Molecular diagnosis of rhino-orbital mucormycosis in a COVID-19 setting

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Abstract

Purpose Mucormycosis is a severe fungal infection caused by species of the order Mucorales. Early and accurate diagnosis is a prerequisite in the management of the disease. In the present study, we evaluated and compared two PCR-based techniques for the diagnosis and identification of mucormycosis in patients with rhino-orbital mucormycosis (ROM) post-COVID-19.

Methods Diagnosed clinically and radiologically, 25 patients of ROM were included in the study and endoscopically or blind collected nasal swabs or orbital tissues were submitted for microbiological evaluation (direct microscopy + culture) and PCR using primers targeting two different loci (ITS and 28S rDNA region) for diagnosis. All PCR products were further processed for species identification using Sanger sequencing whenever possible.

Result Of the 25 samples included in the study, 16 samples were positive for presence of fungal filaments by Smear suggestive of Mucorales sp., but only 7/25 grew in culture. ITS-based PCR was able to identify mucormycosis in 7/25 (28%) samples and 28S rDNA PCR showed positivity for 19/25 (76%) samples. Rhizopus oryzae was found to be the predominant species in our study. The sensitivity and specificity of 28S rDNA PCR compared to culture were found to be 85.71% and 77.78%, respectively, while for ITS-based PCR, they were 42.86% and 77.78%, respectively.

Conclusions 28S rDNA-based PCR is a reliable and sensitive method for early diagnosis of mucormycosis. Molecular techniques have shown a promising future to provide quick and effective treatment by accurately identifying the aetiologic agent.

Keywords COVID-19 · Mucormycosis · Polymerase chain reaction · ITS · 28S · Sanger sequencing

Introduction

Mucormycosis is an aggressively life-threatening infection characterized by extensive angioinvasion...
caused by fungi belonging to the order Mucorales [1]. It primarily affects the patients who are immunocompromised and patients with poorly controlled diabetes mellitus [2–4]. Although once classified as a rare infection, its incidence rose significantly during the second wave of COVID-19 and is now considered as emerging pathogen with the global incidence rate of 0.005 to 1.7 per million population [5]. In India, the incidence rate was significantly high reaching up to 0.14 per 1000 and coping with multiple infections could be challenging for developing countries like India due to economic crisis, healthcare infrastructure inadequacy and awareness reach [5]. These factors along with favourable environment for fungus growth could be the reason for increased incidence in India. Globally, Rhizopus species, Cunninghamamella, Rhizomucor and Lichtheimia species are most frequent while in India, Rhizopus is predominant [5, 6]. The spores present in the environment could be ingested, inhaled or directly inoculated through wounds which could further germinate into angioinvasive hyphae [7, 8]. The signs and symptoms of the patients with rhino-orbito-cerebral mucormycosis (ROCM), the most common manifestation of the disease, are facial pain, lethargy, diplopia, ophthalmoplegia, proptosis, ptosis and rarely neurological involvement. Due to the appearance of invaded necrosed tissue as black eschar, the condition earned its name as ‘Black fungus’ [2, 9]. Depending on the severity of the infection, surgical decisions such as Exenteration, TRAMB, Debridement or Debulking have been considered. The disease progression is rapid, and an effective disease management strategy is crucial to prevent further advancement.

The management of mucormycosis depends on rapid identification of the causal agent. Radiological methods lack sensitivity in this regard, because it is difficult to differentiate imaging findings of mucormycosis from aspergillosis, a disease that is treated differently from mucormycosis [7, 10]. Traditionally, diagnosis of fungal infection has relied primarily on direct microscopic examination of clinical samples, histopathology and culture [11].

Classical laboratory diagnosis involving culture methods can be non-specific and tedious, and when it becomes apparent that the patient has mucormycosis, it is often too late to administer effective treatment. As a result, the number of mucormycosis cases that have been undiagnosed since the last decade has increased [3]. Therefore, the routine use of molecular techniques has helped in the accurate identification of Mucorales fungi in tissue samples especially, when the cultures are negative. In the recent decade, PCR-based molecular techniques have shown high potential for accurately diagnosing mucormycosis infection in the early stages of the disease [2, 12, 13]. These techniques help to anticipate the diagnosis of mucormycosis and to distinguish between infections that are caused by Aspergillus and Mucorales very early. Moreover, they can be performed in every patient, whatever the clinical status, and they are becoming essential in improving patient outcome. In our study, the utility of diagnostic PCR for two different loci (ITS-2 and 28 s rRNA gene) was tested in orbital specimens or nasal biopsy samples, collected from patients suspected with ROM after COVID-19 and subjected to Sanger sequencing for validation and identification at the species level.

Materials and methods

Ethics

A written informed consent was received from each patient after recording clinical details prior to study participation following study approval by the Institutional Review Board. The study was performed as per the principles of the Declaration of Helsinki.

Clinical presentation and collection of specimens

All the patients suspected with rhino-orbital mucormycosis were presented to our institute which is a tertiary eye care referral centre in South India. The study included 25 clinical specimens, all suspected of rhino-orbital mucormycosis (ROM) post-COVID-19 seen between June and August 2021 in LV Prasad Eye institute, Hyderabad. COVID-19 was previously diagnosed using real-time polymerase chain reaction (RT-PCR) assays from nasopharyngeal or oro-pharyngeal swabs and CT chest scan (HRCT). Majority of the patients presented symptoms such as facial pain, diplopia, ophthalmoplegia, proptosis, ptosis and sometimes neurological involvement. The patients also presented black eschar due to extensive haemorrhage and necrosis of the surrounding tissue along with chemosis, periorbital cellulitis and sudden vision
loss. Tissue samples such as orbital mass, necrotic material and nasal biopsy were collected and sent for microbiological work-up. One half of the samples were processed for routine testing by direct microscopy (potassium hydroxide with calcofluor white stain, KOH + CFW) and culture (blood agar and Sabouraud Dextrose agar) while the other half were processed for molecular diagnosis.

DNA extraction and PCR amplification

Following the cryogenic grinding of fresh tissue samples in liquid nitrogen, 200 µl of lysis buffer was added to the 1.5-ml tube and incubated at 37 °C for 3 h. Genomic DNA was extracted using commercially available kits of QIAamp DNA minikit (Qiagen, Germany) as per manufacturer’s instruction. Briefly, samples were treated with proteinase K and lysis buffer and incubated at 70 °C for 20 min and DNA was precipitated with absolute alcohol. Samples were then transferred to a spin column, subjected to several washing and eluted in 30 µl in elution buffer.

Followed by extraction, DNA was amplified using panfungal primers ITS 1 and ITS 4, targeting ITS-2 regions as well as primers (5'-GTGAAATTGTGG AAAGGGAA-3') and (5'-GACTCCCTGGTCCGT GTT-3’) targeting the 28s region of rDNA. Both PCRs were set up for 25 µl reaction mixture containing 100 M deoxy nucleotide triphosphates, 10 M each primer, 1x PCR buffer with 2.0 mM MgCl2, 2 µl of template DNA sample and 1 U of Taq polymerase (Taq DNA Polymerase from Thermus aquaticus, Sigma). The ITS PCR involved an initial denaturation at 95 °C for 5 min, followed by 30 cycles in series of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, with a final step of one cycle at 72 °C for 5 min to final extension. The 28S rDNA PCR involved an initial denaturation at 95 °C for 5 min, followed by 30 cycles in series of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min, with a final step of one cycle at 72 °C for 5 min to final extension. For all PCRs, negative and positive controls were used. Amplification products of both the ITS and 28 s rDNA regions were visualized on 1.2% agarose gel by electrophoresis at product size 600–700 bp and 340 bp. Prior to sequencing, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Germany).

Sanger sequencing

To determine the complete sequences of the ITS region and 28 s rDNA region, the amplicons were sequenced using BigDye terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific) by using the following temperature profile: 2 min at 96 °C, this is followed in series by 32 cycles step of 10 s at 96 °C, 6 s at 60 °C and 4 min at 60 °C and the final step for one cycle at 72 °C for 5 min, followed by a 4 °C soak, and all sequencing reaction mixtures were run on thermocycler (Bio-Rad C1000 touch thermocycler). And the sequencing was carried out on an Applied Biosystems model automated DNA sequencer (ABI Prism 3130 Genetic Analyzer). The obtained sequences were then subjected to BLAST (www.ncbi.nlm.nih.gov/BLAST) to identify the fungal species, and sequences were uploaded onto the NCBI GenBank database. The GenBank accession numbers for the sequence of amplicons of ITS and 28S rDNA-based PCRs of the clinical samples in this study are provided in Table 1.

Statistical analysis

The statistical analysis was performed using 2×2 contingency table (for sensitivity and specificity) using the R software.

Results

Demographic details

A total of 25 patients with suspected ROM were included in the study having a mean age of 48 ±14 (range 14–76) years. Majority of the patients were male (84%, n=21). Overall the widely affected age groups were between 40 and 60 years.

Microbiology

In this cohort, the presence of infection was confirmed by culture in 7/25 (28%) when grown in culture media, while 16/25 (64%) cases showed the demonstration of aseptate ribbon-like hyphae under direct microscopy when stained with KOH+CFW and identified as *Rhizopus* species. The details are elaborated in Table 1.
### Table 1: Clinical and molecular details of all the patients included in the study

| SL no | Culture                  | Direct microscopy (KOH/CFW) | PCR ITS (KOH/CFW) | Sequencing (ITS) | PCR 28 s | Sequencing (28 s) |
|-------|--------------------------|-----------------------------|-------------------|------------------|-----------|------------------|
| 1     | Rhizopus sp.             | Positive for Mucorales      | Negative          | NA               | Negative  | NA               |
| 2     | Rhizopus sp.             | Positive for Mucorales      | Negative          | NA               | Positive  | Rhizopus oryzae (ON209361) |
| 3     | Aspergillus niger        | No organisms                | Positive          | Unidentified     | Positive  | Rhizopus microsporus (OM904072) |
| 4     | Rhizopus sp.             | Positive for Mucorales      | Negative          | NA               | Positive  | Rhizopus delemar (OM904075) |
| 5     | Rhizopus sp.             | Positive for Mucorales      | Positive          | Rhizopus microsporus (OM700183) | Positive  | Rhizopus microsporus (OM904073) |
| 6     | No growth                | Positive for Mucorales      | Negative          | NA               | Negative  | NA               |
| 7     | No growth                | Positive for Mucorales      | Negative          | NA               | Negative  | NA               |
| 8     | Rhizopus sp. + Aspergillus flavus | Positive for Mucorales | Negative          | NA               | Positive  | Unidentified     |
| 9     | No growth                | Positive for Mucorales      | Negative          | NA               | Positive  | Unidentified     |
| 10    | No growth                | Positive for Mucorales      | Negative          | NA               | Positive  | Rhizopus oryzae (ON209363) |
| 11    | Scopulariopsis sp.       | No organisms                | Negative          | NA               | Positive  | Unidentified     |
| 12    | No growth                | No organisms                | Negative          | NA               | Positive  | Rhizopus oryzae (OM98021) |
| 13    | Staphylococcus epidermidis | No organisms               | Negative          | NA               | Positive  | Unidentified     |
| 14    | Rhizopus sp.             | Positive for Mucorales      | Positive          | Rhizopus oryzae (OM883926) | Positive  | Rhizopus oryzae (OM980723) |
| 15    | No growth                | Positive for Mucorales      | Positive          | Rhizopus oryzae (OM883927) | Positive  | Rhizopus oryzae (OM980724) |
| 16    | No growth                | No organisms                | Negative          | NA               | Positive  | Rhizopus oryzae (OM980724) |
| 17    | No growth                | Positive for Mucorales      | Negative          | NA               | Positive  | Rhizopus delemar (OM904074) |
| 18    | No growth                | No organisms                | Negative          | NA               | Positive  | Unidentified     |
| 19    | Alternaria sp.           | Positive for Mucorales      | Positive          | Unidentified     | Positive  | Rhizopus oryzae (OM980722) |
| 20    | No growth                | Positive for Mucorales      | Negative          | NA               | Negative  | NA               |
| 21    | No growth                | No organisms                | Negative          | NA               | Positive  | NA               |
| 22    | No growth                | Positive for Mucorales      | Negative          | NA               | Negative  | NA               |
| 23    | Rhizopus sp.             | Positive for Mucorales      | Positive          | Rhizopus microsporus (ON261600) | Positive  | Rhizopus microsporus (ON167520) |
| 24    | No growth                | No organisms                | Positive          | Unidentified     | Positive  | Rhizopus oryzae (OM980724) |
| 25    | No growth                | No organisms                | Negative          | NA               | Negative  | NA               |

*The sequences could not be identified by sequencing due to mixed contamination with other fungal species*
PCR analysis

Amplification of DNA samples with primer pairs ITS1-ITS4 resulted in the fragment of approximately 600 bp. ITS sequencing identified mucormycosis in 7/25 (28%) cases and included five microscopy positive samples, two samples which were negative by smear and culture examination, and one sample which was culture negative. Direct sequencing confirmed the presence of Mucorales in 4/7 of ITS and identified as *Rhizopus oryzae* (2) and *Rhizopus microsporus* (2) (Table 1). In comparison, the DNA samples amplified with 28S rDNA primer pairs resulted in the fragments of approximately 340 bp in 19/25 (76%) mucor cases, which included 13 culture negative samples and 8 samples negative for smear examination (KOH+CFW). Direct sequencing confirmed the presence of Mucorales in 13/19 of 28 s positive samples and identified as *R. oryzae* (7), *R. microsporus* (3), *Rhizopus delemar* (2) and *Rhizopus arrhizus* (1) (Table 1). The remaining sequences could not be identified due to contamination with *Aspergillus* sp. which were also present in the samples.

Comparing the diagnostic performance of ITS versus 28S rDNA PCR, with culture we found the sensitivity and specificity to be 42.86%/85.71% and 77.78%/27.78%, respectively. Similarly, when compared to direct microscopy, the sensitivity and specificity of ITS versus 28S rDNA PCR were 31.25% and 68.75% versus 77.78% and 11.11%, respectively. Additionally, we have also calculated the sensitivity and specificity by comparing molecular results (Sanger sequencing) as true positives with culture and found that the sensitivity of culture in comparison with 28 s PCR was 71% and specificity was 55%. Similarly, the sensitivity and specificity of culture compared to ITS PCR were found to be 42% and 94%, respectively. Interestingly, the sensitivity and specificity of direct microscopy in comparison with 28 s PCR were 56% and 55%, respectively, while in comparison with ITS PCR they were 25% and 100%, respectively (Fig. 1).

Discussion

The global pandemic of COVID-19 has resulted in a rapid curve shift of infected patients, increasing death rates, economic burden and widespread mobilization of medical resource across the globe [14, 15]. It has been reported that that the risk for severe illness with COVID-19 increases with age and chronic disease [16] along with vaccine hesitancy and resistance [17]; hence, we saw a spurt of ROM during the pandemic. Definitive diagnosis of rhino-orbital mucormycosis is frequently delayed because of its current reliance on clinical examination and radiographic features [1, 13]. These features are only possible to detect at an advanced stage of the disease and in many cases are non-specific. Even when samples are obtained from

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**Fig. 1** Microscopic image of Mucorales and amplified products of DNA from patient orbital samples. **A** Microscopic image of aseptate, broad fungal filaments showing ribbon-like aseptate hyphae with calcofluor white stain under fluorescence microscope. Magnification 400×. **B** 1.2% gel showing amplification of a fragment of the 28S rDNA from Zygomycetes. Lane 1 DNA marker; Lane 2 positive control; Lane 3 *Rhizopus microsporus* (L-1778); Lane 4 negative control, **C** 1.2% gel showing amplification of ITS region from Zygomycetes. Lane 1 DNA marker; Lane 2 positive control (DNA from *Rhizopus* sp. grown on culture); Lane 3 *Rhizopus microsporus* (L-1968); Lane 4 *Rhizopus oryzae* (L-1880); Lane 5 PCR negative (L-1584); Lane 6 *Rhizopus microsporus* (L-1778); Lane 7 negative control.
patients, the definitive diagnosis is reliant on culturing methods or histopathological examination. It is known that the culture of biological samples from patients with mucormycosis often yields negative results because of hypha fragmentation during sample processing [12, 13]. Even when culturing the organism from a biological sample, the information is rarely sufficient to establish mucormycosis diagnosis because Mucorales are ubiquitous, may colonize healthy people and are frequent laboratory contaminants [12, 18]. Moreover, microscopic/histopathological examination requires some level of expertise to identify Mucorales as ribbon-like aseptate hyphae branching at 90° angles [1, 19, 20]. Finally, unlike the galactomannan and β-1,3-d-glucan tests that are commonly used to diagnose aspergillosis [10, 19, 21, 22], there is no approved serological test for mucormycosis. Alternatively, a recent article described the use of a serum disaccharide by mass spectroscopy showing a high degree of detection of this biomarker in nine of ten mucormycosis serum samples. However, this assay does not discriminate between invasive mucormycosis, aspergillosis or candidemia because of its panfungal nature [10]. Timely diagnosis of mucormycosis and distinction from invasive aspergillosis is critical as clinical signs and underlying conditions of mucormycosis and invasive Aspergillosis are similar, but the antifungal treatments may differ [3, 10, 13]. For these collective reasons, definitive mucormycosis diagnosis cannot be established in many cases and a simple, reliable and rapid assay of mucormycosis diagnosis is required.

Recently, there have been a fundamental change in the management of these conditions which includes the development of molecular methods [7]. Molecular diagnosis provides opportunity for understanding the presence and diversity of fungi in clinical specimens, and it is being explored in multiple studies to overcome the low sensitivity, difficulties in identification and long turnaround time of the classical diagnosis [1, 11, 13]. In the present study, we evaluated the PCR-based technique for the rapid and accurate detection of ROM in orbital samples post-COVID-19. While ITS-2 region amplification and sequencing was able to identify mucormycosis in 16% of cases, 28 s rDNA-based PCR was able to identify in 52% of the clinically suspected cases, compared to 7/25 samples (28%) that grew in culture. Even though ITS-2 PCR is generally superior to other fungal primers, because of its panfungal nature it can show lower sensitivity in fungal detection, as shown our study for Mucorales. In comparison, 28S rDNA primer showed higher sensitivity (85.71%), but despite its higher positivity rate, the 28S rDNA-based primer could not identify some of the samples, possibly due to low load or mixed infection with Aspergillosis. Additionally, we calculated sensitivity taking molecular result as true positive comparing it with culture and our result showed that the sensitivity of 28 s and ITS was found to be 71% and 42%, respectively. This could also be traced back to the fragile and delicate nature of the aseptate hyphae of Mucorales, which can be easily destroyed during tissue processing [13, 20]. Additionally, we also found in our study group, one patient with confirmed mucormycosis by microbiological work-up (culture and smear), but was PCR negative by both methods, indicating presence of PCR inhibitors in these samples. In accordance with the previous studies, R. oryzae was the major causative agent for mucormycosis in our study [13, 20] followed by R. microsporus. Limitations of the study include cost of molecular identification and inability to distinguish between mixed fungal infections. Nevertheless, 28S rDNA-based PCR is a promising tool for improved and efficient diagnosis of ROM, although further clinical studies are needed for a comprehensive validation to assess its utility in routine ocular microbiology laboratories. The health crises caused by COVID-19 should focus on consistent cooperation between the developed, developing and the underdeveloped countries to overcome this challenging pandemic [23].

**Conclusion**

The goal of this study was to evaluate a diagnostic workflow for improved fungal identification (mucormycosis) using molecular techniques. When compared to the classical and ITS method of identification, 28S rDNA-based PCR stands as a reliable and sensitive method for early diagnosis of mucormycosis. Molecule-based diagnostic assays can be recommended for identifying the presence and diversity of fungi in clinical specimens, and after proper standardization, it can readily be integrated gradually for full laboratory automation.
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Author contributions Conception or design of the work - 2 and 5Sample collection - 3 and 4Data analysis and interpretation - 1Primary draft of the manuscript - 1Supervision of the study - 5Final validation and manuscript editing - 2, 3, 4 and 5

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Declarations

Conflict of interest All authors declare that they have no conflict of interest.

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