Early Clinical and Laboratory Diagnosis of Invasive Pulmonary, Extrapulmonary, and Disseminated Mucormycosis (Zygomycosis)

Thomas J. Walsh,1 Maria N. Gamaletsou,1,5 Michael R. McGinnis,2 Randall T. Hayden,4 and Dimitrios P. Kontoyiannis3

1Transplantation-Oncology Infectious Diseases Program, Weill Cornell University Medical Center, New York, New York; 2Department of Pathology, University of Texas at Galveston Medical Center, and 3Division of Internal Medicine, University of Texas MD Anderson Cancer Center, Houston; 4Department of Pathology, St Jude Children's Research Hospital, Memphis, Tennessee; and 5Department of Medicine, University of Athens, School of Medicine, Athens, Greece

Early diagnosis of invasive mucormycosis is important for timely therapeutic intervention, improved survival, and reduced morbidity. Given the importance of an accurate and rapid diagnosis of invasive mucormycosis to guide the timely initiation of amphotericin B and possible surgical intervention, a coordinated multidisciplinary approach of clinical assessment, diagnostic imaging, and laboratory assessment is necessary. Laboratory assessment for mucormycosis includes the conventional methods of direct examination and culture of tissue, respiratory secretions, bronchoalveolar lavage fluid, and other fluids. However, because conventional diagnostic tools are limited in their sensitivity, advanced molecular amplification systems, antigen detection assays, proteomic profiles, and metabolite detection may complement existing approaches to improve the rate of early diagnosis of invasive mucormycosis.

PRINCIPLES OF DIAGNOSIS OF MUCORMYCOSIS

Invasive mucormycosis causes severe morbidity and mortality in hematopoietic stem cell transplant and solid organ transplant recipients and in patients with hematological malignancies, diabetes mellitus, burns, trauma, and low birth weight [1–3]. Timely initiation of treatment improves the outcome of invasive mycoses. Similarly, early treatment is predicated on early diagnosis. Early diagnosis of mucormycosis is important, and prompt therapeutic intervention may prevent progressive tissue invasion and its sequelae (Table 1). These sequelae include (1) angioinvasion and direct tissue injury of the respiratory tract, (2) direct extension from lungs into the great vessels, (3) invasion from the paranasal sinuses into the orbit and brain, and (4) hematogenous dissemination to central nervous system tissues. Early diagnosis may also reduce the need for or extent of surgical resection, disfigurement, and suffering. Finally, early diagnosis may improve outcome and survival. Supporting the importance of early diagnosis of invasive mucormycosis, Chamilos and colleagues demonstrated the impact of delayed amphotericin B–based therapy on outcome among 70 consecutive patients with hematological malignancy and mucormycosis [4]. Delayed therapy resulted in a 2-fold increase in mortality at 12 weeks, compared with early treatment (82.9% vs 48.9%). Moreover, delayed treatment of invasive mucormycosis was an independent predictor of poor outcome in multivariate analysis (odds ratio, 8.1 [95% confidence interval, 1.7–38.2]; P = .008).

IMPROVING EARLY DIAGNOSIS OF INVASIVE MUCORMYCOSIS

If early initiation of antifungal therapy is beneficial in the outcome of invasive mucormycosis, then development of improved diagnostic methods should become an
important focus for study. This can be achieved by the recognition of host factors, careful assessment of clinical manifestations, early use of computed tomography (CT) and magnetic resonance imaging modalities, expert evaluation of histological and cytological preparations, optimal use of clinical microbiological methods, and implementation of advances in molecular detection.

Recognition of host factors contributes significantly to the assessment of a patient’s risk for invasive mucormycosis. Among the classic host factors are diabetic ketoacidosis, prolonged glucocorticosteroid therapy, and persistent neutropenia. However, additional insights attained over the last decade underscore the expanding population at risk for invasive mucormycosis, such as patients with type 2 diabetes, solid organ and hematopoietic stem cell transplant recipients, patients with autoimmune disorders, neonates, users of illicit intravenous drugs, and patients with burns, trauma, or surgical wounds. Patients with graft-vs-host disease are at high risk of mucormycosis because of the immunosuppression needed to control graft-vs-host disease and the inherent immunodysregulation associated with this process. Concomitant exposure to voriconazole prophylaxis in immunosuppressed patients with hematological malignancy or in bone marrow transplant recipients is associated with a significantly increased risk of invasive mucormycosis [5]. This is particularly true in those with diabetes and malnutrition who develop pulmonary infiltrates or sinusitis.

No clinical history is completely specific for the diagnosis of invasive mucormycosis. However, there are several examples of initial clinical manifestations, which, in the proper host, carry a potentially high predictive value. Diplopia in a diabetic patient, particularly one who may be hyperglycemic or ketoacidotic, usually signifies involvement of the extraocular muscles of the nerves innervating the extraocular muscles. Hyperglycemia in diabetic patients may also produce blurring of vision but typically does not produce diplopia. Invasive mucormycosis, particularly involving the ethmoid sinus, may breach the lamina papyracea and invade the medial rectus muscle or it may extend along the emissary veins to the ethmoid and cavernous sinuses, encroaching on cranial nerves III, IV, V (1, 2), and VI. Thus, diplopia in a diabetic patient should be evaluated aggressively for possible invasive mucormycosis. A necrotic eschar in maxillary, facial, or sino-orbital tissues in an immunocompromised host may also be an early sentinel marker of invasive mucormycosis. Pleuritic pain in a neutropenic host may signify an angioinvasive filamentous fungus, the most common of which may be *Aspergillus fumigatus*. However, invasive mucormycosis may also be associated with pleuritic pain. Other clinical manifestations that may provide early recognition of invasive mucormycosis are discussed in greater depth in the article by Petrikkos et al in this supplement [6].

Necrotic cutaneous lesions in immunocompromised patients may also be caused by mucormycosis. Most cases of cutaneous mucormycosis are associated with direct inoculation rather than hematogenous dissemination [1]. The differential diagnosis includes infections caused by other angioinvasive pathogens, such as *Aspergillus*, *Fusarium*, *Pseudallescheria*, and *Scedosporium* species. *Pseudomonas aeruginosa* and occasionally Enterobacteriaceae organisms also cause ecthyma gangrenosum that may resemble cutaneous mucormycosis. A biopsy and wet mount of tissue may reveal characteristic hyphal structures.

As a broader measure of awareness in the medical community, early recognition of host factors and clinical manifestations can be improved through adequate training and education, including publications, symposia, and specialized fellowships. Expanding the number of clinical laboratory directors and microbiology technologists, together with increasing the numbers of physicians with specialty training in clinical mycology, will probably strengthen institutional expertise in the diagnosis of mucormycosis.

### EXPANDING ROLE OF DIAGNOSTIC IMAGING

Early detection of pulmonary or sinal lesions by CT has been a key advance over conventional sinus and chest radiographs. One should be aware that in neutropenic patients the development of pulmonary infiltrates already signifies tissue injury, angioinvasion, thrombosis, necrosis, hemorrhage, and edema. For immunocompromised patients, who are at high risk for invasive pulmonary mucormycosis, early CT findings may reveal pulmonary or sinal lesions, in the absence of radiological findings in conventional radiographs, even before localizing symptoms. An often overlooked limitation of serial or screening CT includes expense and cumulative radiation exposure associated with the scan. The radiation exposure associated with serial CT includes expense and cumulative radiation exposure associated with the scan. The radiation exposure associated with serial CT includes expense and cumulative radiation exposure associated with the scan. The radiation exposure associated with serial CT includes expense and cumulative radiation exposure associated with the scan [7].

CT scans are able to detect lesions that are more characteristically associated with angioinvasive filamentous fungi. Such lesions include nodules, halo signs, reverse halo signs, cavities, wedge-shaped infiltrates, and pleural effusions associated with pleuritic pain. These lesions are characteristically correlated with

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<td>Prevention of angioinvasion</td>
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<td>Prevention of direct tissue injury of lung, brain, and sinuses</td>
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<td>Prevention of extension into critical sites: eyes, brain, great vessels</td>
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<td>Prevention of progression to dissemination</td>
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<td>Reduced need for disfiguring surgery</td>
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angioinvasive organisms, including *Aspergillus* spp., *Scedosporium* spp., *Fusarium* spp., Mucorales organisms, and *P. aeruginosa*. Furthermore, invasive pulmonary mucormycosis may be associated with a wide array of early manifestations, including bronchopneumonia, particularly in nonneutropenic, immunocompromised patients. Additionally, lesions of pulmonary mucormycosis may vary over the course of time and can include early small nodules, which may become confluent to form a bronchopneumonia; more-discrete solitary pulmonary nodules; and cavitation with pleural effusion. Although the recognition of risk factors, clinical manifestations, and radiological findings may increase the probability of an early recognition, a definitive diagnosis must be established by culture and biopsy.

**DIRECT EXAMINATION OF INFECTED TISSUE, BRONCHOALVEOLAR LAVAGE FLUID, AND OTHER STERILE FLUIDS**

The symptoms, signs, and radiographic manifestations of mucormycosis are nonspecific, and a definitive diagnosis requires direct identification of the characteristic hyphae or recovery of the organism in culture from specimens obtained from the site of infection (Table 2). Direct examination of sputum, paranasal sinus secretions, or bronchoalveolar lavage (BAL) fluid is frequently nondiagnostic, but isolation of Mucorales organisms from such specimens in a susceptible host with corresponding clinical manifestations should be considered a priori as compelling evidence for infection [8, 9]. Establishing a diagnosis of pulmonary mucormycosis may also reveal concomitant infections caused by other organisms, including *Aspergillus* spp. and other fungal pathogens [10].

Samples for direct microscopy by wet mount, cytopathological, or histopathological examination may be collected by radiographically guided percutaneous needle aspirate and by transbronchial or direct biopsies of lesions. Although histopathological examination is specific and reliably establishes the diagnosis of mucormycosis in most cases in which characteristic hyphae are observed, obtaining biopsy material from deep tissue sites is frequently difficult in patients with thrombocytopenia or coagulopathy.

Direct microscopic examination is performed on all materials sent to the clinical laboratory. When possible, BAL fluid and sterile body fluids should be submitted for examination by clinical microbiology and cytopathology laboratories. Hyphae of Mucorales organisms are typically broad (diameter, 6–16 μm), ribbonlike, and irregularly shaped, nonseptate (coenocytic), or sparsely septate, with branches often arising nondichotomously at “right angles.” The hyphae may be difficult to observe on an unenhanced potassium hydroxide wet mount and may not stain well with conventional Gram stain. The use of chitin-binding stains, such as calcofluor, Fungi-Fluor, or blancofluor, may be used with a fluorescent microscope to identify hyphal elements on potassium hydroxide wet mounts [11]. McDermott and colleagues recently reported the use of calcofluor-stained tissue as a rapid technique for intraoperative diagnosis and assessment of clean resected margins in lieu of frozen sections by pathology [12].

Mucorales organisms are usually morphologically distinguishable from other filamentous fungi, such as *Aspergillus* spp., *Fusarium* spp., and *Pseudallescheria boydii*, which typically appear as slender dichotomously branching septate hyphae [13]. Distinction by direct examination may allow amphoterin B treatment and other potentially life-saving therapeutic interventions to be initiated. One should note, however, that in some cases (particularly where mold-active therapy has commenced before biopsy), morphological features may be atypical, reducing the ability to definitively differentiate Mucorales species from other filamentous fungi.

The histological detection of Mucorales organisms in tissue and their interpretation may be difficult. These organisms are typically difficult to observe on hematoxylin-eosin stains. On the other hand, periodic acid–Schiff and Gomori methenamine silver stains may be used for a fully characterized appearance of the organism. Unfortunately, only fragments may be seen, even with the use of cell wall staining. Therefore, the use of immunohistochemical stains or the possibility of fluorescent and in situ hybridization or in situ polymerase chain reaction (PCR) may also be used to characterize and distinguish genera within the order of Mucorales. Hayden and colleagues developed and applied in situ hybridization for characterization filamentous

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**Table 2. Laboratory Methods for Detection and Diagnosis of Mucormycosis**

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<thead>
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<th>Method</th>
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<td>Direct examination</td>
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<td>Cytopathological examination</td>
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<td>Gomori methenamine silver stain</td>
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<td>Histopathological examination</td>
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<td>Immunohistochemistry analysis</td>
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<td>Antigen detection</td>
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<td>Molecular methods</td>
<td>Direct sequencing of cultured organism or formalin-fixed tissue</td>
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<td></td>
<td>Fluorescent in situ hybridization</td>
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<td>Quantitative PCR of blood, BAL fluid, or tissue</td>
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Abbreviations: BAL, bronchoalveolar lavage; PCR, polymerase chain reaction.
fungi [14]. This molecular diagnostic approach may ultimately improve the rapid and early detection of mucormycosis, but it, among other methods, such as immunohistochemistry, remains investigational at this time. Further work on optimization, standardization, and validation of these assays for use in tissue, cytological preparations, and BAL is warranted. Antigen assays for diagnosis of mucormycosis are investigational but undergoing active development.

**CULTURE OF MEDICALLY IMPORTANT MUCORALES**

Identification of Zygomycetes organisms to the genus and species level continues to rest on colonial and microscopic morphology. Most medically important Zygomycetes are thermotolerant and are able to grow rapidly at temperatures of ≥37°C. Zygomycetes species grow rapidly on virtually any carbohydrate substrate. They are commonly found in soil and in decaying organic matter, including food items such as fruits, bread, and seeds.

Members of the order Mucorales are identified to the genus or species level according to colonial morphology, microscopic morphology, and growth temperature. Microscopic characterization of nonseptate hyphae, rhizoids, columellae, sporangia, and sporangiospores help to define genus and species within the order Mucorales [15, 16]. Because a detailed taxonomic description of Mucorales organisms is beyond the scope of this chapter, the reader is referred to a more in-depth review by Dr June Kwon-Chung in this supplement [17].

*Rhizopus oryzae* is the most commonly reported single species [1]. Other medically important *Rhizopus* species include *Rhizopus rhizopodiformis* and *Rhizopus microsporus*. After the genus *Rhizopus*, the genus *Mucor* in this study was the second most commonly reported. *Rhizopus* spp. and *Mucor* spp. are then followed by *Cunninghamella bertholletiae*, *Apophysomyces elegans*, and *Lichtheimia corymbifera* (formerly *Absidia corymbifera* and *Mycoladus corymbifera*) in frequency of reporting.

Identification of Mucorales organisms to the genus or species level carries valuable epidemiological, therapeutic, and prognostic implications [18]. For example, *R. oryzae* is the most common zygomycete recovered from clinical specimens but tends to exhibit in vitro resistance to posaconazole; *Mucor circinelloides* is less commonly isolated but shows greater susceptibility to posaconazole. *Cunninghamella* tends to have higher minimum inhibitory concentrations to amphotericin B and a higher associated overall mortality. *A. elegans* is associated with traumatic inoculation, especially in arid climates [19].

To optimize growth, clinical specimens should be inoculated onto appropriate media, such as Sabouraud glucose agar, and incubated at room temperature and 37°C. Grinding or homogenization of tissue specimens may destroy the delicate hyphae, rendering culture results negative. Recovery in culture is enhanced if tissue is sliced or minced into small pieces before inoculation onto media. Close collaboration between clinicians and the microbiology laboratory is essential to ensure proper handling of the specimen. Although Mucorales species are angiinvasive, blood culture results are rarely positive, unless there is luminal involvement of a vascular catheter. Zygomycetes organisms are better recovered in culture when incubated at 37°C than at 25°C [20]. Antifungal susceptibility of Mucorales species can be performed rapidly with reproducible minimum inhibitory concentrations at 8-12 hours, as measured by the XTT assay, that parallel results at 24 hours obtained by Clinical and Laboratory Standards Institute methods [21].

Colonies typically appear within 24-48 hours unless residual antifungal agents, such as amphotericin B, are present, which can suppress growth. The colonial appearance and growth pattern in culture help distinguish Mucorales from Entomophthorales organisms. Most mucoraceous species fill a culture dish within 3-5 days and demonstrate a grayish-white, aerial mycelium with a wooly texture. The colonies readily separate from the agar surface. By comparison, 3-5 days after inoculation with Entomophthorales organisms, the colonies are gray to pale yellow in color, flat and waxy, adhere to the plate surface, and often have radial groves. They are currently no biochemical, nucleic acid or serological means to aid in species determination in routine operations of clinical microbiology laboratories.

**MOLECULAR DIAGNOSIS OF MUCORMYCOSIS**

Although direct examination by wet mount and classical culture techniques are the standard methods for detection of Zygomycetes species in clinical microbiology laboratories, recovery of these organisms from BAL, fluid and tissues can be difficult. Fungal elements may not be abundant and, depending on the representative region of tissue sampled, organisms causing the infection may not be seen. Moreover, aggressive tissue grinding may render the fragile, coenocytic organisms nonviable. Many suspected infections are not confirmed until postmortem examination. Additionally, discrimination between the histological features of Zygomycetes organisms and those of other filamentous fungi may not be clear.

Given the increase in the number of these infections in recent years, molecular approaches for the detection of the medically important Mucorales species may increase the sensitivity and rapid diagnosis, thus, resulting in earlier, directed therapy. Studies have shown that molecular identification of zygomycosis is accurate, using the internal transcribed spacer (ITS) region as a first-line sequencing target for the identification of Zygomycetes organisms in pure culture [22]. Recently, in a patient with chronic myeloid leukemia and bone marrow transplant *R. oryzae* was identified from sinus cultures by sequencing of the ITS of...
the ribosomal RNA gene establishing the diagnosis of invasive mucormycosis [23].

Our laboratory has developed 2 real-time, quantitative PCR (qPCR) assays, targeting the 28S ribosomal RNA gene, for the diagnosis of zygomycosis caused by *Rhizopus*, *Mucor*, *Rhizomucor*, and *Cunninghamella* species [24]. The amplicons of the first qPCR assay (qPCR-1) from *Rhizopus*, *Mucor*, and *Rhizomucor* species were distinguished through melt curve analysis. The second qPCR assay (qPCR-2) detected *Cunninghamella* species, using a different primer-probe set. For both assays, analytic sensitivity for detection of hyphal elements from germinating sporangiospores in BAL fluid and lung tissue homogenates from rabbits was 1–10 sporangiospores/mL. Four unique and clinically applicable models of invasive pulmonary mucormycosis served as surrogates of human infections, facilitating validation of these assays for potential diagnostic utility. For qPCR-1, 5 of 98 infarcted lung specimens were positive by qPCR and negative by quantitative culture. None were positive only at quantitative culture. Among 23 BAL fluid samples, all were positive by qPCR, and 22 were positive by quantitative culture. qPCR-1 demonstrated *Rhizopus* and *Mucor* DNA in 20 (39%) of 51 serial plasma samples as early as day 1 after inoculation. Similar properties were observed for qPCR-2, which showed greater sensitivity than quantitative culture in BAL fluid (100% vs 67%; \(P = .04; n = 15\)). The assay detected *Cunninghamella* DNA in 58% of serial plasma samples as early as day 1 after inoculation. As these qPCR assays are sensitive and specific for detection of *Rhizopus*, *Mucor*, *Rhizomucor*, and *Cunninghamella* species in clinically applicable animal models, they may be applicable for the study and detection of invasive mucormycosis in patients. PCR-restriction fragment length polymorphism–based methods targeting the 18S ribosomal gene of Zygomycetes organisms on DNA extracted from human specimens also may provide clinicians with a rapid and definitive diagnosis of mucormycosis [25].

A 2-PCR assay system in paraffin wax embedded tissue samples targeting the 18S ribosomal DNA of Zygomycetes species was found to support the histopathological diagnosis of zygomycosis. Of note, *Aspergillus fumigatus* DNA was identified from one case that was histopathologically diagnosed as zygomycosis [26]. A recent study of the interlaboratory reproducibility of molecular diagnostics of *R. oryzae*, *R. microsporus*, *L. corymbifera*, *Rhizomucor pusillus*, and *Mucor circinelloides* in formalin-fixed paraffin-embedded kidney and brain tissues from mice demonstrated a reproducibility of 100% in the ITS sequencing for the identification of Zygomycetes species. After PCR amplification, the ITS1 region was amplified with the fungal universal primers ITS1 and ITS2 and subsequently sequenced. Notably, 93% of the specimens infected by *M. cirrinielloides* yielded negative results; thus, this species was excluded from the study [27]. In a recent report of pulmonary invasive mucormycosis and aspergillosis in a 66-year-old renal transplant recipient, *A. fumigatus* was initially identified with cytological examination and culture of BAL, whereas *R. microsporus* was subsequently identified by culture from a pulmonary tissue specimen. PCR performed on deparaffinized tissue sections, using specific primers for detection of aspergillosis and mucormycosis, confirmed the findings from histological analysis of lung tissue of both aspergillosis and mucormycosis. [28]. Molecular diagnosis of invasive mucormycosis is feasible and warrants further study of standardization, broader spectrum primers, and carefully designed clinical studies.

The early diagnosis of mucormycosis has become increasingly important, as disease incidence has grown and new treatment modalities have become available. To a large extent, diagnostic options remain limited to clinical and radiographic findings, together with staining and culture. Although these methods are tremendously useful, they are limited in sensitivity, time to detection, and ability to provide rapid results. Newer molecular diagnostic techniques, notably in situ hybridization and PCR, show promise in providing rapid and increasingly sensitive results, potentially improving the outcome in this critically ill patient population.

Notes

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