Laboratory diagnosis of mucormycosis: Present perspective

Munesh K Gupta¹, Nilesh Kumar², Neeraj Dhameja³, Arti Sharma¹, Ragini Tilak¹

Departments of ¹Microbiology, ²General Medicine, ³Pathology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

ABSTRACT

Upsurge in mucormycosis cases in the second wave of SARS CoV2 infection in India has been reported. Uncontrolled diabetes is the major predisposing risk factor for these cases. The early diagnosis and surgical intervention with medical treatment may result in good clinical outcomes. The glycaemic control in diabetic patients also favours better treatment outcome in patients suffering from mucormycosis.

Keywords: Diabetes, GMS, KOH wet mount, Rhizopus arrhizus, RSA protein, steroid

Introduction

Mucormycosis, a life-threatening fungal infection, is caused by the members of order Mucorales.⁴ Mucormycosis presents as fulminant fungal infection in debilitated patients, primarily involving the rhino-facialocranium, lungs, gastrointestinal tract, skin and kidney. Among these, rhino-orbital mucormycosis is the most common presentation where rapid tissue necrosis with black discolouration occurs at the affected site. The member of order Mucorales, genera Rhizopus, Rhizomucor, Mucor, Lichthemia, Apophysomyces, Cunninghamella, and Saksenaea, result in human infections. Rhizopus arrhizus (Rhizopus oryzae) followed by Rhizomucor spp is the leading cause of mucormycosis.⁵⁶

Mucorales are widely distributed in an environment as saprotrophs, where they live on the dead decaying material and in soil.⁶ The spores of these Mucorales enter the human body through inhalation, where they primarily colonize and infect the paranasal sinuses. In immunocompetent individuals, there is no clinical manifestation.⁷ In contrast, neutropenia, uncontrolled diabetes, steroid therapy, hematogenous disorder promote spore germination into the coenocytic hyphae that invade the blood vessels & tissues.⁸ The inhaled spores may also result in pulmonary mucormycosis in neutropenic patients.⁹ Sometimes, the spores are directly inoculated at the abraded skin, especially in a burn, dressing, and traumatized patients, resulting in cutaneous mucormycosis.¹⁰

During the second wave of COVID 19, an upsurge in the number of mucormycosis cases has been observed across India.¹¹ This upsurge is a consequence of pre-existing uncontrolled diabetes, steroids induced hyperglycaemia, IL6 inhibitors (Tocilizumb), high fungal spores in the environment, especially at the construction sites,¹²¹³ Non-judicious use of antifungal agents, especially voriconazole as a prophylactic agent, further enhances the possibility of mucormycosis.¹⁴ COVID-19 infection itself is a predisposing risk factor where it causes lymphopenia and endothelitis, favouring the fungal invasion. Uncontrolled
diabetes and steroid use further increase the chances of invasive fungal infections. Hyperglycemia has been attributed to the high β-hydroxybutyric acid, resulting in a reduced iron-binding capacity of transferrin, which manifests as high serum Iron that favours the rapid growth of Mucorales. The high load of fungal spores, especially at the construction site, is a significant risk factor for mucormycosis in COVID-19 care facilities where the ICU admitted patients with co-morbidities, who are already on steroid, IL6 inhibitor, and multiple antibiotics, are colonized initially by these spores. Subsequently, these spores germinate and result in localized/disseminated mucormycosis. As a consequence of angio-invasion, they result in black discoulouration of the affected site.

This fungal infection has higher mortality, especially in untreated/delayed treatment conditions. Thomas et al. in 2014 has reported 92% mortality in patients suffering from mucormycosis, which is reduced to 46% in patients having COVID associated mucormycosis, with 68% and 31% mortality in disseminated and cutaneous mucormycosis respectively. Among the survivors, vision loss followed by facial deformity are the frequently encountered consequences of mucormycosis. Thus, it is vital to have a high index of suspicion of mucormycosis in COVID 19/recovered patients. Therefore, this review will emphasise pathogenesis, clinical manifestations, lab diagnosis and management of mucormycosis.

**Clinical Presentation**

Mucormycosis presents itself as rhino-orbito-cerebral, pulmonary, cutaneous, gastrointestinal, isolated renal and disseminated in the patients. In rhinocerebral mucormycosis, facial pain, headache, brownish-blackish/blood-tinged nasal discharge is observed, whereas, in conditions with extension to surroundings, it presents as palatal ulcer, proptosis, periorbital swelling, and orbital pain [Figure 1]. CNS involvement primarily affects the frontal lobe and cerebellum where cranial nerve palsy, localized brain abscess, orbital apex syndrome can be observed later.

In pulmonary mucormycosis, the patient usually complains of fever, cough with brown-coloured sputum, chest pain, breathlessness, and haemoptysis. In cutaneous mucormycosis, clinical manifestations vary from pustule or vesicle to non-healing wound with necrotic edges. Sometimes, a cottony like growth is observed over the lesion surface (hairy pus). In Gastrointestinal mucormycosis, the stomach is the primarily affected site, where ulceration of gastric mucosa with associated blood vessel thrombosis occurs.

**Diagnosis**

The tissue necrosis resulting from angio-invasion is a classical feature of mucormycosis but, the other fungi, like *Aspergillus*, *Lomentospora* (*Scedosporium*), and *Fusarium* spp, also result in tissue necrosis. In such cases, a joint approach is made by clinicians, histopathologist, microbiologist and radiologist. A high index of suspicion is needed for early diagnosis of mucormycosis in patients having multiple predisposing risk factors. Endoscopy mediated nasal cavity examination, especially the middle turbinate and sinuses, may provide the initial evidence for mucormycosis. NCCT and MRI of the paranasal sinuses and orbit help to diagnose mucormycosis where the hyperinflamed denser sinus mucosa with bone erosion, including the periosteum, is observed [Figure 2]. These imaging techniques are essential to define the extent of the lesion. These clinic-radiological features provide a high index of suspicion for mucormycosis, whereas the diagnosis is confirmed by microbiological and histopathological examination. In cases of respiratory mucormycosis, reverse halo sign (RHS), multiple (≥10) nodules, pleural effusion, central necrosis, and air-crescent sign are observed on HRCT of the thorax.

**Microbiological Examination**

A microbiological examination is vital for early diagnosis of mucormycosis with identification of the causative pathogen. For this, the clinician should collect the nasal discharge, excised tissue...
by endoscopy or during surgery in the sterile universal container containing normal saline. The clinicians should not use the swabs to collect nasal secretions/discharge as the cotton fibres interfere in the identification and cultivation of the pathogenic fungi. The collected specimen is never kept at a low temperature as the Mucorales does not survive at this temperature. The collected specimen is transported immediately to the Mycology lab, where the tissue samples are teased/cut into multiple small pieces.

Care must be taken while processing the sample as grinding and vortexing of the sample interfere in the viability of highly fragile hyphae of Mucorales. It is vital to select an appropriate portion of the sample for direct microscopic examination as Mucorales has a property of angioinvasion and perineural invasion. Thus, the black-coloured area of the excised tissue is used for direct microscopic examination and cultivation. Different diagnostic tools are being used to diagnose mucormycosis.

A) Potassium hydroxide (KOH) wet mount:

It is a rapid presumptive test for diagnosing the fungal infection. A small piece of the excised tissue or BAL/sputum are kept in the 20% KOH, which dissolves the proteinaceous and other substances. We can fasten the process of dissolving by keeping the mount at 37°C. In KOH wet mount, the Mucorales hyphae reveal coenocytic broad aseptate/sparsely septate hyphae with right-angle branching resembling ribbon-like appearance. It is essential to differentiate the Mucorales hyphae from the Aspergillus hyphae, which also causes rhinosinusitis and angioinvasion. Thin, septate, regular acute angle dichotomous branched hyaline hyphae of Aspergillus are observed in clinical samples. The sensitivity of KOH wet mount varied as the results on the direct wet mount are highly subjective. Therefore, it is essential to differentiate the fungal hyphae from artefacts, especially the cotton fibres.

B) Calcofluor white (CFW) stain:

It is a non-specific fluorochrome dye used widely to diagnose fungal pathogens in clinical samples. It binds specifically to the β1-3, β1-4 glycoside chain of the chitin, an essential fungal cell wall component. Once the sample is stimulated with UV light in a fluorescent microscope, the fungal pathogen appears as the apple green or bluish against the white background, depending on the filter used. The addition of KOH in CFW stain increases the visibility of the fungal elements in clinical samples. Care should be taken while examining the clinical samples, especially the cotton fibres; when present, they fluoresces as bluish as the CFW stain also bind the β1-3, β1-4 glycoside of cellulose.

C) Culture:

Culture is an essential diagnostic tool to determine the causative fungal pathogens with their antifungal susceptibility. Sabouraud’s Dextrose agar (SDA) is most widely used to cultivate the fungal pathogen. Briefly, the received sample is cut into multiple small pieces, directly inoculated over SDA and Potato dextrose agar (PDA) and incubated at 25°C in BOD. Mucorales grow rapidly at 25-37°C, where cottony fluffy growth is usually observed within 72hrs. The causative fungal pathogen is identified by standard mycological procedures including colony characteristics, morphological features on lactophenol cotton blue mount (LPCB), and growing at different temperature. The following morphological characteristics; presence/absence of rhizoid, length and branching pattern of sporangiophore, apophysis, columella, collarette, the shape of the sporangium, size & shape of the sporangiospores, zygospore are observed on LPCB mount. Care should be taken while reporting the cultivated fungi as it may be due to contamination. In such cases, other supporting tests aid in the diagnosis.

The primary concern about the culture is its low sensitivity, as in more than 50% of mucormycosis cases, usually, there is no growth. This low culture positivity may be attributed to different factors ranging from sample collection, storage to sample processing. The storage of samples at 4°C affects the viability of Mucorales. The tissue sample processing, including grinding or homogenization, also affects the viability of these fungal pathogens.
Rhizopus arrhizus (R. oryzae) is the predominant cause of mucormycosis. It rapidly grows at room temperature on SDA medium, where cottony fluffy growth with black dots, resembling salt-paper, is observed [Figure 5]. On LPCB wet mount well-developed rhizoid, opposite to long unbranched sporangiophores, with apophysis, collarette, hemispherical columella and globose hyaline-dark brown sporangium having numerous striated sporangiospores are observed [Figure 6].

D) Molecular methods:

These methods are widely used to diagnose mucormycosis from clinical samples and to identify the causative pathogen from the growth. Different molecular methods, semi-nested PCR, nested PCR with RFLP, real Time PCR targeting the ITS region or specific primers targeting a restricted number of mucoralean genera/species, are being used to diagnose mucormycosis. Among these, the 18S ribosomal RNA gene is the most common target, but others; 28S rDNA, the mitochondrial gene rnl, the cytochrome b gene or the Mucorales-specific CotH gene are also being targeted. Once the targeted region is amplified, it is sequenced to determine the causative fungal pathogen. These molecular methods aid the diagnosis where the fungal load is low and the conditions where other diagnostic tools fail. Care should be taken while interpreting the results of molecular-based diagnostic tools as they don’t differentiate between the actual pathogen/colonization or contamination.

Amplification of different target genes may generate varied results for mucormycosis as Zaman et al. has reported that only 54% (27/54) of the ROCM cases were confirmed by ITS2 amplification with subsequent sequencing. In contrast, Mucorales-specific PCR amplified DNA in all 50 samples, subsequently identified as Mucorales species. This difference may be due to the amplification of all fungal elements present in clinical samples, as ITS 2 is present in all fungal species. It probably generated the sequence of all fungi present in clinical samples during amplification, resulting in a non-specific sequence. In contrast, amplification of Mucorales-specific PCR DNA results in the only generation of Mucorales specific products. Thus selecting a panfungal target in non-sterile sites may result in the identification of non-specific fungi, which can be easily overcome by selecting a specific target. Currently, real-time quantitative PCR targeting ITS1/ITS2 region with specific probes for R. arrhizus, R. microsporus, and Muoer spp., qPCR with specific primers targeting cytochrome b gene, 28S rDNA are also being used to diagnose the causative Mucorales in fresh/FFPE tissues.

E) Histopathological examination:

Histopathological examination of the excised tissue is essential to determine the tissue reactions and the causative fungal pathogens. For this, the excised tissue is to be sent in a sterile container having 10% formalin, which preserves the tissue’s architecture. Initially, a gross examination of the excised tissue is done subsequently, it is cut into multiple small pieces which undergo paraffin embedding through dehydration, clearing and infiltration. Subsequently, the embedded paraffin block is cut into multiple 4-5 micrometre thickness parts, stained by Hematoxylin and Eosin (H&E) staining. On microscopic examination, tissues from the suspected case of mucormycosis show necrosis, inflammatory infiltrate rich in neutrophils and fungal hyphae. The fungal hyphae appear basophilic one, broad, aseptate and show right-angle branching. The fungal hyphae are well observed on the H and E stained section; however, special stains like PAS and GMS can be done if required. Sometimes granuloma, angioinvasion or perineural invasion can be seen in tissue samples.

F) Hematoxylin and Eosin staining:

H&E staining is most widely used for histopathological examination. It is a combination of two dyes, Hematoxylin and

![Figure 5: Cottony-fluffy growth of Rhizopus arrhizus on SDA agar](image)

![Figure 6: LPCB wet mount showing Rhizoid (a), sporangiophore (b), Apophysis (c), columella (d), collarets (e), sporangium (f), 400x](image)
Eosin. In the presence of mordant, aluminium salts, Hematoxylin, a basic dye, stains the nucleus purple, whereas Eosin, an acidic dye, stains the cytoplasm pink. It demonstrates the inflammatory response against fungal pathogens by identifying hyaline/phaeoid fungi [Figure 7]. The major drawback of this staining is that it fails to detect fungal pathogens when they are sparsely present. While interpreting the staining, it is also difficult to distinguish the fungal element from the small blood vessels in CNS and lung tissues.

G) Periodic acid Schiff (PAS) staining:

PAS staining detects the carbohydrate component of the fungal cell wall, rich in β 1-3, β 1-4 D-glucan. The periodic acid results in the oxidation of carbohydrate components into the aldehyde, which is detected by the addition of Schiff reagent, where the fungal elements reveal as red or pink or magenta. [Figure 8]. The protein and nucleic acid remain unstained.

H) Grocott-Gomori's Methenamine Silver staining (GMS) staining:

GMS is a unique stain used to detect the fungal element in the clinical samples. It is considered to be best as it provides better contrast for screening. Compared to PAS, it also stains the old and non-viable fungal organisms in tissue samples. GMS staining also detects the carbohydrate (glucan) component of the fungal cell wall, which appears dark and black coloured [Figure 9]. It also stains the filamentous bacteria, Actinomycetes & Nocardia. The major drawback of this staining is that it may overstain the fungi, which can obscure the internal structures. The GMS stain also does not properly study the host response to fungal infection.

I) Serological test:

Galactomannan and β-D glucan tests are widely used to diagnose fungal infection. This β-D glucan test is considered a panfungal antigen test except for the Mucormycosis and cryptococcosis, whereas the Galactomannan test is specific for the Aspergillosis.[50,51] These tests have higher negative predictive value as false-positive galactomannan, and β-D glucan antigen can be observed in various settings. Similarly, the positive galactomannan and β-D glucan test exclude the mucormycosis diagnosis. Presently, ELISA testing targeting the serum R. arrhizus, Rhizomucor pusillus antibody, western immunoblotting targeting R. arrhizus antigen, Cytoplasm, hyphae walls and septate, of R. arrhizus WSSA are available for diagnosis of Mucormycosis.[52-54] Recently, ELISA (ELISpot) or immunocytofluorimetric assay targeting Mucorales specific IFN-γ-producing T cells have been assessed.[55] In 2017, Kanako Sato et al. have reported the unique protein antigen, protein RSA of 23 kDa, in the serum and lung homogenates of R.arrhizus infected mice compared to uninfected mice.[56]

The diagnosis of Mucormycosis by a serological test is a challenging task as human beings are frequently exposed to the Mucorales spores, resulting in antibody titre. The sensitized T lymphocyte may be an essential tool in diagnosing mucormycosis. Again, it is not easy to distinguish between the colonization and
true Mucorale pathogen as these spores readily colonise the non-sterile body site.

J) Treatment:

A high index of suspicion is to be kept in each case of mucormycosis as these cases have higher mortality and severe morbidity among survivors. Mucormycosis cases are managed by extensive surgery, reversal of the predisposing risk factors, and early administration of antifungal drugs in appropriate doses.\[57\]

Extensive surgery of the affected region is the primary treatment. The delay in surgery results in a bad prognosis. Amphotericin B is considered a drug of choice where the liposomal form is given to prevent nephrotoxicity and ototoxicity.\[58,59\] These Mucorales are usually resistant to fluconazole, itraconazole and voriconazole; in such conditions, posaconazole and isavuconazole are used as supportive/salvage therapy.

Strict glycemic control is required to manage mucormycosis, which is achieved by appropriate insulin dosing. Sodium bicarbonate can be used to neutralize the ketoacidosis.\[60\] However; all these measures may result in electrolyte imbalance, which needs to be corrected immediately.

Conclusion

Mucormycosis cases have higher morbidity and mortality among survivors. Therefore, the primary care physicians should have a high index of suspicion in patients presenting with blackish nasal discharge, and sinus pain as better prognosis has been reported in patients presenting in the early stages of infections. Thus, in the background history of diabetes and immunosuppressed states, prompt diagnosis with integrated team management can reduce the morbidity and mortality among the patients suffering from mucormycosis.

Key message

1. The primary care physician should have a high index of suspicion for mucormycosis in patients with uncontrolled diabetes, presenting as blackish nasal discharge or facial pain.
2. Prompt diagnosis of mucormycosis can be made by KOH wet mount of the excised tissue.
3. Mucormycosis cases should be managed by multidisciplinary team comprising otorhinolaryngologist, ophthalmologist, physicians, radiologist and microbiologist.
4. The Black fungus, a clinical terminology of mucormycosis, is not an appropriate synonym as the causative fungal pathogens are hyaline.

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Conflicts of interest

There are no conflicts of interest.

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