

Original Article

Molecular phylogenetic analysis of paranasal sinus tissue-associated fungal pathogens acquired from COVID-19 patients

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ABSTRACT

Objectives: During the second wave of the COVID-19 pandemic, a rapid increase in cases of mucormycosis (MM), or black fungus-associated infection, has been reported throughout India. Many of the immunocompromised individuals with substantial use of steroids, oxygen therapy, and patients with comorbidities such as diabetes mellitus, hypertension, ischemic heart disease, and pneumonia were at high risk of getting MM as a serious co-infection along with COVID-19. Even after the hype around MM and other fungal pathogens in COVID-19 patients, they still largely remain neglected and least studied infections.

Materials and Methods: Here, we report the *internal transcribed spacer (ITS)-5.8S* ribosomal DNA (rDNA) region-based molecular phylogenetic characterization of ten pure fungal cultures isolated from paranasal sinus tissue samples of COVID-19 patients clinically and radiologically suspected of invasive fungal infection. From all the fungal isolates, whole genomic DNA was extracted, polymerase chain reaction (PCR) amplified, and subjected to sequencing with primers specific for the *ITS* gene.

Results: All ten sequences generated were above 550 base pairs in length, and these quality-checked sequences were deposited in the National Center for Biotechnology Information GeneBank. Molecular phylogenetic analysis revealed that the ten fungal sequences (accession numbers OR140558–OR140567) generated during the current study belonged to four broad clusters of *Rhizopus delemar*, *Rhizopus microsporus*, *Flavodon* spp., and *Aspergillus flavus*.

Conclusion: We suggest that ITS-5.8S rDNA region-based molecular identification tools, such as PCR and Sanger sequencing, are suitable for early diagnosis and identification of fungal pathogens in most of the immunocompromised patients during the viral epidemic and/or pandemic situations. Increased awareness amongst clinicians and public health providers about including these molecular techniques for early and accurate diagnosis of invasive fungal infections is required.

Keywords: Aspergillosis, COVID-19, Immunocompromised patients, *Internal transcribed spacer* gene, Mucormycosis, Phylogenetic analysis

INTRODUCTION

The pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that started in late 2019 in Wuhan, China, has still not been completely controlled globally; however, hospitalization of patients and the intensity of symptoms caused by SARS-CoV-2 infection have reduced. On May 5, 2023, the World Health Organization declared that COVID-19 is no longer a global health emergency.^[1] More than 600 million COVID-19 confirmed cases have been reported globally, with around 6 million confirmed deaths. In India, the first wave began in March 2020 and lasted till November 2020, while the second wave of the coronavirus pandemic, which emerged between March and May 2021 in India, has been reported to have the most

severe consequences in the form of an increasing number of cases, deficiencies in essential treatments, and consistently rising mortality rate, particularly in young patients.^[1,2] These severe consequences were observed to be accompanied by an increase in the cases of opportunistic infections triggered by fungi, including the COVID-19-associated pulmonary aspergillosis (CAPA) and Coronavirus-associated mucormycosis (CAM).^[3-6] In a matter of three months, during the second wave of coronavirus disease, about 47,000 cases of CAM were reported in India.

Despite the absence of any clear or certain host factor, fungal co-infections such as endemic mycoses, oropharyngeal candidiasis, fusariosis, invasive pulmonary aspergillosis, and mucormycosis (MM) were notably observed in

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COVID-19 patients during the second wave of the pandemic throughout the Indian subcontinent.^[7-9]

MM is a set of infections produced by saprophytic, ubiquitous, and filamentous fungi belonging to the order Mucorales. MM has been reported to cause angioinvasive infections, which can cause non-suppurating necrosis and severe tissue damage.^[10-13] Another most commonly observed fungal co-infection in COVID-19 patients is the CAPA. Most of the CAPA cases reported are in the form of case reports, and the real burden or prevalence of CAPA in patients has not been well identified.^[3,14,15] High mortality rates have been reported to be associated with CAM and invasive aspergillosis. CAM can also be mistaken for CAPA, specifically in patients with COVID-19-induced acute respiratory distress syndrome. In such patients, accurate and timely diagnosis becomes critical for preventing the fatal outcome.^[16]

The methods that are currently utilized for the diagnosis of fungal infections in most clinical laboratories are traditional, based on staining and morphological identification of cultures, which may have technical limitations.^[17,18] Thus, the use of molecular methods based on amplification and sequencing of the ribosomal DNA (rDNA) *internal transcribed spacer* (ITS) gene has been recognized as a complementary alternative for the diagnosis of these clinical samples, which were previously discarded as unidentifiable specimens due to the lack of phenotypic characterization.^[18,19] The primary advantage of techniques based on molecular biology is the accurate identification of fungal and bacterial isolates at the genus and species level, which contributes to a better understanding of the epidemiology of these clinically significant isolates.^[18,19] Thus, the current study was conducted to isolate the pure fungal cultures from COVID-19 patients, which were further subjected to molecular identification by polymerase chain reaction (PCR) and Sanger sequencing.

MATERIALS AND METHODS

Sample collection and clinical presentations

A total of 48 endoscopic paranasal sinus tissue samples, which were clinically and radiologically suspected of having post-COVID-19 fungal infections, were sent to the Department of Microbiology, Government Medical College and Hospital, Miraj, for microscopy and culture identification. Detailed clinical history and demographic characteristics of all the patients were recorded separately.

Culture separation and Staining

All the samples received were immediately processed for microscopy using 10% potassium hydroxide (KOH) wet mount test. These wet mounts were examined microscopically using $\times 10$ and $\times 40$ objectives. Cultures were performed on Sabouraud's dextrose agar (SDA) medium

after incubation for 3–5 days at 37°C; all the cultures were stained with lactophenol cotton blue stain and were observed under a compound microscope. Only the pure fungal cultures showing clear visible growth were further processed for molecular identification.

Ethical consideration

Before the onset of the study, written informed consent was taken from all the patients included in the current study, and detailed information regarding clinical, demographic characteristics, and comorbidities/risk factors involved was collected separately from all the patients. Our current study was a public health response study and was designed and performed in compliance with the Helsinki Declaration of 1975, as revised in the year 2000.

DNA extraction and PCR amplification

Approximately 1/cm² area from all the fungal cultures was removed from the agar slants and added to 2 mL of 0.9% sterile saline solution. All these samples were vortexed and kept for incubation at room temperature for 1 h. 120 μ L of this saline suspension was used further for DNA extraction. Whole genomic DNA from each of the fungal isolates was extracted using the standard phenol/chloroform extraction method. Extracted genomic DNA was subjected to PCR amplification using universal fungal primers of ITS1 [5'-TCC GTA GGT GAA CCT TGC GG -3'] and ITS4 [5'-TCC TCC GCT TAT TGA TAT GC -3'], targeting the ITS-5.8S rDNA region.^[18] PCRs were set up in duplicate with a 25 μ L reaction volume of PCR Master Mix (Promega Corp., Madison, WI, USA), 1 μ L of each ITS1 and ITS4 primer (10 μ M), and 1.5 μ L of template DNA sample. PCR reactions were set with an initial denaturation at 95°C for 5 min, followed by 38 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 10 min. Before sequencing, the PCR products were purified by the PEG-NaCl precipitation method.

Sanger sequencing

To determine the sequences of the ITS region, the amplicons were sequenced using the BigDye terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific). The sequencing procedure was carried out on an Applied Biosystems model automated DNA sequencer (ABI 3730 XL DNA Analyzer) at the National Center for Cell Science (NCCS), Pune, as per the manufacturer's instructions. Essentially, sequencing was carried out from both ends so that each position was read at least twice. Assembly of all the sequences was carried out initially using the Laser-Gen package; all these sequences were used for further analysis and interpretation.

Sequence analysis and construction of a phylogenetic tree

All the good quality sequences were subjected to the National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST) to identify the closest member of the fungal genus and species. For a better understanding of the phylogeny of these isolates, five homologous sequences per sample were downloaded from GeneBank. The total set of nucleotide sequences was aligned using the Muscle tool of the MEGA version 7.0 software.^[18,19] To generate the phylogenetic tree, the Tamura 3-parameter method was applied. The evolutionary relationship was inferred using the Neighbor-Joining method with 1000 bootstrap replications and the Tamura-Nei model for nucleotide substitution. After clear understanding and identification, the good quality sequences were deposited in the NCBI GenBank database, and accession numbers for these sequences were obtained from the NCBI.

RESULTS

Clinical and demographic details

Biopsy samples from 48 patients suspected of post-COVID-19-associated paranasal sinus tissue fungal infections were received at the Department of Microbiology for culture identification and microscopy. Demographic details for all the patients were recorded, and as per the observations, the mean age of the patients with fungal infection was observed to be 52 years (range 25–75 years). The male population was predominantly affected, and 37 (77.08%) out of 48 patients manifesting paranasal sinus tissue-associated fungal infections were male, and 11 (22.91%) were female. As per the clinical characteristics, majority of the patients presented with symptoms such as nasal crusting 30 (62.5%), headache 18 (37.5%), periorbital edema 12 (25%), dental pain 8 (16.66%), loss of vision 7 (14.58%), proptosis 6 (12.5%), ptosis 6 (12.5%), body ache 6 (12.5%), fever 5 (10.41%), breathlessness 4 (8.33%), numbness 4 (8.33%), and maxillary pain 2 (4.16%).

Details about comorbidities involved in these 48 patients were also recorded, and it was observed that 41 (85.41%) patients had diabetes mellitus as the most common comorbidity, followed by obesity 19 (39.58%), hypertension 14 (29.16%), cardiovascular disease 12 (25%), kidney disease 06 (12.5%), and liver dysfunction 02 (4.16%); however, none of these 48 patients were observed to be suffering from influenza co-infection or cancer [Table 1]. 35 patients out of 48 (72.91%) were observed to be above 50 years of age while 26 (54.16%) patients were provided with oxygen masks, corticosteroids were prescribed for 20 (41.66%) patients, and 02 (4.16%) patients were put on mechanical ventilation during the hospitalization period. These parameters were counted as risk factors involved in the progression of the paranasal sinus tissue-associated fungal infections.

Table 1: Number of patients manifesting post-COVID-19 rhino-orbital fungal infection affected with different comorbidities* and/or risk factors#.

Sr No	Type of comorbidity/risk factor	Patients with comorbidities out of the total 48 patients	Positivity percentage
1	Diabetes mellitus*	41	85.41
2	Advanced age (Above 50)#	35	72.91
3	Use of oxygen Mask#	26	54.16
4	Use of corticosteroids#	20	41.66
5	Obesity*	19	39.58
6	Hypertension*	14	29.16
7	Cardiac disease*	12	25
8	Kidney disease*	6	12.5
9	Liver dysfunction*	2	4.16
10	Use of mechanical ventilator#	2	4.16
11	Influenza co-infection*	0	0
12	Cancer*	0	0

*: Co-morbidities, #: Risk factors

Microbiological findings

All 48 tissue samples received at the department were immediately processed microscopically to observe the fungal growth. 33/48 (68.75%) samples demonstrated aseptate ribbon-like fungal hyphae with wide-angle branching at irregular intervals in the KOH mount; however, only 10 (20.83%) samples showed visible fungal growth on SDA slants after 3–5 days of incubation at 37°C. The number of fungal cultures that could be grown on SDA culture medium was less compared to the fungal filaments observed in the KOH mount test; this could have happened due to the excess grinding or homogenization of the tissue sample, which may have damaged the fungal hyphae, and some fungal genera may require special culture conditions for their growth.

Sequencing and phylogenetic analysis

Ten pure fungal cultures that had shown visible growth on SDA medium were selected for further molecular studies. Whole genomic DNA was extracted from these ten fungal cultures, and each culture was subjected to PCR amplification with ITS1-ITS4 primer pairs, which resulted in 600–700 base pairs (bp) of PCR products. Each PCR product was purified using the PEG-NaCl precipitation method and was subjected to Sanger sequencing. Sequencing of all ten PCR products was performed at NCCS Pune as per the manufacturer's instructions.

The sequences generated were uploaded to the NCBI BLAST, and accession numbers for these sequences were obtained

(accession numbers OR140558–OR140567). The NCBI BLAST results revealed that the sequence similarities for all the sequences analyzed were above 99%. The Similarity percentage and the closest match for the isolates are shown in Table 2.

As per the phylogenetic analysis of the ITS-5.8S rDNA, sequences were clustered under four different clades [Figure 1] of *Rhizopus delemar/arrhizus*, *Rhizopus microsporus*, *Aspergillus flavus/oryzae*, and *Flavodon Flavus*. Seven out of ten sequences were clustered under the genus *Rhizopus*, whereas two sequences were under the genus *Flavodon* and one sequence under the genus *Aspergillus*. The genus *Rhizopus* shows a separation into two different clades with strong bootstrap support.

DISCUSSION

The multiple waves of the COVID-19 pandemic have disturbed the healthcare and financial systems worldwide. Symptomatically, COVID-19 has been reported to be similar to influenza virus infection, whereas advancing age and excessive use of corticosteroids have been related to the increased severity of COVID-19 disease, which may also have been the reason behind the rapid increase of fungal co-infections in these patients during the pandemic.^[5,6] As per our findings, comorbidities such as diabetes mellitus, obesity, hypertension, cardiovascular disease, and kidney-related disease were the most common comorbidities contributing to the invasive fungal infections in COVID-19 patients.

In general, the diagnosis of paranasal sinus tissue-associated fungal infections is delayed because the current diagnosis procedures depend on the radiological findings and clinical examination of the patients. Even if the tissue samples are collected, the definitive diagnosis is dependent on culture and histopathological examination.^[18,19] It is a known fact that most of the fungal hyphae are delicate and can get disintegrated during the processing of samples, which may not yield positive results in the culture identification method. Moreover, there is no specific serological test for the detection of most of these fungal infections, including MM.^[18] For these collective reasons, a reliable and rapid test for the diagnosis of fungal infections during epidemic or pandemic situations is required. Molecular diagnosis has recently emerged as a rapid and reliable technique for the identification and characterization of fungal pathogens from clinical specimens.^[18-20] In the current study, we evaluated the PCR and sequencing-based molecular diagnostic techniques for accurate detection of invasive fungal isolates from patients with post-COVID-19-associated paranasal sinus fungal infections.

As per our sequencing and phylogenetic analysis, *R. delemar* was identified as the major causative agent for the paranasal sinus tissue-associated MM, followed by *R. microsporus*. Seven out of ten isolates were identified as belonging to the *Rhizopus* genus. *Rhizopus* has been reported to grow rapidly in patients with diabetic conditions. In such patients, phagocytosis is significantly impaired, which may help in the colonization and survival of invasive infectious agents.

One out of ten isolates from our study clustered with the *A. flavus* group of sequences, while the other two showed alignment with the *F. flavus* group. Opportunistic fungal species, including *Aspergillus* and *Flavodon* species, are generally harmless to the host.^[14] With the abnormal changes in the patient's immune conditions, these opportunistic fungi become pathogenic and can further cause invasive fungal infections. *Aspergillus* species are known to cause a series of pulmonary infections, such as pulmonary aspergillosis, CAPA, allergic bronchopulmonary aspergillosis, fungal asthma, and a few others.^[16] Our clinical and molecular findings related to the CAM and CAPA are in accordance with previous reports.

We further suggest that a more detailed and comprehensive study must be designed to understand the effect of fungal co-infections in comorbid and immunocompromised individuals so as to develop a comprehensive management plan that would help general physicians provide appropriate treatment and reduce the mortality rate in such patients.

Table 2: NCBI-BLAST-based sequence similarity and closest match analysis for all ten fungal isolates.

Sr No	Isolate code	Closest match to the isolate	NCBI BLAST sequence similarity percentage
1	Mu_CoV_1	<i>Aspergillus flavus</i>	99% and above
2	Mu_CoV_2	<i>Rhizopus microsporus</i>	99% and above
3	Mu_CoV_3	<i>Rhizopus delemar</i>	99% and above
4	Mu_CoV_4	<i>Rhizopus arrhizus</i>	99% and above
5	Mu_CoV_5	<i>Ceriporia sp. BAB-5052</i>	99% and above
6	Mu_CoV_6	<i>Rhizopus microsporus</i>	99% and above
7	Mu_CoV_7	<i>Rhizopus arrhizus</i>	99% and above
8	Mu_CoV_8	<i>Rhizopus microsporus</i>	99% and above
9	Mu_CoV_9	<i>Rhizopus delemar</i>	99% and above
10	Mu_CoV_10	<i>Flavodon flavus</i>	99% and above

NCBI: National center for biotechnology information, BLAST: Basic local alignment search tool

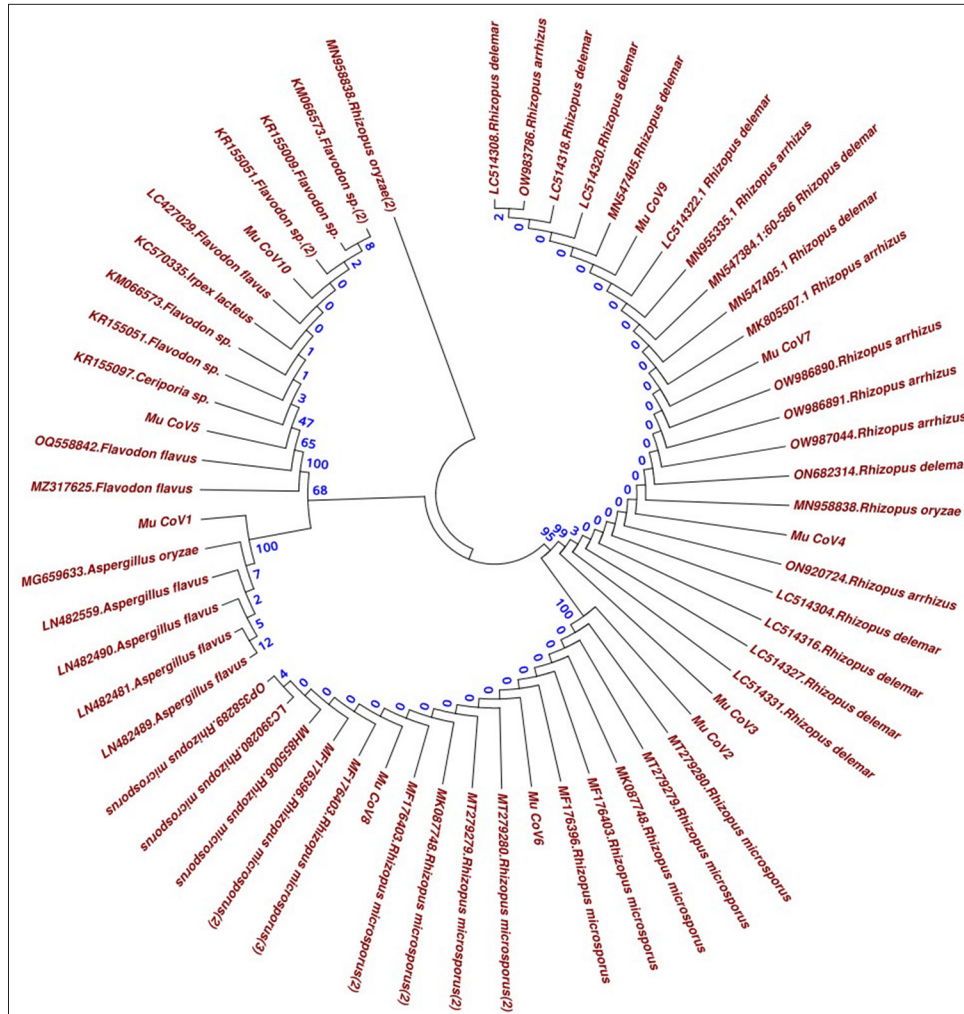


Figure 1: Cladogram showing the evolutionary relationship of ten sequences or taxa generated during the current study as compared with their corresponding sequences retrieved from the National Center for Biotechnology Information GeneBank. The evolutionary history was inferred using the Neighbor-Joining method.

Limitations of the study

All the tissue specimens reported in this study were collected during or immediately after the second wave of COVID-19; at that point in time, it was difficult to maintain a specific sample volume and sample quality. Further, due to some logistics issues prevailing during that period, uniformity in the collection and flow of the samples was challenging to manage, due to this we were not able to collect larger number of samples. Moreover, during the COVID-19 pandemic, due to some financial constraints, it was decided to study the molecular characterization for only those samples that showed positive results on the KOH mount and also showed substantial growth as a pure culture on the SDA medium.

CONCLUSION

The rise in COVID-19 cases around the world, specifically in developing countries, bears an additional risk for the inception of bacterial and fungal co-infections, such as the CAM and CAPA infections. Through our current study, we intend to assess a diagnostic workflow for the precise and rapid identification of fungal isolates obtained from patients with post-COVID-19-associated paranasal sinus fungal infections using PCR-based molecular techniques. The ITS-5.8S rDNA region-based molecular identification stands as a sensitive and reliable method for early diagnosis of various fungal pathogens from clinical specimens, including the CAM. Thus, we recommend the integration of molecular diagnostic assays as a regular laboratory practice for the

identification of fungi from clinical specimens, which will serve the general physicians and doctors in curbing the morbidity and mortality in patients during an epidemic and/pandemic situation.

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Ethical approval: The study was approved by the Institutional Review Board at Government Medical College Miraj, number GMC/IEC/C-5/2022, dated 7th October 2022.

Declaration of patient consent: The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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