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SPECIAL REPORT

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The potential for rapid antigen testing for mucormycosis in the context of COVID-19

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ABSTRACT

Introduction: Mucormycosis is a highly aggressive angio-invasive disease of humans caused by Mucorales fungi. Prior to the COVID-19 pandemic, mucormycosis was a rare mycosis typically seen in immunocompromised patients with hematological malignancies or in transplant recipients. During the second wave of the pandemic, there was a dramatic increase in the disease, especially in India where a unique set of circumstances led to large numbers of life-threatening and disfiguring rhino-orbital-cerebral mucormycosis (ROCM) infections.

Areas covered: The review examines mucormycosis as a super-infection of COVID-19 patients, and the risk factors for COVID-19-associated mucormycosis (CAM) that drove the ROCM epidemic in India. The limitations of current diagnostic procedures are identified, and the measures needed to improve the speed and accuracy of detection discussed.

Expert opinion: Despite increased awareness, global healthcare systems remain unprepared for further outbreaks of ROCM. Current diagnosis of the disease is slow and inaccurate, negatively impacting on patient survival. This is most evident in low- to middle-income countries which lack suitably equipped diagnostic facilities for rapid identification of the infecting pathogens. Rapid antigen testing using point-of-care lateral-flow assays could potentially have aided in the quick and accurate diagnosis of the disease, allowing earlier intervention with surgery and Mucorales-active antifungal drugs.

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Mucorales; Rhino-orbital-cerebral mucormycosis (ROCM); COVID-19-associated mucormycosis (CAM); rapid antigen test; Point-of-care test (POCT); lateral-flow technology

1. Introduction

Mucormycosis is a highly aggressive angio-invasive disease of humans and animals caused by Mucorales fungi, and is second only to invasive pulmonary aspergillosis (IPA) as a life-threatening fungal infection [1,2]. Mucorales fungi are ubiquitous molds present in soil and rotting vegetation and in the indoor environment [3], and their asexual air-borne spores generated in sporangiophores allow dispersal throughout the natural environment (Figure 1). These propagules are potentially infective and, when inhaled by humans with impaired immunity, can lead to rapidly progressive and fatal rhino-orbital-cerebral, pulmonary, cutaneous, and disseminated infections [4–6]. Additional manifestations can include gastrointestinal and osteoarticular mucormycosis [7–9].

Unlike IPA, which is mainly caused by a single species of fungus in the genus *Aspergillus* (*Aspergillus fumigatus*), 25 species from 11 different genera of Mucorales fungi are able to cause mucormycosis in humans [10]. While *Rhizopus arrhizus* is the principal global agent of rhino-orbital-cerebral mucormycosis (ROCM) and is a major cause of pulmonary, gastro-intestinal, cutaneous, and disseminated infections in humans [11–13], other species within the *Rhizopus* genus (most importantly *Rhizopus microsporus*), and species within the genera *Apophysomyces*, *Cunninghamella*, *Lichtheimia*, *Mucor*, *Rhizomucor*, *Saksenaea*, and *Syncephalastrum*, are also able to cause the myriad manifestations of mucormycosis in humans [1,4]. This is further complicated by geographical differences in species distribution and prevalence as disease-causing agents. While *Rhizopus* and *Lichtheimia* species are the most common causes of pulmonary, disseminated, and ROCM in Europe [14] and *Rhizopus* is the most common cause of cutaneous infections in Europe, North America, and South America, *Apophysomyces* is the most common cause of cutaneous mucormycosis in India [15,16]. Differential thermotolerance also contributes to disease type; *Rhizopus* and *Lichtheimia* are thermotolerant and can grow at 37°C, *Rhizomucor* is thermophilic and can grow up to 55°C, while *Mucor* species are commonly mesophilic, unable to grow at temperatures exceeding 34°C. This means that *Mucor* species are typically restricted to cutaneous infections, while *Lichtheimia*, *Rhizomucor,* and *Rhizopus* species cause pulmonary, ROCM, gastrointestinal, and disseminated infections [17,18].

2. Risk factors for COVID-19-associated mucormycosis (CAM)

Innate immunity provided by alveolar macrophages and neutrophils is critical to the destruction and clearance of inhaled fungal spores [19,20] and so mucormycosis, prior to the COVID-19 pandemic, was typically seen in patients with hematological malignancies [6,21,22] and in hematopoietic stem cell and solid organ transplant recipients [4,6] where prolonged neutropenia as a consequence of leukemia, aggressive

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Article highlights

- Mucormycosis is the second-most common mold disease of humans after invasive aspergillosis, with life-threatening infections typically seen in immunocompromised patients.
- The second wave of the COVID-19 pandemic saw a dramatic increase in mucormycosis as a super-infection especially in India where uncontrolled diabetes and indiscriminate use of corticosteroids contributed to an epidemic of life-threatening rhino-orbital-cerebral infections.
- The paucity of rapid, accurate, and sensitive tests for the disease, especially in low- to middle-income countries, contributed to the high rates of mortality and morbidity in patients with COVID-19-associated mucormycosis (CAM).
- Rapid antigen testing using lateral-flow technology may have enabled point-of-care detection of rhino-orbital-cerebral mucormycosis (ROCM) in resource-limited settings.
- A lateral-flow test that incorporates a monoclonal antibody specific to *Rhizopus arrhizus*, the principal global agent of ROCM, has been developed that could be rapidly deployed as a POCT for the disease.

anti-cancer treatment, or immunosuppression provides an ideal opportunity for lung infections by opportunistic air-borne pathogens, such as *Aspergillus* and Mucorales fungi. While fatal Mucorales infections are mainly confined to patients with impaired immunity, necrotizing infections can also occur in ostensibly immunocompetent humans following traumatic implant of spores into the skin following combat injuries, natural disasters, and burns [23]. Dissemination from the site of infection can then lead to infections of the lungs, liver, spleen, heart, and other organs.

Mucormycosis is a rare disease, but one that is reported in underdeveloped and developed regions of the world [12,24]. Disease prevalence varies from 0.01 to 0.2 per 100,000 population in Europe and the United States of America, and with a prevalence of ~ 910,000 cases worldwide prior to 2019 [24]. However, even before the advent of the COVID-19 pandemic, the incidence of mucormycosis in India at 14 per 100,000 population was as much as 70 times higher than the global average [25–27]. A major independent risk factor for mucormycosis is a history of poorly controlled or undiagnosed diabetes mellitus [28], a disease that is increasing globally, and which is expected to rise in India from 77 million individuals in 2019 to over 134 million by 2045 [29]. In the presence of hyperglycemia and low pH, which is found in patients with diabetic ketoacidosis (DKA), phagocytes (macrophages and neutrophils) are dysfunctional and so are unable to contain and kill inhaled spores, leading to pulmonary and hematogenously disseminated Mucorales infections [19].

During the Coronavirus pandemic, there was a further dramatic spike in mucormycosis cases, especially ROCM, in patients with active COVID-19 infections and in those who had recovered from the virus [30]. This surge in infections, whilst seen in Europe and elsewhere [17,31,32], was most pronounced in India [33], especially during the second wave [34,35], and mainly affected the maxillary sinus with the involvement of maxillary teeth, orbits, and ethmoidal sinuses [28,33,36–38]. The reasons for this are multifaceted and comprehensively reviewed elsewhere [38], but were compounded by the high background prevalence of diabetes and the overuse of corticosteroids (e.g. methylprednisolone and dexamethasone) which while reducing the inflammatory response to the SARS-CoV-2 virus and the likelihood of respiratory failure [28], increases susceptibility to fungal super-infections [28,39–42]. Furthermore, steroids raise the blood glucose level by causing insulin resistance, thereby reducing insulin action. This melding of risk factors for mucormycosis [43] resulted in 41,512 cases and 3,554 deaths from the disease in India during the period May 5 to 12 July 2021 [27]; over 80% of patients had diabetes as a co-morbidity, with 82% receiving corticosteroids [31]. In addition, a meta-analysis of 958 CAM patients showed that ~ 9%

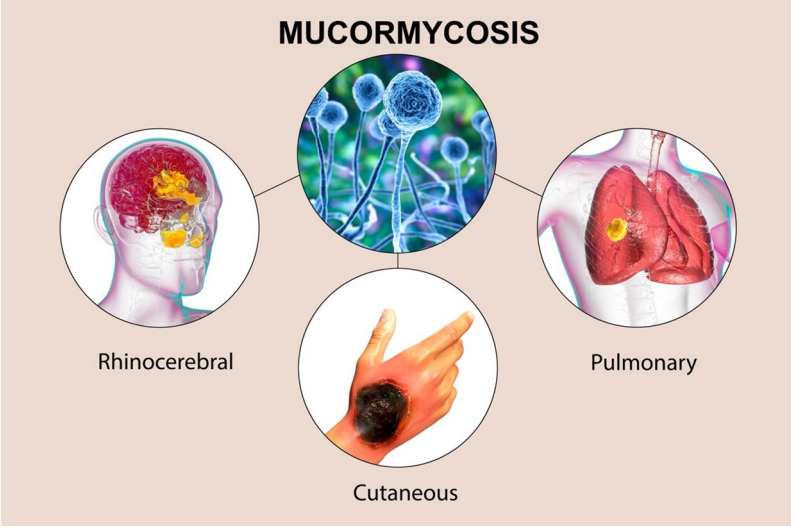

Figure 1. Clinical manifestations of infection by Mucorales fungi. Following inhalation of air-borne sporangiospores released from sporangia (blue structures), rapidly progressive angio-invasive infections of the paranasal sinuses by invasive hyphae can lead to rhino-orbital-cerebral mucormycosis (ROCM). ROCM is categorized as: localized sinus, localized orbital, localized cerebral, sino-orbital, sino-cerebral, rhino-cerebral and generalized ROCM. Inhalation of spores into the lungs can result in localized and deep extension (infections involve the lungs, chest wall, heart, artery or aorta) pulmonary mucormycosis, while necrotizing cutaneous mucormycosis caused by traumatic implant of infective propagules in the skin can lead to disseminated disease of the lungs, liver, spleen, heart, and other organs following hematogenous spread of Mucorales fungi from the sites of infection. Image courtesy of Shutterstock.

had mixed *Aspergillus* and Mucorales co-infections, with a significant delay in the detection and treatment of mucormycosis due to the challenge of differential diagnosis [44]. This is especially the case in low- or middle-income countries where detection of mucormycosis and aspergillosis is reliant on slow and insensitive culture of the infecting organisms from invasive biopsy samples.

3. Current methods for mucormycosis detection

The Mucorales are a group of ancient early-diverging fungi in the sub-phylum Mucoromycotina, and are in a basal position with respect to Basidiomycota and Ascomycota in the fungal tree of life. They have a different cell wall structure and composition to other fungal lineages [45]; their cells walls contain both chitin and chitosan (a deacetylated version of chitin), whereas the basidiomycetes and ascomycetes contain chitin as the main structural component, alongside β-D-glucans and mannans. The human fungal pathogen *A. fumigatus*, an ascomycete, releases immunogenic antigens during invasive hyphal growth that make ideal targets as circulating biomarkers of infection present in serum and bronchoalveolar lavage fluid (BALf). One of these is the cell wall carbohydrate galactomannan (GM) which, along with other *Aspergillus* glycoconjugates [1], contains the highly immuno-reactive epitope galactofuranose or galf [46]. The detection of galf-containing carbohydrates or peptidoglycans using monoclonal antibodies (mAbs) forms the basis of laboratory-based ELISA [47,48] and point-of-care lateral-flow tests [1,49] for IPA detection, and molecular imaging of *Aspergillus* lung diseases in leukemia patients using immuno-positron emission tomography (Immuno-PET) [50].

Unlike *A. fumigatus*, Mucorales cell wall polysaccharides comprise an unusual abundance of fucose-based glycans [45], and an unusually low amount of β-D-glucan, and so pan-fungal tests that detect fungal β-D-glucans in serum are of limited use in the diagnosis of mucormycosis, other than to rule-out infections by other fungi (e.g. *A. fumigatus*) that possess this cell wall carbohydrate [51]. Diagnosis of mucormycosis therefore relies on lengthy and insensitive culture of Mucorales species from tissue samples or from invasive BALf, and speciation by a skilled medical mycologist [52]. However, the fragile nature of Mucorales hyphae means that clinical isolates are often unculturable from biopsy samples and so direct examination of tissues for characteristic broad ribbon-like hyphae is needed, further increasing the turn-around time for diagnosis. Technologies to improve the identification of infecting species in tissue samples include polymerase-chain reaction (PCR), immunohistochemistry (IHC), *in situ* hybridization (ISH), and next-generation DNA sequencing from formalin-fixed paraffin-embedded (FFPE) tissues [53,54]. Identification of isolates recovered from biopsy and growing in culture is achievable using Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF), which allows species identification and delineation of pathogenic Mucorales [55]. Polymerase chain reaction of serum and BALf samples has also shown promise for the detection of mucormycosis, particularly where recovery of Mucorales from biopsy samples has been unsuccessful [52,56]. It is important to note, however, that the vast majority of CAM cases occurred in low- to middle-income countries [27] where appropriately equipped and well-funded medical mycology facilities are absent.

Due to the paucity of quick, sensitive, and specific tests for mucormycosis, diagnosis is often only achieved at autopsy [21,53,54], with an overall mortality rate from disseminated infections of 50% [11]. Higher rates of mortality are particularly associated with dual disseminated aspergillosis and mucormycosis, especially in COVID-19 patients [39,57]. The ability to detect and differentiate mixed infections is of paramount importance since front-line antifungal drugs used to treat *Aspergillus* infections such as voriconazole and echinocandins are inactive or show limited efficacy against Mucorales fungi due to intrinsic resistance [58]. The inability to identify these mixed infections leads to ineffective drug regimens and to break-out mucormycosis [22]. Furthermore, amphotericin B, the antifungal drug of choice for the treatment of mucormycosis is not used for the treatment of aspergillosis where avoidable due to nephrotoxicity [59].

4. The potential for rapid antigen testing for mucormycosis

Significant improvements have been made in the speed of IPA detection with the recent introduction of two commercial lateral-flow tests, the OLM AspLFD test and the IMMY GM-LFA. These tests were developed for the diagnosis of IPA in immunocompromised patients using serum and BALf [1], but have also now found some utility [60–63] in the detection of COVID-19-associated pulmonary aspergillosis (CAPA), a newly recognized *Aspergillus* lung disease affecting previously immunocompetent, mechanically ventilated, intensive care unit patients with severe viral pneumonia. Consequently, the potential for rapid antigen testing for human mycoses in the context of COVID-19 has been demonstrated.

Unlike aspergillosis, there are no commercially available lateral-flow tests for the detection of Mucorales antigens and which allow for point-of-care detection of mucormycosis. A hallmark of mucormycosis is extensive angio-invasion with accompanying thrombosis [64]. This provides an opportunity for the detection of infectious biomarkers (nucleic acids and antigens) circulating within the bloodstream. The performance of a commercial PCR assay (MucorGenius®) has recently been compared to an in-house qPCR assay to detect Mucorales DNA in spiked serum samples, with lower analytical sensitivity compared to the in-house test [65]. Another commercial PCR test, the MycoGENIE *Aspergillus* spp./Mucorales spp. real-time duplex PCR kit, which simultaneously targets both the 28S rDNA regions of the *Aspergillus* genus and the Mucorales order, has also recently been prospectively tested on serum samples from patients with mucormycosis or aspergillosis or with *Aspergillus*/Mucorales co-infections [66]. Sera from 744 patients were analyzed including 35 with IPA, 16 with mucormycosis, and four with dual infections. Sensitivity varied from 85.7% in probable/proven IPA to 28.6% in CAPA. All disseminated mucormycosis were positive, including four *Aspergillus* co-infections, but sensitivity fell to 33.3% with localized forms of infection.

Molecular diagnostic approaches employing PCR show real promise for IPA and mucormycosis detection and differentiation, but are not currently translatable to rapid point-of-care detection. For rapid diagnosis in resource-limited settings, assays based on lateral-flow technology are needed. A recent advance has been made in this area with the development of a mAb, KC9, and a lateral-flow device (LFD) for the detection of a *Rhizopus arrhizus*-specific antigen [11]. The antigen is a 15-kDa extracellular polysaccharide (EPS) that is secreted by the growing fungus. While the identity of the antigen is currently unknown, its abundance as an extracellular biomarker makes it an ideal candidate for the detection of *R. arrhizus*, the principal global agent of mucormycosis in humans, and responsible for > 80% of ROCM. Furthermore, the ability of KC9 to differentiate *Rhizopus arrhizus* from other invasive mold pathogens such as *Aspergillus* and *Scedosporium* species is of paramount importance, especially where the clinical presentation of rhinosinusitis overlaps during co-infections in COVID-19 patients [39,67–69]. Importantly, the KC9 LFD is compatible with human serum and BALF, which means it may be suitable for the detection of disseminated and pulmonary mucormycosis, respectively. It should be noted that the test has yet to be validated in the clinic, but its development offers an important first step toward point-of-care testing for the disease.

While BALf is an appropriate fluid for biomarkers of pulmonary mucormycosis, and serum for biomarkers of hematogenous dissemination, fluid from the sino-nasal cavity or saliva might serve as a better source of antigenic biomarkers for rapid detection of ROCM, the most abundant form of mucormycosis witnessed during the recent spike in CAM infections. An examination of 113 Indian CAM patients within the sino-nasal, rhino-sino-orbital, and ROC mucormycosis categories showed the greatest diagnostic yields of culturable Mucorales from nasal mucosa and middle turbinates, and that *R. arrhizus* accounted for 86% of infections overall [70]. Furthermore, the 'black turbinate' sign has been shown to be an early indicator of sino-nasal and rhino-cerebral mucormycosis in magnetic resonance imaging [51,71–73]. Consequently, swabs of the nasal mucosa and mid-turbinates, akin to those used for the rapid antigen detection of SARS-CoV-2 [74], may have acted as a similarly suitable source of antigenic biomarker for KC9 LFD detection. Alternatively, given the oral and dental manifestations of ROCM [38], saliva might act as a noninvasive biofluid for point-of-care detection of infectious disease [75]. Identification of *R. arrhizus* through rapid point-of-care detection of the signature 15-kDa biomarker may have helped to improve the speed and accuracy of ROCM detection and to have lessened the burden of severe facial disfigurement and blindness caused by tissue necrosis and the aggressive surgical debridement needed to prevent disease progression. This is particularly true for countries and regions where access to well-equipped diagnostic facilities was lacking, and which led to delayed diagnosis and treatment.

5. Conclusion

To summarize, mucormycosis, while a rare fungal disease, has always been a threat to immunocompromised individuals, second only to aspergillosis as a life-threatening disease of patients with hematological malignancies and bone marrow and solid organ transplant recipients. A history of poorly controlled diabetes mellitus is a major independent risk factor for the disease, demonstrated by the recent spike in ROCM that emerged during the second wave of the COVID-19 pandemic in India. There, and elsewhere, COVID-19 collided with injudicious use of corticosteroids, diabetes, and other factors to produce a perfect storm of conditions for ROCM [76,77]. The lack of easily accessible, cheap, and rapid tests for ROCM led to high rates of mortality or disfigurement due to delayed recognition and treatment. The recent development of a lateral-flow device for the detection of the principal global agent of mucormycosis, *R. arrhizus*, holds promise for the rapid point-of-care detection of this devastating disease.

6. Expert opinion

Despite their major impact on human health, causing over 13 million infections and more than 1.5 million deaths worldwide each year [25,78], the lack of recognition of fungi as agents of lethal diseases continues. The World Health Organisation has only just recognized the importance of fungi in human disease, publishing a fungal priority pathogens list (the WHO FPPL) in 2022 [79]. This is the first global effort to systematically prioritize fungal pathogens and to highlight the unmet needs of medical mycology, with mucormycosis ranked as a high priority disease grouping. Key amongst the priorities is the prevention of antifungal resistance which, similar to antibiotic resistance, is a serious and growing problem in the clinic, driven to a large extent by inadequate diagnostics which has led to sustained misuse of the limited arsenal of mold-active drugs. Remarkably, even despite the major advances in diagnostic technologies over recent years, the gold standard procedure for the detection of human mycoses remains culture of the infecting pathogen from an invasive biopsy. Culture is an insensitive and lengthy diagnostic procedure, but is often the only option in low- to middle-income countries that lack sophisticated diagnostic equipment. This is amply demonstrated by the recent surge in cases of ROCM in India during the second wave of the COVID-19 pandemic. The majority of cases of ROCM cases were diagnosed by culture, further delayed by co-infections of Mucorales and *Aspergillus* species which have different antifungal susceptibility profiles.

Early and accurate detection and differentiation of invasive fungal infections is critical for patient survival, with prognosis worsening significantly in the absence of rapid identification and treatment with antifungal drugs. Insensitive and time-consuming culture delays detection, and while PCR assays for fungal infections are quick and accurate, they cannot easily be performed at the patient bedside. Lateral-flow tests meet the requirements of speed, accuracy, and low cost and can be performed at point-of-care, meeting the ASSURED criteria for diagnostics for the developing world [80]. The widespread use of rapid antigen home tests for COVID-19 shows how adaptable the technology is and how easily rapid antigen tests can be performed. The time is right for policymakers,

public health professionals, and other stakeholders to support the integration of point-of-care lateral-flow tests for mucormycosis and other life-threatening mycoses into diagnostic workstreams, provided they have sufficient specificity and sensitivity. This, after all, is a key action point of the WHO FFPL. At present, the research and development of lateral-flow tests for human mycoses, their validation, accreditation, and commercialization has been met by a very small number of SMEs at their own cost and risk with limited financial support from government funding bodies. The landscape needs to change if we hope to keep pace with the continuing rise in fungal diseases that maim and kill millions of people worldwide each year.

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Declaration of interest

The author is affiliated with ISCA Diagnostics Limited.

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