagnosis of IFI.5

**Microbiologic and Histopathologic Techniques**

Several relevant fungal pathogens can be isolated from peripheral blood, but the sensitivity of fungal blood cultures is low. For example, blood cultures remain negative in half of patients with disseminated candidiasis.20 Blood cultures are practically never positive in disseminated aspergillosis or mucormycosis.21 In patients with pulmonary IA, Aspergillus can be isolated from sputum, but in only 8% to 34% of cases; the diagnostic yield is increased to 45% to 62% when bronchoalveolar lavage (BAL) is performed and biomarker assays are performed on BAL samples (discussed later).22–24 In pulmonary mucormycosis, sputum cultures are positive in only 25% of cases and BAL does not increase the yield.25 The possibility of colonization by environmental fungi (such as Aspergillus spp) may lead to overdiagnosis and the risk of a false-positive diagnosis, particularly when upper respiratory secretions are used for diagnostic purposes. Identification of fungal elements invading tissue with associated inflammation or necrosis is considered to be a gold standard. Tissue biopsies of affected visceral organs typically do not produce false-positive results; however, these biopsies are frequently difficult to obtain because of the critical condition of the patient and the increased risk of bleeding because of concomitant severe thrombocytopenia, and false-negative results still occur because of inadequate sampling. In the presence of cutaneous involvement, punch biopsy of skin lesions often results in identification of the fungal pathogen, but fungal staining is important.

Visualization of yeasts or hyphae in tissue through microscopic examination should be followed by a culture to identify a specific organism, because morphologic identification alone is not sufficiently specific. Aspergillus is characterized by narrow, acute angle branching septated hypha, and in culture growth is visible within 3 days if the inoculum size is adequate. The hyphae of Fusarium in tissue look similar to those of Aspergillus. In tissue, the agents of mucormycosis have wider, ribbon-like, aseptate or pauci-septate hyphae, and these features help differentiate this pathogen from Aspergillus spp. Obtaining positive cultures from tissues infected by molds, particularly mucormycosis, is challenging given the difficulty of extracting viable fungi from infected tissues.26

**Fungal Biomarker Assays**

Because of the limitations of microbiologic techniques, noncultural methods such as galactomannan (GM) antigen; β-(1-3)-D-glucan (BDG) antigen; and PCR-based assays have been introduced,27–29 and the serum GM assay has been evaluated in prospective studies. Both of these tests now are accepted as important diagnostic tests for the early detection of IFIs in patients with HMs and HSCT recipients.5

GM is a major component of the cell wall of Aspergillus spp and is released in the bloodstream during rapid growth of hyphae invading tissue. In patients with IA, serum GM positivity often precedes the development of clinical signs, radiologic abnormalities, or fungal growth in culture by up to a week.27 Besides Aspergillus spp, the serum GM assay also detects Penicillium spp, which rarely causes IFIs in humans in the Western Hemisphere. The validity of serum GM antigen monitoring has been evaluated in several retrospective and prospective studies with a wide range of reported sensitivities depending on the study population and definitions of the test positivity.27,30–38 In patients with HMs and HSCT recipients, the cutoff value of the serum GM antigen index has been established at 0.5 based on receiver operator characteristic analyses.39 In a meta-analysis, the GM assay in patients with HMs had a pooled specificity of 70% and sensitivity of 92%, and in HSCT recipients a pooled specificity of 86% and sensitivity of 82%.40 These estimates of sensitivity and specificity are based on serial testing of serum GM; the value of a single GM test result in patients with suspected IA is less accurate. Thus, the serum GM assay should be performed twice weekly prospectively in patients at high risk for IFI.13,41 The risk period for monitoring would correspond to neutropenia for patients with acute leukemia and the first 100 days after HSCT, or longer in the presence of persistent GVHD.

The serum GM assay also can provide prognostic utility. Very high GM levels at the beginning of antifungal therapy are associated with poorer
prognosis than those with lower levels of positivity.\textsuperscript{42} Patients with IA whose GM tests become negative during the first 2 weeks of therapy have better survival compared with those with persistent GM positive tests.\textsuperscript{43}

Although the biomarker tests offer great promise, they have limitations of both false-positive and false-negative test results. The accuracy of the GM assay can be affected by simultaneous use of piperacillin-tazobactam\textsuperscript{44,45} and amoxicillin-clavulanate\textsuperscript{46} because of the presence of trace GM in these antibiotic formulations, and the GM assay may remain falsely positive up to 5 days after discontinuation of these antibiotics. However, recent studies indicate fewer problems with false positivity with these antibiotics, presumably because of changes in manufacturing.\textsuperscript{47,48} The GM assay can also be falsely positive during an infection with \textit{Histoplasma capsulatum}\textsuperscript{49} or \textit{Blastomyces dermatitidis}\textsuperscript{50} because these microorganisms share cross-reactive antigens. Alternatively, the use of antifungal prophylaxis with agents having anti-mold activity could lead to falsely negative GM tests.\textsuperscript{31}

In addition to serum, GM can be detected in other body fluids that come in contact with infected tissues.\textsuperscript{51} The utility of the GM assay has been best studied in BAL samples. In patients with proven or probable IA, BAL GM has a high sensitivity of 90\% to 94\% and specificity of 79\% to 94\%,\textsuperscript{23,52} which exceeds the sensitivity and specificity of culture and microscopy\textsuperscript{23} and serum GM.\textsuperscript{24,53} This is important because historically bronchoscopy has

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\caption{Suggested diagnostic algorithm for evaluation of suspected pulmonary IFI in patients with HMs and HSCT recipients. Abbreviations: BAL, bronchoalveolar lavage; GM, galactomannan; GMI, galactomannan index; HMs, hematologic malignancies; HSCT, hematopoietic stem cell transplant; IFI, invasive fungal infection.}
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a poor diagnostic yield for pulmonary IA, and the addition of transbronchial biopsy does not significantly increase the diagnostic yield but substantially increases the risk of complications, such as for bleeding or pneumothorax. However, studies indicate that the diagnostic yield of bronchoscopy can be increased from 22% to 30% up to 45% to 54% if the GM test is performed on BAL fluid.23,24 In the authors’ experience, one-third of patients who underwent bronchoscopy for suspected IA had the diagnosis confirmed by the BAL GM, despite negativity of all other mycological tests, including the serum GM.24 BAL and serum testing for Aspergillus biomarkers provide complementary information. Infection in some patients is detected through the serum GM and not BAL GM, and vice versa; sometimes GM is detected in both serum and BAL. This should not be surprising, because some forms of IA are angioinvasive, with GM principally secreted into the bloodstream, whereas others are airway, with GM principally secreted into respiratory secretions.

In contrast to the GM assay, the BDG assay detects the presence of numerous fungal pathogens, including Candida spp, Aspergillus spp, Pneumocystis spp, and Fusarium spp, with acceptable specificity and high sensitivity. However, like the GM assay, the BDG cannot detect the various agents of Mucormycosis spp or Cryptococcus spp.29 The strength of the BDG assay is the ability to detect a variety of the most common relevant fungal pathogens in patients with HMs and HSCT recipients; its weakness is its inability to discriminate which pathogen is detected. A recent meta-analysis, which included patients with HMs, showed that 2 consecutive positive BDG test results have the sensitivity of 50% and specificity of 99%, with positive and negative predictive values of 84% and 95%, respectively.54

**Molecular Techniques**

Molecular techniques most widely studied to date are primarily PCR assays for Aspergillus-specific DNA sequences or consensus sequences for a variety of fungal pathogens. A meta-analysis of PCR assays for IA showed that the sensitivity varied from 0.75 to 0.87 and the specificity varied from 0.87 to 0.75 depending on whether 1 or 2 consecutive positive samples were required, respectively.55 The data suggested that a single PCR-negative result could be sufficient to exclude a diagnosis of IA; however, 2 positive tests are required to best confirm the diagnosis. Despite good prospects for PCR assays, molecular techniques are not yet available for clinical use because of numerous methodological issues, particularly widespread environmental fungal contamination, difficulties in DNA extraction, and lack of standardization.56 PCR results are subject to significant variation depending on the choice of primers, DNA extraction techniques, and testing conditions, which adversely affect the reliability of test performance in different laboratories. Attempts to standardize PCR techniques for IA are

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**Figure 2** Suggested diagnostic algorithm for evaluation of suspected sinus or orbital IFI in patients with HMs and HSCT recipients.

*Chest CT is recommended if suspicion of IFI is high.

Abbreviations: HMs, hematologic malignancies; HSCT, hematopoietic stem cell transplant; IFI, invasive fungal infection.
now underway in Europe, and the expectations are that new molecular diagnostic techniques for several fungal pathogens will be available in upcoming years.

**Practical Considerations and Conclusions**

Early therapy for IFI improves outcomes. Therefore, when IFI is suspected, antifungal therapy should be promptly initiated and not delayed until a definitive diagnosis of IFI is made. Alongside initiating presumptive therapy, diagnostic assessment should be aggressively pursued, with appropriate use of imaging, microbiology, and serologic studies. Figures 1 and 2 offer a suggested diagnostic pathway. Because bacterial and fungal respiratory infections cannot be conclusively distinguished through imaging alone, antibiotics to cover gram-positive and gram-negative pathogens should also be started in acutely ill patients with HMs and HSCT recipients while diagnostic assessment proceeds. Once the diagnostic assessment is completed, adjustments to therapy should be made, and if bacterial causes are excluded, antibiotics can be discontinued. Clinicians must clearly understand the capabilities and limitations of each diagnostic approach for IFIs to appropriately use these methodologies (Table 1).

The authors recommend using the new noninvasive biomarker diagnostic methods with serial monitoring along with clinical information to guide further evaluation and treatment decisions in patients suspected of IFI. The high negative predictive value of the biomarker assays makes it appropriate to withhold antifungal treatment in high-risk patients with persistent febrile neutropenia alone if they are receiving *Candida* prophylaxis, lack biomarker positivity, and have no clinical signs or im-

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Abbreviations: BAL, bronchoalveolar lavage; BDG, β-(1-3)-D-glucan; CXR, chest radiography; GM, galactomannan; HMs, hematologic malignancies; HSCT, hematopoietic stem cell transplant; IA, invasive aspergillosis; IFI, invasive fungal infection; PCR, polymerase chain reaction.
aging abnormalities suggestive of IFI. However, any high-risk patient with a nodular pulmonary infiltrate should be presumptively treated for mold IFI while definitive diagnostic assessment is being performed. The authors recommend bronchoscopic examination for testing of BAL samples for multiple pathogens and to include testing for GM. Addition of BAL GM testing dramatically improves the yield of bronchoscopy for diagnosis of IA and in patients with HMs and HSCT recipients with nodular pulmonary infiltrates. The authors do not ordinarily perform a lung biopsy. However, for sites other than lungs, tissue is often required for diagnosis if the serum markers are negative. Endoscopic examination of the sinuses with aspiration and biopsy, biopsy of dermal or subcutaneous lesions, or other invasive biopsy techniques should be considered as appropriate to establish or exclude the diagnosis of IFI and identify copathogens to guide treatment decisions, because radiologic findings can suggest but not establish the cause. However, even if invasive approaches fail to establish the diagnosis of IFI, in patients in whom strong clinical suspicion exists and relevant imaging abnormalities are seen, presumptive antifungal therapy should be considered for continuation, in the authors’ view if another cause cannot be established, given the diagnostic limitations of the microscopic examination and cultures, until a definitive diagnosis is established (Table 1).

Because of significant technical difficulties and disadvantages of invasive tissue biopsies and limitations of fungal microbiologic and histopathologic techniques, further development and clinical use of noninvasive assays have a great promise for improving the accuracy and promptness of IFI diagnosis in patients with HMs and HSCT. In the future, the hope is that molecular diagnostic techniques will help in diagnosing IFIs. The simultaneous use of different types of methodologies can offer complementary information to increase diagnostic accuracy, but is associated with higher costs and may require extra expertise, which may limit their use by clinical laboratories.

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