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Then and Now: Present Perspective Developments in Diagnosis of Mucormycosis

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Abstract:

Mucormycosis is a life-threatening condition, caused by a group of filamentous Molds belong to order Mucorales and class Zygomycetes. This invasive fungal infection has gained significant attention due to its increasing incidence with COVID 19. Early diagnosis is crucial for timely initiation of appropriate treatment and improving patient outcomes. This review summarizes recent advances in the diagnosis of mucormycosis, focusing on clinical manifestations, imaging techniques, laboratory tests, and emerging diagnostic modalities. Additionally, challenges and future directions in mucormycosis diagnosis are discussed.

Keywords:

mucormycosis; epidemiology, clinical diagnosis; advanced diagnostics, treatment

INTRODUCTION:

Mucormycosis is an uncommon, life-threatening, invasive fungal infection with high mortality and morbidity. *Mucormycetes* belong to the order Mucorales and the subphylum *Mucoromycotina*. As saprotrophs, microscopic arthropods occur in a variety of environments, where they reside in soil and on decaying materials. [1] *Mucormycosis* is primarily caused by inhalation of fungal sporangiospores or direct inoculation into the skin or gastrointestinal tract mucosa, with most infections occurring between August and November.[3, 7]

The predisposing factors for mucormycosis in patients with hematologic cancer are similar to those encountered in other opportunistic mold infections, such as aspergillosis, including profound and

protracted neutropenia and monocytopenia, chronic high-dose corticosteroids, reactivation of opportunistic herpes viruses (especially cytomegalovirus), severe graft-versus-host disease and its treatment, active hematologic malignancy and its associated functional neutropenia, as well as high-risk SCT transplantation. In addition, uncontrolled hyperglycemia, frank diabetes mellitus and/or diabetic ketoacidosis, and iron overload. [2, 5]

The disease was first described in 1876 when Furbinger described in Germany a patient who died of cancer and in whom the right lung showed a hemorrhagic infarct with fungal hyphae and a few sporangia. The first instance of disseminated mucormycosis was reported by Arnold Paltauf in 1885, and he named it "*Mycosis mucorina*." The appearance of sporangiophores and rhizoid-like structures in his illustrations of the etiologic agents led to the conclusion that *Lichtheimia corymbifera* was likely the source of the infection.[7]

The incidence of *mucormycosis* has increased recently, especially in India, as a consequence of the COVID-19 pandemic. The number of cases documented worldwide has increased within the past few decades. [4]

Depending on the clinical presentation, it is classified as rhinocerebral, pulmonary, cutaneous, gastrointestinal, disseminated, or other, which includes uncommon, rare forms such as endocarditis, osteomyelitis, peritonitis, renal, etc.

Mucormycosis is becoming more common, yet it's still hard to diagnose. *Mucormycosis* and other frequent invasive mold diseases, like *aspergillosis*, are easily misdiagnosed radiographically and clinically. For diagnosis, histopathology is the "gold standard." It is not conceivable to determine species with histopathologic identification of Mucorales in tissue specimens, as this requires a high level of pathological expertise. [8]

Molecular assays can be used either for detection or identification of *mucormycetes*, and they can be recommended as valuable add-on tools that complement conventional diagnostic procedures. [7]

This fungal infection has a higher mortality rate, especially in untreated or delayed treatment conditions. Among the survivors, vision loss followed by facial deformity is one of the frequently encountered consequences of *mucormycosis*. [3]

Clinical presentation:

Based on anatomical localization, mucormycosis can be divided into 6 forms: (a) Rhino-orbital-cerebral mucormycosis, (b) pulmonary, (c) cutaneous, (d) gastrointestinal, (e) disseminated; and (f) miscellaneous mucormycosis. [3]

In rhino cerebral mucormycosis, facial pain, headache, and brownish-blackish/blood-tinged nasal discharge is observed, whereas, in conditions with extension to the surroundings, it presents as palatal ulcer (Fig. 1), proptosis, periorbital swelling, and orbital pain. CNS involvement primarily affects the frontal lobe and cerebellum, where cranial nerve palsy, localized brain abscess, and orbital apex syndrome can be observed later. [3,4]

In pulmonary *mucormycosis,* the patient usually complains of fever, cough with brown-coloured sputum, chest pain, breathlessness, and hemoptysis. Clinical signs of cutaneous *mucormycosis* might range from non-healing wounds with necrotic margins to pustules or vesicles. On the surface of the lesion, a growth that resembles cotton sometimes appears (hairy pus).

In Gastrointestinal mucormycosis, the stomach is the primarily affected site, where ulceration of gastric mucosa with associated blood vessel thrombosis occurs. [3]

In Hematologic patients, lung is the most prevalent site of infection. Other common sites include the paranasal sinuses, the brain, skin, digestive tract, or disseminated disease with more than one affected site. [2]

Diagnosis:

Conventional diagnostic tools

- Clinical diagnosis:

Clinical diagnosis of *mucormycosis* can be challenging due to the low sensitivity and specificity of this diagnosis. The hallmark of *mucormycosis* is tissue necrosis, which results from angioinvasion and thrombosis. However, after the disease has progressed to an advanced level, it helps raise suspicion, start laboratory testing, and reveal clinical indicators of the condition. [9]
The primary manifestations of *mucormycosis* are dermal, respiratory, and rhino cerebral *mucormycosis*, with clinical signs including oral ulceration, black lesions on the bridge of nose, nasal discharge containing blood, paranasal sinus infection, perforations in the palate, paraesthesia, and facial cellulitis. These symptoms can overlap with other systemic disorders such as invasive *aspergillosis, fusariosis, nocardiosis*, Wegener granulomatosis, and other malignancies, making clinical diagnosis a non-specific procedure.[9]
In patients with diabetes and sinusitis, a "red flag" algorithm for diagnosing and treating rhino-orbito-cerebral *mucormycosis* is proposed by Corzo-Leon et al., which includes cranial nerve palsy, diplopia, sinus pain, proptosis, periorbital swelling, orbital apex syndrome, or palatine ulcers. Finding these signs should prompt immediate further testing, including blood tests, imaging, ocular and/or sinus surgery, endoscopic revision, and initiation of antifungal treatment.[10]
Pulmonary mucormycosis most often occurs in neutropenic patients, with clinical features that cannot be easily distinguished from those of pulmonary aspergillosis or fusariosis. In countries where

tuberculosis is endemic, the two infections may coexist. Prolonged fever, nonproductive cough, haemoptysis, pleuritic chest pain, and dyspnoea are common symptoms [9,10]

● Histopathology staining

A definitive diagnosis of *mucormycosis* relies on detecting fungal hyphae in biopsies or bronchoalveolar lavage (BAL). Histopathology plays a crucial role, distinguishing the fungus as a pathogen and determining blood vessel invasion. Mucorales genera produce distinctive wide, thin-walled, ribbon-like hyphae with right-angle branching, unlike other fungi. Hematoxylin and eosin (H&E) staining commonly used for histopathology reveals inflammatory responses against fungal pathogens but may fail to detect sparse fungal presence and can be challenging to distinguish from small blood vessels. [6] (Fig. 2)

Stains like Grocott methenamine-silver (GMS) and periodic acid-Schiff (PAS) stains highlight fungal walls. PAS staining detects fungal cell wall carbohydrates, oxidizing them into aldehydes, while GMS staining reveals dark, black-stained fungal cell walls. However, GMS staining may overstain, obscuring internal structures, and it's less effective in distinguishing old or non-viable fungi compared to PAS staining. [6, 11]

● Direct microscopy

Direct microscopy of clinical specimens, particularly with optical brighteners like Blankophor and Calcofluor White, is a cost-effective and valuable method for diagnosing *mucormycosis*. *Mucorales hyphae* exhibit variable width, irregular ribbon-like appearance, and wide-angle branching under microscopy. Although this method is recommended by reputable medical mycology organizations, it cannot identify fungi to the genus level. [3]

Potassium hydroxide (KOH) wet mount is another rapid presumptive test for fungal infections. *Mucorales hyphae* in KOH wet mount display coenocytic broad aseptate/sparsely septate hyphae with right-angle branching, resembling a ribbon-like appearance. It's crucial to distinguish *Mucorales from Aspergillus hyphae*, which also cause similar conditions. *Aspergillus hyphae* appear as thin, septate, regular acute angle dichotomous branched hyaline hyphae in clinical samples. [3, 6]

Calcofluor White (CFW) stain is a non-specific fluorochrome dye used to diagnose fungal pathogens in clinical samples. It specifically binds to the β 1 3, β1 4 glycoside chain of chitin in the fungal cell wall. When examined under a fluorescent microscope with UV light, fungal pathogens appear as apple green or bluish against a white background. However, caution is needed to differentiate fungal elements from other substances like cotton fibers, which also fluoresce bluish due to the staining of cellulose. [3, 6, 11]

● Culture:

Mucormycosis is primarily caused by the fungus Rhizopus arrhizus (R. oryzae), a thermotolerant organism capable of rapid growth at 37°C on various substrates. Diagnosis is based on colonial and microscopic morphology, as well as growth temperature. [3]

Sabouraud's Dextrose agar (SDA) and potato dextrose agar (PDA) are commonly used for cultivating Mucorales, with rapid growth typically observed at 25°C within 72 hours, appearing as cottony fluffy growth. Standard mycological procedures, including colony characteristics, morphological features on lactophenol cotton blue mount (LPCB), and growth at different temperatures, aid in identifying the causative pathogen. [3]

On SDA medium, R. arrhizus exhibits rapid growth at room temperature, presenting as cottony fluffy growth with black dots resembling salt paper. Microscopic examination of LPCB wet mounts reveals well-developed rhizoids, opposite long unbranched sporangiophores, with apophysis, collarette, hemispherical columella, and globose hyaline dark brown sporangium containing numerous striated sporangiospores. [3, 6]

In cases where cultures are negative, immunohistochemistry using monoclonal antibodies against R. arrhizus can assist in diagnosis and differentiate aspergillosis from *mucormycosis*. This method has gained moderate recommendation in recent guidelines. [6]

● Molecular methods:

Molecular methods play a crucial role in diagnosing *mucormycosis*, offering various techniques such as semi-nested PCR, nested PCR with RFLP, real-time PCR targeting the ITS region, and specific primers targeting mucoralean genera/species. These methods typically target genes like *18S ribosomal RNA, 28S rDNA, mitochondrial gene rnl, cytochrome b gene,* and the *Mucorales-specific Cot H gene*. [3, 6]

While effective in detecting low fungal loads and in cases where other diagnostic tools fail, molecular methods do not always differentiate between actual pathogen colonization and contamination. For instance, while Mucorales-specific PCR may identify Mucorales species in clinical samples, it may not always confirm *mucormycosis* cases due to the potential amplification of non-specific sequences. [3]

Amplification of different target genes can yield varied results; for instance, only 54% of rhino-orbito-cerebral *mucormycosis* (ROCM) cases were confirmed by ITS2 amplification with subsequent sequencing. Real-time quantitative PCR targeting the ITS1/ITS2 region, qPCR with specific primers targeting the cytochrome b gene, and 28S rDNA are also utilized for diagnosing Mucorales in fresh or formalin-fixed paraffin-embedded tissues. These molecular methods offer valuable diagnostic tools in the identification of *mucormycosis*, but careful interpretation and consideration of potential limitations are necessary. [6]

● Serological test:

Galactomannan and β-D-glucan tests are commonly employed for diagnosing fungal infections, with β-D-glucan being specific for Aspergillosis. However, these tests may have a higher negative predictive value due to false-positive results, particularly with galactomannan and β-D-glucan antigens. [6, 11]

For *Mucormycosis* diagnosis, ELISA testing, western immunoblotting, and ELISpot assays are available. In 2017, a novel protein antigen, protein RSA of 23 kDa, was discovered in serum and lung homogenates of mice infected with R. arrhizus. However, diagnosing *Mucormycosis* via serological tests poses challenges due to human exposure to Mucorales spores, leading to antibody titres. Although sensitized T lymphocytes may be crucial for diagnosing *Mucormycosis,* distinguishing between colonization and true Mucorales pathogens remains challenging due to spores' ability to colonize non-sterile body sites. [6, 11]

● Radiology.

Imaging plays a critical role in the early diagnosis, initiation of antifungal therapy, and monitoring of treatment responses for invasive fungal infections. (Fig 3) MRI is considered the gold standard due to its superior contrast resolution in soft tissue and marrow abnormalities, while CT imaging is often used in conjunction. [11]

A key symptom indicative of invasive fungal infections like rhinocerebral *mucormycosis* is the lack of contrast enhancement of the invading mucosa, known as the "black turbinate" sign, resulting from small artery occlusion. [11]

In chest radiographs, major nodules or peri-nodular halos may indicate fungal infections invading blood vessels. Multiple nodules and pleural effusion are more common in *mucormycosis*, with a reverse halo or multiple nodules accompanied by lung effusion suggesting infection by Mucorales mould. [11]

On CT scans, the "reverse halo sign" is another characteristic feature of *mucormycosis*, observed in 94% of cases within the first week of illness. Initial CT scans of immunocompromised patients with pulmonary *mucormycosis* commonly show a nodule/mass or consolidation with a surrounding ground-glass opacity halo. Follow-up CT scans may reveal morphological changes like the reverse halo sign, central necrosis, and air-crescent sign. Sequential morphological changes have been linked to the absolute neutrophil count in patients. These imaging findings aid in the diagnosis and management of *mucormycosis* and other invasive fungal infections. [11, 9]

● Serology.

Serology is a valuable diagnostic tool used to detect antibodies to fungi, aiding in the identification of fungal infections. Various techniques are employed, including lateral flow tests, radioimmunoassays, enzyme immunoassays, immunodiffusion, counter immunoelectrophoretic, complement fixation (CF), immunoassays using antibodies, and agglutination techniques. [6]

Future advancements in molecular technologies may improve serological techniques, but they require direct tissue collection, standardization, technological advancements, and cost reduction. Monoclonal antibody (2DA6) has shown high reactivity with purified fucomannan of Mucor species using sandwich ELISA. Lateral flow immunoassays (LFIA) have proven convenient for testing serum, urine, and tissues. [6, 11]

However, serological investigations have limitations, such as being time-intensive and technically challenging. Immunocompromised patients may exhibit a lower antibody response, reducing the test's utility. Additionally, serology faces challenges in discriminating between current and past infections, leading to unreliable test interpretation. Enzyme-linked immunosorbent assays (ELISA), immunoblots, and immunodiffusion tests have been evaluated with varying degrees of success. Ongoing research and technological advancements are crucial for improving the accuracy and reliability of serological tests for fungal infections. [6, 11]

- **Molecular assays**

Molecular-based assays for the detection and identification of Mucorales include conventional polymerase chain reaction (PCR), restriction fragment length polymorphism analyses (RFLP), DNA sequencing of defined gene regions, and melt curve analysis of PCR products. These assays commonly target the internal transcribed spacer or the 18S rRNA genes. [6, 11]

Studies utilizing formalin-fixed, paraffin-embedded, or fresh tissue samples have shown varying performance of these assays, with sensitivity ranging from 70% to 100% and specificity not consistently calculated to 100%. However, limitations such as the small number of patients studied and lack of comprehensive clinical evaluation hinder the widespread adoption of in-house molecular assays as standalone diagnostic tools in routine clinical practice. [6, 11]

Recent efforts focusing on molecular-based diagnosis from blood and serum have shown promising clinical data. Molecular-based diagnosis from serum has demonstrated earlier detection compared to culture, ultimately confirming culture-proven cases. While molecular-based diagnostic assays are valuable complementary tools to conventional diagnostic procedures, further research and clinical validation are needed to fully integrate them into routine clinical practice. [6]

Advanced diagnostic techniques

- **Advanced serological tests.**

Mucormycosis diagnosis has historically relied on techniques like ELISA, immunoblots, and immunodiffusion tests, each with varying effectiveness. Recent advancements in serological methods have improved specificity and sensitivity, aided by antisera directed against fungal antigens. Gel

precipitation, a longstanding technique, is commonly used to identify various immunoglobulin types using in-house antibodies from fungal cultures. Distinct Mucorales T cells have been observed in invasive *mucormycosis* using enzyme-linked immune spot (ELISpot) tests, though more research is needed to determine their diagnostic utility. [11]

The monoclonal 2DA6 antibody, examined in ELISA for new serological test targets, has shown strong reactivity with Mucor species. However, drawbacks include reduced specificity due to cross-reactivity and delayed appearance of antibodies in peripheral blood, which may hinder early detection. Accurate titre settings are crucial to prevent incorrect outcomes, especially in early-stage infections. [6, 11]

Despite these limitations, serology diagnostic tests remain accessible, non-invasive, and rapid, providing valuable information to aid medical professionals in making more precise and timely diagnoses. Ongoing research and improvements in serological techniques hold promise for enhancing their diagnostic accuracy and utility in the future. [11]

- ## Nucleic acid-based diagnostics

PCR methods have undergone significant advancements for diagnosing fungal infections, including multiplex PCR, nested PCR, reverse transcription quantitative PCR (RT-qPCR), targeting internal transcribed spacer regions and ribosomal DNA, PCR ELISA, conventional PCR, and direct DNA sequencing. These techniques provide diagnostic specificity but may have concerns regarding responsiveness and reproducibility, particularly in generating false-negative results. [11]

Traditional PCR is rapid and can enhance sensitivity, but variations in results may occur due to the lack of standardized techniques. Modified nested PCR techniques have been developed to improve both specificity and sensitivity, offering enhanced accuracy in detecting fungal pathogens. [6, 11]

Despite these advancements, challenges remain in achieving consistent and reliable results, particularly in clinical settings. Further standardization and validation of PCR methods are necessary to address these concerns and ensure their effectiveness in diagnosing fungal infections accurately. [11]

Future diagnostic tools

- ## Biosensors

Biosensors are integrated receptor-transducer systems designed to provide selective quantitative or semi-quantitative analytical information using biological recognition elements. They typically comprise a transducer, an identification element (biological recognition element), and a signal processor. In the medical field, there are various types of biosensors, including wearable biosensors, aimed at enhancing patient quality of life.

Electrochemical biosensors are capable of detecting fungi such as Candida albicans and Aspergillus fumigatus, while optical biosensors are utilized for detecting Candida species. These biosensors offer rapid and sensitive detection methods for fungal pathogens.

The integration of biosensor technology into fungal diagnostics is expected to advance research in medical mycology by employing methodologies not traditionally utilized in the field. Biosensors have the potential to revolutionize fungal diagnostic approaches, offering improved sensitivity, specificity, and speed compared to conventional methods. Additionally, biosensors may enable point-of-care testing, facilitating timely diagnosis and treatment of fungal infections. Continued research and development in biosensor technology hold promise for enhancing fungal diagnostic capabilities and improving patient outcomes. [11]

● Micro-needle-based diagnostics

Micro needles are miniature, flexible devices designed for skin penetration and bioanalysis. They can be shaped into various forms and are widely favored for their minimally discomforting nature. Historically, infectious diseases such as tuberculosis have been diagnosed using micro needle-based platforms. Various diagnostic systems have been devised to either collect or detect biomarkers in the skin.

Research into micro needle-based diagnostics for communicable diseases stands to benefit from specialized knowledge gained from other diseases. Integrated lab-on-a-chip transdermal drug delivery devices hold promise for overcoming bottlenecks and accessibility issues associated with centralized test facilities.[11]

By leveraging micro needle technology, diagnostic tests for communicable diseases can become more accessible, efficient, and patient-friendly. These advancements have the potential to revolutionize the field of infectious disease diagnostics, enabling rapid and accurate detection even in resource-limited settings. [11]

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● Metabolomics-Breath Test

The study conducted by Koshy et al. utilizing an experimental murine model of invasive *mucormycosis* with three Mucorales species—Rhizopus arrhizus var. arrhizus, R. arrhizus var. delemar, and R. microsporus—revealed distinct breath profiles of the volatile metabolite sesquiterpene. This discovery holds significant potential for non-invasive diagnosis of fungal infections and monitoring therapy response, particularly in high-risk populations.

By identifying unique breath profiles associated with fungal infections, this method offers a promising approach to diagnose *mucormycosis* without invasive procedures. Moreover, the ability to monitor therapy response through changes in breath profiles could enhance treatment management and patient care.

Non-invasive diagnostic methods are particularly valuable in high-risk populations where invasive procedures may pose additional risks or challenges. This innovative approach has the potential to revolutionize the diagnosis and management of invasive fungal infections, offering a safer and more efficient alternative to traditional diagnostic techniques. Further research and validation of this method in clinical settings are warranted to fully realize its potential in improving patient outcomes. [6,10]

MANAGEMENT

Successful treatment of *mucormycosis* typically involves four steps:

1. Early diagnosis: Initiating antifungal therapy promptly after diagnosis significantly improves survival rates. Treatment should ideally begin within five days of diagnosis to maximize efficacy. [12]

2. Reversal of underlying predisposing risk factors: It is crucial to address and reverse any underlying conditions or factors that predispose individuals to *mucormycosis.* This may involve reducing or discontinuing immunosuppressive medications, particularly corticosteroids, and aggressively managing conditions such as diabetic ketoacidosis to restore normal physiological status. [12]

3. Surgical management: Surgical debridement of necrotic tissues is often necessary for complete eradication of the fungal infection. Studies have shown that surgery is an independent predictor of favorable outcomes in *mucormycosis* cases, and patients who do not undergo surgical intervention have higher mortality rates. [13]

4. Antifungal therapy: Primary treatment typically involves polyene antifungals such as amphotericin B. The optimal dosages for antifungal agents are not definitively established, but starting dosages commonly used for adults and children include 1 mg/kg/day for amphotericin B deoxycholate and 5–7.5 mg/kg/day for liposomal amphotericin B and amphotericin B lipid complex. Salvage therapy options such as deferasirox or posaconazole may be considered for patient's refractory to or intolerant of polyene therapy. [13]

Strict glycemic control is essential in managing *mucormycosis*, along with appropriate insulin dosing to regulate blood glucose levels. Sodium bicarbonate may be used to neutralize ketoacidosis, but electrolyte imbalances resulting from treatment measures should be promptly corrected to ensure patient safety. By following these comprehensive treatment steps, healthcare providers can effectively manage *mucormycosis* and improve patient outcomes. [12, 13]

CONCLUSIONS

Mucormycosis is indeed a serious fungal infection that can have devastating consequences, particularly for individuals with predisposing conditions such as diabetes, immunocompromised

states, or iron overload treatments. The disease presentation can vary widely, ranging from sinusitis to disseminated infections, and early detection is crucial for improving patient outcomes. [6, 15]

In developed countries, hematological malignancies, particularly acute myeloid leukemia, are commonly associated with *mucormycosis.* On the other hand, in developing countries, factors like post-pulmonary tuberculosis and chronic kidney disease are emerging as risk factors, highlighting the need for awareness and vigilance across different healthcare settings. [3]

One of the key challenges in managing *mucormycosis* is the difficulty in early diagnosis. Traditional diagnostic methods may not always be sensitive or specific enough, leading to delays in treatment initiation. However, advances in molecular diagnostics, such as PCR-based tests, are improving our ability to detect the fungus even at low fungal loads, which can be critical for early intervention. [14]

Serological assays, including ELISA and immunoblotting, are also valuable tools for aiding in early diagnosis. These tests can detect specific antibodies or T lymphocytes associated with *mucormycosis,* helping to complement clinical findings and guide treatment decisions. [17]

Looking ahead, the development of biosensors holds promise for more rapid and accurate diagnosis of *mucormycosis*. These devices can target specific fungal biomarkers, potentially providing point-of-care testing options that are both sensitive and species-specific. Additionally, micro-needle diagnostics and other innovative methodologies may further expedite the diagnostic process, improving patient outcomes. [19, 18]

Overall, while significant progress has been made in *mucormycosis* diagnostics, ongoing research and validation of new testing modalities are essential for enhancing our ability to identify and treat this potentially life-threatening infection effectively. [6]

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