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Original Article

Microbiology Section

Microbiological Genus Characterisation, Clinical Features, and Outcome in Patients of COVID-19 Associated Mucormycosis: A Single-centre Cross-sectional Study

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ABSTRACT

Introduction: Mucormycosis is a known invasive fungal infection, its prevalence increased during the Coronavirus Disease-2019 (COVID-19) pandemic, particularly during the second wave. The most common clinical form is rhino-orbital-cerebral, but other forms such as pulmonary, gastrointestinal, cutaneous, and disseminated forms also exist. There have been very few Indian studies exploring the various aspects of COVID-19 Associated Mucormycosis (CAM).

Aim: To describe and characterise the genus of the causative agents of mucormycosis, an invasive fungal infection, and to analyse the clinical features and outcomes in the study participants.

Materials and Methods: This was a cross-sectional study conducted in a dedicated COVID-19 Care Hospital at Dr. Vasantrao Pawar Medical College in Nashik City, Maharashtra, India. The study duration was one year, from February 2021 to January 2022. The universal sampling strategy included all clinically diagnosed cases of mucormycosis (n=104). The study variables included demographic factors, presence of risk factors, organ involvement, and in-hospital mortality. Diagnosis was based on fungal Potassium Hydroxide (KOH) mount and fungal culture. Genus characterisation was performed using Lactophenol Cotton Blue (LPCB) mount. Data were analysed using Statistical Package for the Social Sciences (SPSS) version 16.0, and the Chi-square test was applied to study the association between qualitative variables.

Results: The majority of patients with the disease were male (77, 74%). The most commonly involved site was isolated paranasal sinuses (48, 46.1%), followed by paranasal sinus with maxilla involvement (24, 23.1%). The KOH report was positive in 100 (96.2%) of patients. Fungal growth in culture was reported in 69 (66.3%) patients, among them *Rhizopus* spp. (66, z95.6%) being the most common fungal genus isolated. Among 25 patients in the age category >60 years, 21 (84%) had a positive culture (p-value=0.041, significant). Diabetic patients had a significantly higher incidence of mucormycosis compared to non diabetics based on culture results (p-value=0.004). The mortality rate in this study was 26 (25%).

Conclusion: Mucormycosis was more prevalent in males and predominantly affected the paranasal sinuses. Risk factors such as age category, sex, and a history of diabetes were significantly associated with the presence of mucormycosis. KOH can be used as a quick diagnostic test to initiate prompt treatment.

Keywords: Diabetes mellitus, Rhino-orbital-cerebral, Risk factors

INTRODUCTION

Mucormycosis is a rare invasive fungal infection. This fatal disease came to the limelight during Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) pandemic when patients with recovered or ongoing severe COVID-19 started developing this invasive fungal infection as a post-COVID-19 complication, especially during the second wave. The disease is caused by Mucormycetes of the order Mucorales, as well as zygomycotic species. Hence, the disease is also called Zygomycosis [1]. Rhino-orbital-cerebral is the most common clinical form, and other clinical forms like pulmonary, gastrointestinal, cutaneous, and disseminated forms also exist. The prognosis for these patients depends on early treatment in the form of aggressive surgical debridement and systemic antifungals. There are many predisposing factors for the development of mucormycosis in COVID-19 patients. These include uncontrolled diabetes mellitus, immunosuppression by steroids, prolonged Intensive Care Unit (ICU) stay, post-transplant/malignancy, and voriconazole therapy, etc., [2,3]. It is a known fact that invasive mucormycosis has a very high mortality rate, and therefore establishing the correct diagnosis in a timely manner is of utmost importance. The diagnosis of mucormycosis is mainly based on clinical features, which are generally non specific, and investigations like radiographic imaging, which can determine the organ involvement and extent of the disease. The definitive diagnosis and identification of the aetiological agent are established only through fungal microscopy, fungal culture, and histopathology on appropriate biopsy samples. According to the "One World One Guideline" by the European Confederation of Medical Mycology (ECMM), appropriate imaging is strongly recommended to document the extent of the disease when mucormycosis is suspected. Biopsy is also strongly recommended for microscopy and culture if mucormycosis is a potential diagnosis [4]. Culture of specimens is also strongly recommended for genus and species identification. Identification at the genus level is strongly supported for improved epidemiological understanding of mucormycosis [4].

There were very few Indian studies that have explored the various aspects of CAM and its risk factors [4,5]. The aim of the study was to describe and characterise the causative agents of mucormycosis, an invasive fungal infection. The objective of the study was to determine the association of various predisposing factors with the disease. This study highlights the most common aetiological agents of mucormycosis and stresses the importance of a multidisciplinary approach for diagnosis, including clinical features, radiographic imaging, fungal microscopic KOH mount, and fungal culture, for timely intervention and treatment of the disease.

MATERIALS AND METHODS

This was a cross-sectional study conducted in a dedicated COVID-19 Care Hospital at Dr. Vasantrao Pawar Medical College in Nashik City,

Maharashtra, India. The study duration was one year, from February 2021 to January 2022. Institutional Ethics Committee permission was obtained before commencing the study (Ref no. Dr.VPMCH&RC/IEC/17/2021-22, dated 25/06/2021). Informed consent was obtained from the study participants. During the study period, the city was reporting many cases of CAM. The patients included in the study were admitted to the institute from April to September 2021. It was a universal sampling approach, as all clinically diagnosed cases of mucormycosis who met the eligibility criteria were included in the study (n=104).

Inclusion criteria:

a) Clinically suspected cases of mucormycosis as per Indian Council of Medical Research (ICMR) Advisory [6] admitted to the ENT ward or presenting to the Ear, Nose and Throat (ENT) OPD or referred from other hospitals, irrespective of age and sex.

b) Past or present documented history of COVID-19 (RT-PCR or Antigen positive).

c) Willingness to participate in the study.

Exclusion criteria: Documented history of mucormycosis in the past was excluded from the study.

Study Procedure

All participants were personally contacted, and interviews were conducted using a predesigned proforma. Moribund patients' indoor records were screened for data collection. Appropriate diagnostic samples were collected from all these patients as per the protocol and sent to the Microbiology Department, where microscopy and fungal culture were performed.

Study variables:

a) Demographic factors: Age, sex, and place of residence of all participants included in the study were analysed.

b) Presence of risk factors [7]: Diabetes mellitus, Systemic corticosteroids, Basal blood sugar levels on admission, supportive oxygen therapy, and ICU admission were the risk factors studied for their association with the disease.

c) Clinical syndromes: The type of organ involvement, as per radiological imaging, such as Rhino-orbital-cerebral, Rhino-orbital, Rhino-cerebral, Sinusitis alone, etc.

d) In-hospital mortality.

Microbiological diagnosis:

Microscopy (KOH Mount): The most common samples obtained were nasal discharge, nasal scrapings, biopsy samples, and postoperative samples.

Culture: Each sample was inoculated on three slants of Sabouraud dextrose agar and incubated at 30°C for 14 days. Colonies were fast-growing, covering the surface of the agar, with dense cottony growth.

Genus: The genus of the isolated fungal agent in culture was subjected to LPCB for genus identification.

STATISTICAL ANALYSIS

All these parameters were analysed using appropriate statistical tools. Descriptive statistics, such as frequency tables, were used for descriptive data. Cross tabulations and Chi-square tests were used to test the association between qualitative variables. A p-value <0.05 was considered as a significant association, while a p-value <0.01 was considered as a highly significant association. Statistical analysis was performed using SPSS version 16 software.

RESULTS

A total of 104 patients were admitted to this hospital for the treatment of CAM during the study period. The results are compiled in the following tables.

The majority of patients were male (77, 74%), and the majority of patients were from rural areas (63, 60.6%). The mean age for males was 49.16±12.53 years, while for females it was 53.7±12.51 years [Table/Fig-1].

Parameters		n (%)
Sex	Male	77 (74)
	Female	27 (26)
Place of residence	Rural	63 (60.6)
	Urban	41 (39.4)
Age category (years)	15-30	5 (4.8)
	30-45	40 (38.5)
	45-60	34 (32.7)
	>60	25 (24)

[Table/Fig-1]: Baseline characteristics of patients with mucormycosis.

The most commonly affected site was the paranasal sinus, accounting for 48 cases (46.1%), followed by the paranasal sinus with involvement of the maxilla, which accounted for 24 cases (23.1%) [Table/Fig-2].

Affected site	n (%)
Paranasal sinus only	48 (46.1)
Paranasal sinus with maxilla involvement	24 (23.1)
Paranasal sinuses with orbital involvement	19 (18.3)
Paranasal sinuses with palatal involvement	2 (1.9)
Paranasal sinuses with intracranial involvement	4 (3.8)
Paranasal sinuses with orbital with intracranial	5 (4.8)
Only orbital involvement	1 (1)
Only intracranial involvement	1 (1)

[Table/Fig-2]: Affected site of mucormycosis among study participants.

The KOH test was positive in 100 participants (96.2%), compared to a culture positivity rate of 69 cases (66.3%). The most common genus isolated in culture was *Rhizopus* spp., accounting for 66 cases (95.6%) [Table/Fig-3].

Variables		n (%)
KOH test	Positive	100 (96.2)
	Negative	4 (3.8)
Culture	Positive	69 (66.3)
	Negative	35 (33.7)
Genus identification (N=69)	*Rhizopus* spp.	66 (95.6)
	Rhizomucour spp.	2 (2.9)
	Lichtheimia spp.	1 (1.5)

[Table/Fig-3]: Laboratory investigation results among study participants.

Among the 25 patients in the age category >60 years, 21 (84%) reported culture positivity, followed by 24 patients (70.6%) in the age group category of 45 to 60 years (N=34). It was observed that as age increases, the culture tends to be positive (p-value=0.041, significant). Among the 77 males, 46 (59.7%) were culture positive, compared to 23 (85.2%) culture positive cases among the 27 females, which was relatively higher. The observed difference was found to be statistically significant (p-value=0.016).

Among the 34 patients with a history of diabetes, 29 (85.3%) reported positive culture results, while out of the 70 non diabetic patients, 40 (57.1%) reported culture positivity. Diabetic patients had a significantly higher incidence of mucormycosis compared to non diabetic patients by culture (p-value=0.004). Although the proportion of culture positive cases was higher, with 36 (72%) patients having higher sugar levels at the time of admission compared to 17 (58.6%) patients with normal sugar levels, the association was found to be statistically non significant. Long-standing steroid use is a major

risk factor for mucormycosis, but in this study, it was found to be statistically not significant, with 35 cases (71.4%) among those who underwent steroid therapy (N=49) compared to 34 cases (61.8%) among those who did not receive steroids (N=55) [Table/Fig-4].

Risk factor		Culture growth present	Culture growth absent	Total	(N=104) p-value*
Age (years)	15-30	2 (40%)	3 (60%)	5 (100%)	0.041 significant
	30-45	22 (55%)	18 (45%)	40 (100%)	
	45-60	24 (70.6%)	10 (29.4%)	34 (100%)	
	> 60	21 (84%)	4 (16%)	25 (100%)	
	Total	69 (66.3%)	35 (33.7%)	104 (100%)	
Sex	Male	46 (59.7%)	31 (40.3%)	77 (100%)	0.016 significant
	Female	23 (85.2%)	4 (14.8%)	27 (100%)	
	Total	69 (66.3%)	35 (33.7%)	104 (100%)	
Place of residence	Rural	40 (63.5%)	23 (36.5%)	63 (100%)	0.445 not significant
	Urban	29 (70.7%)	12 (29.3%)	41 (100%)	
	Total	69 (66.3%)	35 (33.7%)	104 (100%)	
History of diabetes	Present	29 (85.3%)	5 (14.7%)	34 (100%)	0.004 highly significant
	Absent	40 (57.1%)	30 (42.9%)	70 (100%)	
	Total	69 (66.3%)	35 (33.7%)	104 (100%)	
					N=79**
Blood sugar level random	High (>200 mg/dL)	36 (72%)	14 (28%)	50 (100%)	0.223 not significant
	Normal (<200 mg/dL)	17 (58.6%)	12 (41.4%)	29 (100%)	
	Total	53 (67.1%)	26 (32.9%)	79 (100%)	
					(N=104)
Steroid use	Present	35 (71.4%)	14 (28.6%)	49 (100%)	0.301 not significant
	Absent	34 (61.8%)	21 (38.2%)	55 (100%)	
	Total	69 (66.3%)	35 (33.7%)	104 (100%)	
On Oxygen during COVID-19 admission	Present	41 (70.7%)	17 (29.3%)	58 (100%)	0.293 not significant
	Absent	28 (60.9%)	18 (39.1%)	46 (100%)	
	Total	69 (66.3%)	35 (33.7%)	104 (100%)	
Ventilator during COVID-19 admission	Present	7 (87.5%)	1 (12.5%)	8 (100%)	0.188 not significant
	Absent	62 (64.6%)	34 (35.4%)	96 (100%)	
	Total	69 (66.3%)	35 (33.7%)	104 (100%)	

[Table/Fig-4]: Association of some risk factors associated with mucormycosis.
*Statistical test used: Chi-square test; **Blood sugar level of 25 patients were not available so N=79

It was found that 23 (33.3%) culture-positive patients among the 69 succumbed, compared to only 3 (8.6%) out of 35 culture-negative patients. There was a significantly strong association (p-value=0.006) between culture positivity and mortality. The proportion of mortality among males was 19 (24.7%) out of 77, which is comparable to females with 7 (25.9%) out of 27. The association between gender and mortality was not significant (p-value=0.897).

The proportion of mortality was highest in the age group > 60 years, with 11 (44%) out of 25 patients, followed by the age group of 45 to 60 years with 8 (23.5%) out of 34 patients, and 7 (17.5%) out of 40 patients in the age group of 30 to 45 years. There was significant association between age group and mortality (p-value=0.043) [Table/Fig-5].

Risk factor		Clinical outcome		Total	(N=104) p-value*
		Death	Discharged/ Recovered		
1. Culture report	Positive	23 (33.3%)	46 (66.7%)	69 (100%)	0.006 highly significant
	Negative	3 (8.6%)	32 (91.4%)	35 (100%)	
	Total	26 (25%)	78 (75%)	104 (100%)	
2. Sex	Male	19 (24.7%)	58 (75.3%)	77 (100%)	0.897 not significant
	Female	7 (25.9%)	20 (74.1%)	27 (100%)	
	Total	26 (25%)	78 (75%)	104 (100%)	
3. Age (years)	15-30	0	5 (100%)	5 (100%)	0.043 significant
	30-45	7 (17.5%)	33 (82.5%)	40 (100%)	
	45-60	8 (23.5%)	26 (76.5%)	34 (100%)	
	>60	11 (44%)	14 (56%)	25 (100%)	
	Total	26 (25%)	78 (75%)	104 (100%)	

[Table/Fig-5]: Factors associated with mortality among study participants.
*statistical test used: Chi-square test

DISCUSSION

COVID-19 has been the most disastrous pandemic, looming over the world for almost two and a half years now. CAM, which is the most devastating fungal disease associated with COVID-19, came into the limelight during the second wave of the pandemic. This fungal infection is not unusual in India, as the case rate in the pre-COVID-19 era was estimated to be 70 times higher compared to the developed world [8,9]. The incidence of mucormycosis started increasing during the first wave itself. A multicentre retrospective study across India, conducted to evaluate the epidemiology and outcomes among cases of CAM during September-December 2020, showed a 2.1-fold rise in mucormycosis during the study period compared to September-December 2019 [9].

In the present study, 104 patients were analysed with CAM admitted and treated in the hospital.

Among the baseline characteristics of the patients, 77 (74%) of the patients affected by CAM were men, 63 (60.6%) were from rural areas, and 40 (38.5%) belonged to the 30-45 year age group, followed by 45-60 years with 34 (32.7%) patients, respectively. The mean age at which males were affected was 49.16 years. Present study findings were similar to a multicentre study conducted by Patel A et al., and a systematic review of the literature by Nagalli S and Kikkeri NS where they found that 74.6% and 77% of males were affected by the disease compared to females, respectively, and the most common age group affected was 56.9 and 54.9 years, respectively [9,10]. This gender predisposition and age group susceptibility could be because males are involved more in outdoor activities, and this fungus is a typically innocuous environmental fungus that is ubiquitous in nature and primarily affects immunocompromised patients.

Based on the anatomical site of involvement, mucormycosis is classified into Rhino-Orbito-Cerebral (ROCM), pulmonary, gastrointestinal, cutaneous, renal, and disseminated [11]. ROCM is the most common form and is often seen in patients with diabetic ketoacidosis or uncontrolled diabetes mellitus. In the present study, paranasal sinuses were the most commonly involved site (98%), either isolated (46.2%) or in combination with other sites such as the maxilla (23.1%), orbit (18.3%), palate (1.9%), intracranial (3.8%), and orbital with intracranial involvement (4.8%). These findings were corroborated by Nagalli S and Kikkeri NS where paranasal sinuses were involved in 79.4% of patients, with the maxillary sinus (47.4%) being the most frequently infected site [10].

It is known that the infection begins in the nose and paranasal sinuses due to the inhalation of fungal spores. The fungus then invades the arteries, leading to thrombosis, which subsequently causes tissue necrosis. Finally, the infection spreads to orbital and intracranial structures either through direct invasion or through the blood vessels.

The microbiological diagnosis of mucormycosis mainly relies on the direct visualisation of fungal structures in KOH mount and fungal culture, which are considered the gold standard tests for diagnosis [12]. In the KOH mount, the hyphae of Mucorales have a variable width (ranging from 6 to 25 μm), are non septate or pauci

septate, and exhibit an irregular, ribbon-like appearance [13]. The angle of branching varies and includes wide-angle bifurcations. Mucorales grow rapidly (3 to 7 days) on most fungal culture media, such as Sabouraud agar and potato dextrose agar, incubated at temperatures at 25°C and 30°C [14]. In this study, fungal microscopy yielded positive results in 96.2% of the tested samples, while culture positivity was found to be 66.3%. When samples are received in the laboratory, they typically undergo microscopy examination first, followed by culture. Preliminary examination of the sample in the form of a simple KOH mount can be an excellent tool for making a definitive diagnosis and can prove to be lifesaving for the patient. A review article by Skiada A et al., states that even when fungal hyphae are seen in microscopy, fungal cultures are positive in only 50% of cases. Hyphae are fragile in nature and may be damaged during tissue manipulation (avoidance of excessive tissue homogenisation is recommended) [15,16]. The most commonly isolated fungal agent on culture was *Rhizopus* spp. (95.6%), followed by *Rhizomucor* spp. (2.9%) and *Lichtheimia* spp. (1.5%), respectively. Identification of Zygomycetes organisms to the genus and species level still relies on colonial and microscopic morphology on culture. Identification carries valuable epidemiological, therapeutic, and prognostic implications. For example, *Rhizopus oryzae* is the most common zygomycete recovered from clinical specimens but tends to exhibit in-vitro resistance to posaconazole [16].

In the present study, the association of some risk factors associated with mucormycosis with fungal culture positivity were analysed. Among the 25 patients in the age category >60 years, 21 (84%) reported positive in culture, followed by 24 patients (70.6%) out of 34 in the age group category of 45 to 60 years. It was observed that as age increases, the likelihood of positive culture results also tends to increase (p-value=0.041, significant). The geriatric age group is more prone to severe disease due to the presence of risk factors such as diabetes mellitus, severe COVID-19, and the use of steroids [7]. Among the 34 patients with a history of diabetes, 29 (85.3%) reported positive in culture, while out of the 70 non diabetic patients, only 40 (57.1%) reported culture positive. Diabetic patients reported a significantly higher rate of mucormycosis compared to non diabetic patients by fungal culture (p-value=0.004). CAM caused a devastating situation, particularly in India, possibly due to the high incidence rate of type 2 diabetes. Mononuclear and polymorphonuclear phagocytes are the first line of defense against the inhaled spores of Mucorales, producing oxidative metabolites and cationic peptide defensins to prevent their invasion into deeper tissues [17]. Steroids and hyperglycaemia impair the ability of phagocytes to respond to invading organisms, leading to impaired chemotaxis, dysfunctional phagocytes, and defective intracellular killing [18]. Ketoacidosis associated with uncontrolled diabetes enhances the growth of mucormycosis due to the ketoreductase enzyme produced by the fungus in diabetic patients, allowing it to utilise the patient's ketone bodies [19]. Additionally, studies have shown that Mucorales utilise iron for growth, and ketoacidosis promotes the release of iron from its protein-bound form [20]. Although long-term steroid use is a major risk factor for mucormycosis, it was not found to be statistically significant in the present study, although the proportion of mucormycosis was 71.4% among those who underwent steroid therapy compared to 61.8% among those who did not. The widespread use of steroids and broad-spectrum antibiotics as part of the COVID-19 treatment regimen is known to exacerbate pre-existing fungal diseases. Authors experience highlights the significant association of uncontrolled blood sugars with the emergence of mucormycosis, even in the absence of long-term steroid therapy. Similar findings were noted in a recently published case series by Sarkar S et al., in which all patients were diabetic with elevated blood sugar levels and received steroids as per guidelines [21].

In the present study, mucormycosis was found to be fatal in 25% of the study participants, while 75% recovered and were discharged from the hospital. A systematic review of cases reported worldwide, including India, reported a mortality rate of 30.7% [22]. An interesting multicentre epidemiologic study of CAM in India showed that the combined 6-week mortality rate was 38.3%, and the 12-week mortality rate was 45.7% [9]. In the present study, the proportion of mortality was highest in the age group >60 years (44%), followed by the age group of 45 to 60 years (23.5%), and 17.5% in the 30 to 45 years age group. There was a significant association between age group and mortality (p-value=0.043), with mortality increasing as age increases. The same multicentre epidemiologic study also reported similar findings [9].

It was found in the present study that 23 culture-positive patients (33.3%) succumbed to the disease compared to only 3 culture-negative patients (8.6%). There was a significantly strong association (p-value=0.006) between culture positivity and mortality. This can be attributed to the fulminant disease and high fungal load in these patients, leading to such a significant association. A high fungal load contributes to increased disease severity and mortality, which can only be addressed through timely diagnosis using clinical features, radiological imaging to determine the extent of infection, and planning appropriate intervention, including fungal microscopy KOH mount, and fungal culture [23]. Therefore, a multidisciplinary approach is crucial for the correct and timely diagnosis of this rare invasive fungal disease. Molecular diagnosis for identifying the fungal agent, along with antifungal susceptibility testing, should also be prioritised to initiate treatment with appropriate antifungals. Furthermore, guidelines should be developed and implemented in all healthcare facilities to enable epidemiological data collection and rapid reporting of any outbreaks. Antifungal stewardship programs should be implemented to ensure appropriate prescription and use of antifungal agents, as well as control and monitoring of infections caused by clinically relevant pathogens in healthcare facilities. Rational use of corticosteroids should also be undertaken and monitored. Finally, strict protective measures must be implemented to prevent the dissemination of this life-threatening fungal pathogen.

Limitation(s)

The present study was a single-centre study, and in order to generalise the results, more centres need to be incorporated with a wider sample size.

CONCLUSION(S)

Mucormycosis tends to be common among men as well as the older age group. The presence of diabetes predisposes patients to mucormycosis, so special care needs to be taken if the patient has diabetes. The paranasal sinuses are most commonly involved, so they should be screened radiologically at the earliest. The preliminary examination of the sample in the form of a simple KOH mount can be an excellent tool for quick and definitive diagnosis of mucormycosis. Culture-positive patients exhibit higher mortality. Thus, a multidisciplinary approach is needed for the correct and timely diagnosis of this rare invasive fungal disease.

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