Original Article

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MALDI-TOF-MS for Rapid Detection of Fungi causing Mucormycosis during COVID-19 Pandemic from a Tertiary Care Hospital in Southern India: A Retrospective Cohort Study

Microbiology Section

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ABSTRACT

Introduction: Mucormycosis is an opportunistic fungal infection that became a public health emergency during the second wave of Coronavirus Disease-2019 (COVID-19). It is an acute, angioinvasive, opportunistic, and emerging mycosis caused by mucormycetes, resulting in a significantly fatal fungal infection among immunocompromised and/or immunosuppressed individuals. Mucormycosis has a specific predilection for blood vessels (angioinvasive) and tissues, leading to extensive necrosis and thromboembolic events. Globally, the incidence rate of mucormycosis varies from 0.005 to 1.7 per million people. Early diagnosis and appropriate therapy are crucial for clinical outcomes.

Aim: To evaluate the mycological profile of mucormycosis during the COVID-19 pandemic using automated Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectroscopy (MALDI-ToF-MS) in comparison with conventional fungal identification methods.

Materials and Methods: A retrospective cohort study was conducted, collecting data from July 2020 to June 2022, involving 1,176 patients from various clinical departments who attended PSG Hospitals affiliated with PSG Institute of Medical Sciences and Research, Coimbatore, Tamil Nadu, India. Data analysis was performed from July 2022 to August 2022. Clinical specimens, including Bronchoalveolar Lavage (BAL), Sputum, Cerebrospinal Fluid (CSF), nasal swabs, tracheal aspirates, pus, blood, corneal scraping, eye discharge, and necrotised tissues, were subjected to Potassium Hydroxide (KOH) mount, conventional culture coupled with micrometry-aided microscopy, and MALDI-ToF-MS among suspected mucormycosis cases. The results were collected in Microsoft Excel, and data analysis was conducted using the International Business Machines Statistical Package for the Social Sciences statistical software (IBM SPSS) version 24.0. The study variables were expressed as descriptive statistics: frequency (N) and percentage (%). The incidence of mucormycosis, mycological profile, and diagnostic utility of conventional micrometry-aided microscopy-assisted culture were compared with the automated MALDI-ToF-MS protein signature method of testing.

Results: Fungal elements were positive in 130 (11.05%) out of a total of 1,176 clinical samples, such as necrotised tissue, biopsy, and sputum, in the KOH mount. Fungal culture was positive in 258 (21.94%) out of 1176 samples, with mucormycosis detected in 73 (28.29%) of the culture-positive samples. The incidence of mucormycosis in this study was 73 (6.21%) out of 1176. A total of 101 (8.59%) samples showed positive results for mucormycetes through KOH, culture, and MALDI-ToF-MS. MALDI-ToF-MS could rapidly and specifically identify and confirm the causative agents of mucormycosis at their genus and species levels compared to KOH or culture.

Conclusion: Simple, low-cost, and less laborious conventional culture coupled with micrometry-aided microscopy can detect Mucorales at the species level, where KOH microscopy could not differentiate between species. MALDI-ToF-MS can be employed for the rapid detection and confirmation of mucormycosis agents at the genus or species level.

Keywords: Aseptate hyphae, Early diagnosis, Fungal culture, Matrix assisted laser desorption ionisation time of flight mass spectrometry, Microscopy, Mucorales

INTRODUCTION

Mucormycosis is an acute, angioinvasive, opportunistic, and emerging mycosis caused by mucormycetes, resulting in a significantly fatal fungal infection among immunocompromised and/or immunosuppressed individuals [1,2]. Factors such as age, uncontrolled Diabetes Mellitus (DM), neutropenia, severe burns, prolonged steroid therapy, intravenous drug abuse, malnutrition, iron overload conditions, and organ transplantation are considered significant risk factors for mucormycosis [1-3]. Other high-risk groups include those living with the Human Immunodeficiency Virus (HIV) and those on immunomodulating drugs such as the antifungal voriconazole [4]. Mucormycosis mainly presents as sino-orbital, rhino-cerebral, pulmonary, cutaneous, gastrointestinal, and disseminated infections [1-3]. Mucormycosis has a specific predilection for blood vessels (angioinvasive) and tissues, resulting in extensive necrosis and thromboembolic events. Globally, the incidence rate of mucormycosis varies from 0.005 to 1.7 per million people. The estimated prevalence of mucormycosis in India, derived by computational-model-based simulation, was found to be 140 per million people as of December 2020 [4-7]. This estimate is about 80 times higher than the prevalence rates of mucormycosis reported in developed countries [8]. The mortality rate of mucormycosis ranges between 20-96%, depending on the site and extent of involvement. The case fatality rate of patients with mucormycosis was 46%, with the highest rates noted in disseminated forms (68%) and the lowest rates found in cutaneous presentations (31%) of mucormycosis [8,9].

The timeline of the first wave of the COVID-19 pandemic extended from April 1, 2020, to January 31, 2021, for a period of 10 months, followed by the second wave, which lasted from March 1, 2021, to June 30, 2021, for a period of three months. India accounted for 71% of global mucormycosis cases from December 2019 to

April 2021 [7]. Mucormycosis cases emerged rampantly during the second wave of COVID-19, with pre-existing diabetes mellitus as the predominant risk factor, making it a notifiable disease causing an incipient mucormycosis epidemic. The Government of India declared an "epidemic of mucormycosis" in many states of India during the second wave of COVID-19 pandemic. The condition of mucormycosis was known as black fungus and considered a "notifiable disease" or "alert" due to its immediate threat to life, declared as a public health emergency [10]. The main cause of the high incidence of mucormycosis was the excessive use of steroids for treating COVID-19 and the immunosuppressive properties of the virus [9,11]. Early diagnosis and appropriate early surgical and/or medical therapy played a significant role in clinical outcomes. Though the culture identification of Mucorales species is not of much importance in terms of therapeutic management, it still provides us with details about individual species. Conventional micrometry-aided microscopy-assisted culture of mucormycosis is an epidemiological tool and is much cheaper, rapid, and does not require very costly equipment, unlike MALDI-ToF-MS, which is the novelty and importance of this study. The conventional techniques for identifying fungi are based on morphological, biochemical, and/or immunological characteristics and can take 2-5 days or longer [12]. For definitive interpretations, it is frequently necessary to combine multiple phenotypic techniques [13]. Due to the biological complexity of fungal agents, research into them is generally challenging, mainly due to the co-existence of multiple fungal phenotypes (hyphae and/or conidia) in the same organism [14]. In these situations, KOH mount is a technique that helps directly examine the ribbon-like structures called hyphae in fungi [15].

The clinical course and outcome can vary among different members and according to the involved anatomical location. Late diagnosis, misdiagnosis, or untreated mucormycosis without necessary surgical debridement of affected parts, along with specific intravenous antifungals, can result in potentially significant and fatal fungal infections. Since mucormycosis is an aggressive and life-threatening infection, it requires quick and accurate identification of the infecting fungus species, followed by treatments such as antifungal medications and surgery [8,15]. The diagnosis of mucormycosis through the KOH mount examination has limitations as it cannot identify fungi at the genus and species level [16,17]. Another emerging technique is MALDI-ToF-MS, which allows for early, more reliable, and rapid laboratory diagnosis of mucormycosis, making it a potential tool compared to other conventional methods [16]. Conventional micrometry-aided Lactophenol Cotton Blue (LPCB) microscopy-assisted culture of mucormycosis seems to be a simple and more precise diagnosis of clinically suspected mucormycosis, though Polymerase Chain Reaction (PCR) is considered the gold standard. MALDI-ToF-MS is also considered an alternative gold standard in cases where infrastructure is available. Histopathological Examinations (HPE) can be very helpful in the direct demonstration of fungi.

The purpose of this study was to evaluate the mycological profile of mucormycosis during the COVID-19 pandemic using automated MALDI-ToF-MS in comparison with conventional fungal identification methods which was the primary objective of the study. The secondary objectives of the study were to identify the agents causing mucormycosis from specimens received for KOH and/or LPCB stain, along with micrometry-aided microscopy of mucormycosis agents from positive mucormycosis cultures during the study period from the mycology lab register, Hospital Information System (HIS), and Medical Records Department (MRD).

MATERIALS AND METHODS

A retrospective cohort study was conducted from July 2020 to June 2022, involving a total of 1,176 samples received from 1,176 patients. Analysis of the data was done from July 2022 to August 2022. Ethical approval for the study was obtained from the Institutional Human Ethics Committee (IHEC) with reference number PSG/IHEC/2022/Appr/Exp/163; Project No. 22/164 dated 30-06-2022. A waiver of consent was applied for as there was no active involvement of patients in the study.

Inclusion criteria: Patients who were tested positive for SARS-CoV-2/COVID-19, had type 2 diabetes mellitus, and/or were under prolonged steroid use and were suspected or diagnosed with mucormycosis were included in the study.

Exclusion criteria: Patients who had been previously diagnosed or treated for mucormycosis within the last six months were excluded from the study.

Sample size calculation and justification:

Z=1.96 for 95% confidence interval and d=5%. Prevalence: 20% [18].

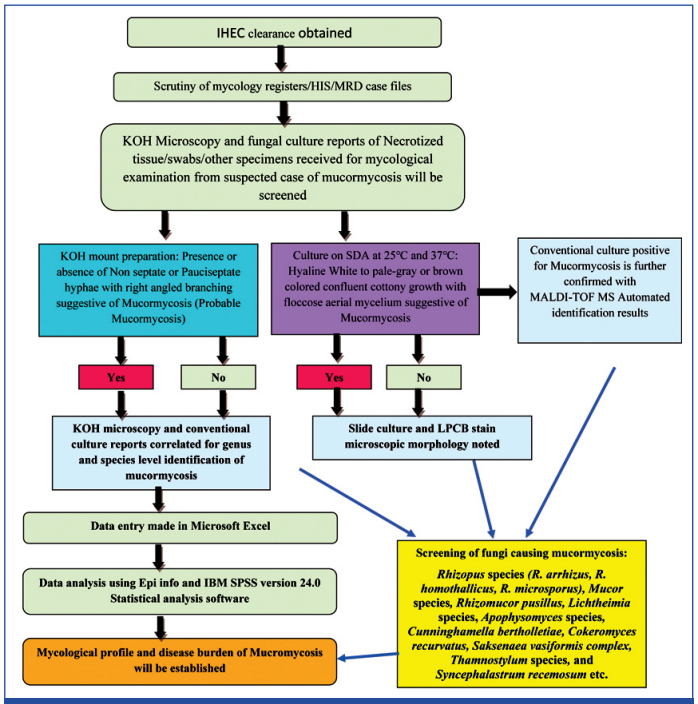
Desired sample size calculation: Probability.

$=1.96^2\times20\times(100-20)/5\times5=3.84\times1600/25=512$.

Therefore, the sample size to be included in this study was determined to be 1000 specimens.

Sample size included: 1176.

These patients, including both inpatients and outpatients, were clinically suspected or diagnosed with mucormycosis and were from various clinical departments such as General Medicine, Ophthalmology, Ear Nose Throat (ENT), and Dermatology at PSG Hospitals affiliated with PSG Institute of Medical Sciences and Research in Coimbatore, Tamil Nadu, India (as shown in [Table/Fig-1]).



[Table/Fig-1]: Diagnostic algorithm for the detection of Mucormycosis.
HIS: Hospital information system; MRD: Medical records department; KOH: Potassium hydroxide

Procedure

The clinical specimens, such as BAL, endo-tracheal aspirate, sputum, CSF, nasal swabs, tracheal aspirates, pus, blood, urine, pleural fluid, peritoneal fluid, drain fluid, corneal scraping, eye discharge, and biopsied tissue or necrotised tissues from affected areas (sinuses, orbit, face, nasal mucosa, nasal cavity, middle meatus, skin, mucosal debris, etc.), were subjected to diagnostic KOH mount, fungal culture and LPCB stain along with micrometry-aided microscopy of mucormycosis agents from positive mucormycosis culture from July 2020 to June 2022. The data collection was conducted during this period. The results of LPCB stain, along with

micrometry-aided microscopy of mucormycosis agents from positive mucormycosis cultures, were noted in Microsoft Excel as part of the data collection process. LPCB microscopy of cultures positive for mucorales showed coenocytic broad aseptate hyaline hyphae or sparsely septate hyphae with right-angle branching [19]. A total of 36 mucormycosis agents, such as *Rhizopus* spp. and *Rhizomucor* spp., isolated from mucorales culture-positive specimens, were further subjected to automated bioMérieux Vitek® MS, a MALDI-ToF-MS technique, for identification according to the manufacturer's instructions. The reports of the MALDI-ToF-MS identification were compared with the culture and KOH reports [20,21]. The bioMérieux Vitek® MALDI-ToF-MS identification had already been performed in the Department of Microbiology, Jawaharlal Nehru Postgraduate Medical Education and Research, Puducherry, India.

Data collection: The KOH mount, culture, and MALDI-ToF-MS results, along with clinical and demographic data of the study population, were collected from the Mycology lab register and HIS for further analysis. Variables such as age, sex, disease diagnosis, and presence of co-morbidity were included in the analysis during the baseline survey. The incidence of mucormycosis, mycological profile, and the diagnostic utility of conventional micrometry-aided LPCB microscopy-assisted culture were compared with automated MALDI-ToF-MS, which is a protein signature method for testing and identifying fungi.

STATISTICAL ANALYSIS

Data analysis was performed using the IBM Statistical Package for the Social Sciences (SPSS) software version 28.0. Descriptive statistics were used to calculate the frequency and percentage of the variables. All analyses were performed using Microsoft Excel 2021 (Office 365, Microsoft Corporation).

RESULTS

Out of a total of 1,176 participants included in the analysis, 720 (61.22%) were males and 456 (38.78%) were females, resulting in a male-to-female ratio of 1.57. The mean age of the participants was 49.28±16.74 years, with a minimum age of 0 years and a maximum age of 90 years. The age-wise distribution of the patients is described in [Table/Fig-2].

Age group (years)	Study participants N (%)	Age group (years)	Study participants N (%)
0-10	18 (1.53)	51-60	282 (23.98)
11-20	37 (3.15)	61-70	178 (15.14)
21-30	109 (9.27)	71-80	102 (8.67)
31-40	176 (14.97)	81-90	20 (1.70)
41-50	254 (21.59)	91-100	0

[Table/Fig-2]: Age wise distribution of the study population with suspected mucormycosis (N=1176).

Preliminary findings showed that 368 (31.29%) participants had fungal pneumonia, 104 (8.84%) were suspected cases of mucormycosis, 93 (7.91%) had fungal meningitis, and 56 (4.76%) had sepsis, as shown in [Table/Fig-3].

Diagnosis	N (%)	Diagnosis	N (%)
Fungal pneumonia	368 (31.29)	RROCM	3 (0.26)
Suspected mucormycosis	104 (8.84)	Rhinosinusitis	3 (0.26)
Fungal meningitis	93 (7.91)	Acute meningitis	2 (0.17)
Fungal rhinosinusitis	121 (10.28)	Candidal infection	2 (0.17)
Sepsis	56 (4.76)	CLD	2 (0.17)
Pneumocystis jirovecii pneumonia	33 (2.81)	COVID pneumonia	2 (0.17)
Post-COVID-19	28 (2.38)	Encephalopathy	2 (0.17)
Tinea corporis	22 (1.87)	Fungal cellulitis	2 (0.17)
Urinary tract infection	20 (1.70)	Fungal mycosis	2 (0.17)
Fungal rhinitis	19 (1.62)	Fungal orbital cellulitis	2 (0.17)
Fungal infection	17 (1.45)	Nasal sinusitis	2 (0.17)
PTB (old case included)	12 (1.02)	Pericardialeffusion	2 (0.17)
Cellulitis	5 (0.43)	Pyopneumothorax	2 (0.17)
Fungal candidiasis	5 (0.43)	Rhinitis	2 (0.17)
Meningitis	5 (0.43)	Sinusitis	2 (0.17)
Orbital cellulitis	10 (0.85)	Fungal pansinusitis	2 (0.17)
Pneumothorax	4 (0.34)	Synovitis	2 (0.17)
Vaginal candidiasis	4 (0.34)	Tuberculosis	2 (0.17)
Oral candidiasis	3 (0.26)	Wound infection	2 (0.17)
Post COVID-19 pneumonia	3 (0.26)	Unspecified	204 (17.35)

[Table/Fig-3]: Clinical condition of study participants.
N: frequency, %: percentage. PTB: Pulmonary tuberculosis; ROCM: Rhino-orbital-cerebral mucormycosis; CLD: Chronic liver disease. The total may not be equal to 100% due to the presence of missing data

Among the participants, 951 (80.87%) reported no co-morbid conditions. However, some patients had co-morbidities, including 76 (6.46%) post-COVID patients, 27 (2.30%) with Diabetes Mellitus (DM), and 14 (1.19%) with Pulmonary Tuberculosis (PTB), as depicted in [Table/Fig-4].

Co-morbid conditions	N (%)	Co-morbid conditions	N (%)
No co-morbid condition	951 (80.87)	Encephalitis	1 (0.09)
Post-COVID	76 (6.46)	Fungal pneumonia	1 (0.09)
Diabetes mellitus (DM)	27 (2.30)	HAP	1 (0.09)
PTB	14 (1.19)	Haematemesis	1 (0.09)
COPD	8 (0.68)	Haemoptysis	1 (0.09)
CVA	8 (0.68)	HIV	2 (0.17)
CKD	7 (0.60)	Hospital acquired infection	1 (0.09)
Type 2 respiratory failure	7 (0.60)	Hypernatremia and AKI	1 (0.09)
DCLD	5 (0.43)	Hyponatraemia	1 (0.09)
Multidrug poisoning	4 (0.34)	ILD	2 (0.18)
Retro viral infection	4 (0.34)		
ICH	3 (0.26)	Intestinal obstruction	1 (0.09)
Sepsis	3 (0.26)	Left CVA	1 (0.09)
Suspected tuberculosis	3 (0.26)	LMN facial nervepalsy	1 (0.09)
Cellulitis	2 (0.17)	Meningo-encephalitis	1 (0.09)
L foot cellulitis	2 (0.17)	Nephrotic syndrome	1 (0.09)
LRTI	2 (0.17)	Obstructive Sleep Apnoea (OSA)	1 (0.09)
Lung abscess	2 (0.17)	Post LSCS	1 (0.09)
AKI	3 (0.26)	Post TURP	1 (0.09)
Posterior urethral valve disorder	2 (0.17)	Prostate abscess	1 (0.09)
Suspected COVID	2 (0.17)	CAP	1 (0.09)
Tuberculosis	2 (0.17)	Pulmonary oedema	1 (0.09)
ABPA	1 (0.09)	Renal failure	1 (0.09)
Acute sinusitis	1 (0.09)	Renal transplant	1 (0.09)
Anorectal malformations	1 (0.09)	Right LL lobe consolidation	1 (0.09)
B/L UL Consolidation	1 (0.09)	Suspected Tuberculosis/ Community acquired pneumonia	1 (0.09)
CLD	1 (0.09)	Hypertension	1 (0.09)
Dental infection	1 (0.09)	Thrombocytopenia	1 (0.09)
Diabetic ketoacidosis	1 (0.09)	Upper gastrointestinal bleeding	1 (0.09)
Diabetic nephropathy	1 (0.09)	Acute pancreatitis	1 (0.09)
DKA	1 (0.09)	Chronic psychosis	1 (0.09)
Anaemia	1 (0.09)	Candidiasis	1 (0.09)

APLA positive	1 (0.09)	Seizure	1 (0.09)
Pancreatitis	1 (0.09)		

[Table/Fig-4]: Co-morbid conditions in the participants.
N: frequency; %: percentage. ABPA: Allergic bronchopulmonary aspergillosis; AKI: Acute kidney injury; APLA: Antiphospholipid antibodies; CKD: Chronic kidney disease; B/L UL: Consolidation bilateral upper lung consolidation; CLD: Chronic liver disease; COPD: Chronic obstructive pulmonary disease; ICH: Intracranial haemorrhage; ILD: Interstitial lung disease; PTB: Pulmonary tuberculosis; CVA: Cerebrovascular accident; TURP: Transurethral resection of the prostate; LL: Left lower; L: Left; DKA: Diabetic ketoacidosis; HAP: Hospital-acquired pneumonia; HIV: Human immunodeficiency virus; LRTI: Lower respiratory tract infection; LMN: Lower motor neuron; OSA: Obstructive sleep apnea; CAP: Community-acquired pneumonia; LSCS: Lower segment caesarean section; DCLD: Decompensated liver disease. The total may not be equal to 100% due to the presence of missing data and/or there was more than one co-morbidity in certain patients

Clinical specimens collected from the participants for KOH mount, culture, and MALDI-ToF-MS included 211 (17.94%) Bronchoalveolar Lavage (BAL) samples, 122 (10.37%) sputum specimens, 108 (9.18%) Cerebrospinal Fluid (CSF) samples, 90 (7.65%) biopsied tissue or necrotised tissues from affected areas, 76 (6.46%) nasal swabs, and 50 (4.25%) tracheal aspirates, among others. A detailed description of the diagnosis, co-morbidities, and specimens collected is provided in [Table/Fig-5].

Specimen	N (%)	Specimen	N (%)
BAL	211 (17.94)	Periorbital tissue	2 (0.17)
Sputum	122 (10.37)	Right brachial artery blood 1	2 (0.17)
CSF	108 (9.18)	Right brachial artery blood 2	2 (0.17)
Tissue	90 (7.65)	Right eye swab	2 (0.17)
Nasal swab	76 (6.46)	Throat swab	2 (0.17)
Tracheal aspirate	50 (4.25)	Ascitic fluid	1 (0.09)
Pus	31 (2.64)	Nasal tissue biopsy	1 (0.09)
Blood	28 (2.38)	Blood CVC1	1 (0.09)
Mini BAL	27 (2.30)	Blood CVC2	1 (0.09)
Urine	25 (2.13)	Corneal scrapings	1 (0.09)
Pleural fluid	24 (2.04)	Drain fluid	1 (0.09)
Skin scrapings	24 (2.04)	Endobronchial biopsy	1 (0.09)
L nasal swab	13 (1.10)	Et aspirate	1 (0.09)
R nasal cavity	12 (1.02)	Eye discharge swab	1 (0.09)
Blood BACTEC unflagged	11 (0.94)	L femoral artery blood	1 (0.09)
Vaginal swab	10 (0.85)	L maxillary sinus fungal Rhino sinusitis	1 (0.09)
Nasal cavity	9 (0.77)	L middle nasal discharge	1 (0.09)
Peritoneal fluid	6 (0.51)	Left knee synovial tissue	1 (0.09)
Nasal mucosa tissue	6 (0.51)	Left nasal swab	1 (0.09)
R nasal swab	6 (0.51)	Lung biopsy	1 (0.09)
Maxillary sinus	5 (0.43)	Mucormycosis	1 (0.09)
R middle meatus	5 (0.43)	Nasal mucosa	1 (0.09)
Tongue scraping	5 (0.43)	Nail clippings	1 (0.09)
L middle meatus	4 (0.34)	Nail scraping	1 (0.09)
L nasal cavity	4 (0.34)	Nasal aspirate	1 (0.09)
Peripancreatic fluid	4 (0.34)	Nasal mucous swab	1 (0.09)
Peritoneal fluid CLD	4 (0.34)	Pigtail fluid	1 (0.09)
ICD drain	3 (0.26)	Pleural biopsy	1 (0.09)
L nasal mucosa	3 (0.26)	Pus from nose post COVID-19	1 (0.09)
Middle ear pus	3 (0.26)	Preputial swab	1 (0.09)
Nasal tissue	3 (0.26)	Pus- left kidney	1 (0.09)
Pericardial fluid	3 (0.26)	Pus swab (face)	1 (0.09)
Wound swab	3 (0.26)	R eye swab	1 (0.09)
Debris mucosa	2 (0.17)	R nasal discharge	1 (0.09)
Endotracheal aspirate	2 (0.17)	Sphenoidal sinus	1 (0.09)
Facial discharge	2 (0.17)	Splenic drain	1 (0.09)
Mucoid pus	2 (0.17)	Buccal swab	1 (0.09)
Nasal cavity tissue	2 (0.17)	Swab from dental tissue	1 (0.09)
Nasal discharge swab	2 (0.17)		
N=1176			

[Table/Fig-5]: Specimens collected from the study participants.
N: frequency, %: percentage. BAL: Bronchoalveolar lavage; CLD: Continuous lumbar drainage; CVC: Central venous catheter; ICD: Insertion and management of chest drains; CSF: Cerebrospinal fluid; L: Left; R: Right; Et: Endotracheal. The total may not be equal to 100% due to the presence of missing data

KOH mount for fungal elements was positive in 130 (11.05%) out of the total 1176 clinical samples, such as necrotised tissue, biopsy, and sputum. Among these positive findings, 47 (36.15%) showed broad aseptate hyaline hyphae with right-angle branching suggestive of Mucorales [Table/Fig-6].

Positive KOH findings	N (%)
Fungal elements ±BY-like organism	80 (61.54)
Broad aseptate hyaline hyphae with right angled branching	47 (36.15)
Thin septate hyaline hyphae with acute angle branching	3 (2.31)
KOH Microscopy Positive for fungal elements Total N (%)	130 (100)

[Table/Fig-6]: Positive findings from the KOH examination.
N: frequency, %: percentage. BY-like organism: Budding yeast-like organism

Fungal culture was positive in 258 out of the total 1176 samples (21.94%). Among these positive cultures, mucormycosis was detected in 73 (28.29%) samples, resulting in an overall incidence of mucormycosis of 6.21% in the study population [Table/Fig-7]. The remaining 185 (71.70%) positive cultures were identified as non-mucorales fungal agents, as depicted in [Table/Fig-8].

Culture findings	N (%)
Rhizopus spp.	51 (69.86)
Rhizomucor spp.	20 (27.40)
Mucor spp.	2 (2.74)
N=73	

[Table/Fig-7]: Positive culture findings of agents causing mucormycosis.
N: frequency, %: percentage

Culture findings	N (%)
Candida tropicalis	46 (24.86)
Candida albicans	57 (30.81)
Candida glabrata	16 (8.65)
Candida parapsilosis	7 (3.78)
Candida guillermondii	3 (1.62)
Candida krusei	3 (1.62)
Candida auris	1 (0.54)
Candida coferi	1 (0.54)
Candida famata	1 (0.54)
Candida lipolytica	1 (0.54)
Candida tropicalis and *Candida albicans*	1 (0.54)
Aspergillus niger	17 (9.18)
Aspergillus flavus	12 (6.49)
Aspergillus fumigatus	5 (2.70)
Aspergillus species	2 (1.08)
Trichophyton species	5 (2.70)
Bipolaris species	2 (1.08)
Dematiaceous fungi	1 (0.54)
Others	4 (2.16)
N=185	

[Table/Fig-8]: Positive culture findings of non-mucormycosis fungal agents.
N: frequency, %: percentage

A total of 101 (8.59%) samples out of 1176 showed positive results for mucormycetes by both KOH mount and culture, while

157 (13.35%) samples showed positive culture results despite initial negative KOH examination. Additionally, 29 (2.47%) samples showed positive results in KOH microscopy but were negative in fungal culture. A comparison of KOH microscopy results with culture is presented in [Table/Fig-9].

	KOH Positive	KOH Negative	Subtotal
Culture positive	101 (8.59%)	157 (13.35%)	258 (21.94%)
Culture negative	29 (2.47%)	889 (75.60%)	918 (78.06%)
Subtotal	130 (11.05%)	1046 (88.95%)	N-1176 (100%)

[Table/Fig-9]: Comparison of KOH and culture findings among the study population.

Out of the 73 mucormycosis-positive cultures, 36 non duplicate mucormycosis culture isolates were selected for MALDI-ToF-MS identification. Among these, 31 were identified as "*Rhizopus oryzae* complex" using MALDI-ToF-MS. Three isolates were identified as *Rhizopus homothallicus* by LPCB, and two isolates were identified as *Rhizopus microsporus*. These isolates were not tested by MALDI-ToF-MS, as shown in [Table/Fig-10]. MALDI-ToF-MS demonstrated rapid and specific identification and confirmation of causative agents of mucormycosis at the genus and species level compared to KOH or culture. A comparison of culture with LPCB versus MALDI-ToF-MS results is depicted in [Table/Fig-10].

S. No.	Blinded serial no.	Conventional micrometry assisted LPCB microscopy of mucormycosis positive cultures	Vitek MALDI-ToF-MS results
1	330	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
2	339	*Rhizopus homothallicus*	*Testing by Vitek-MS was not necessary and not done as LPCB Microscopy could easily fetch the right results
3	340	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
4	341	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
5	343	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
6	348	*Rhizopus homothallicus*	*Testing by Vitek-MS was not necessary and not done as LPCB Microscopy could easily fetch the right results
7	354	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
8	357	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
9	362	*Rhizopus homothallicus*	*Testing by Vitek-MS was not necessary and not done as LPCB Microscopy could easily fetch the right results
10	364	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
11	373	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
12	381	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
13	383	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
14	399	*Rhizopus microsporus*	*Testing by Vitek-MS was not necessary and not done as LPCB Microscopy could easily fetch the right results
15	395	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
16	403	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
17	404	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
18	405	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
19	411	*Rhizopus microsporus*	*Testing by Vitek-MS was not necessary and not done as LPCB Microscopy could easily fetch the right results
20	412	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
21	419	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
22	430	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
23	434	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
24	435	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
25	443	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
26	444	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
27	451	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
28	465	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
29	469	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
30	470	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
31	493	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
32	518	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
33	529	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
34	542	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
35	551	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex
36	570	*Rhizopus oryzae* complex	*Rhizopus oryzae* complex

[Table/Fig-10]: Comparison of culture and LPCB vs MALDI-ToF-MS for the detection of mucorales.

DISCUSSION

COVID-19 has increased the risk of invasive fungal infections, such as mucormycosis, aspergillosis, and candidiasis, among affected patients. During India's second wave of the pandemic, there was an epidemic of mucormycosis, leading to delayed diagnosis and significant morbidity and mortality [10,22]. In routine clinical practice, direct HPE of BAL or necrosed tissue is used for definitive lab diagnosis of mucorales. A presumptive diagnosis can be made using KOH wet mount examination. Conventional fungal culture at 37°C is used for isolation, speciation, and susceptibility testing of mucorales [23,24]. Micrometry-aided LPCB microscopy of culture-positive samples is a rapid and cost-effective tool for diagnosis and antifungal stewardship. While molecular detection by PCR is the gold standard, MALDI-ToF-MS identification of cultured Mucorales is an excellent confirmation method [25]. Appropriate sampling, careful sample handling, and effective communication between clinicians and lab personnel are crucial for optimal diagnosis. Treating physicians face the challenge of early diagnosis and initiating appropriate therapy, including surgical resection of infected areas and antifungal treatment with drugs like Liposomal Amphotericin, Isavuconazole, or Posaconazole, for COVID-19 Associated Mucormycosis (CAM).

This study aimed to rapidly detect mucormycosis through culture and identify the causative agents using conventional culture and automated MALDI-ToF-MS protein signature detection. Most participants in the study were between 41 and 60 years old, and the male-to-female ratio was 1.57, slightly lower than reported in other studies [26,27]. The common clinical presentations in the study population included fungal pneumonia, fungal meningitis, suspected cases of mucormycosis, fungal rhinosinusitis, and sepsis, which aligned with a previous study conducted in India in 2021 [10]. Other studies have reported new risk factors, such as chronic kidney disease and post-PTB, in Asian countries [2]. Single-center studies on the incidence of mucormycosis have shown varying numbers of cases over the years, which could be attributed to improved awareness, advancements in diagnosis, and technical expertise [25-28]. Incidence rates range from 9.5 to 129 cases per year [29,30]. A multicentre study in Indian Intensive Care Units (ICUs) reported a 12% prevalence of mucormycosis [31]. Among various anatomical locations, rhino-orbital-cerebral mucormycosis (ROCM) and pulmonary mucormycosis were the predominant forms in this study. Previous studies in India have also reported ROCM as the most common form (45-74%), followed by cutaneous (10-31%) and pulmonary (3-22%) cases [2,27,28,32,33]. Out of the culture-positive isolates, a total of 73 cases (28.29%) were identified as mucormycosis using microbiology-aided diagnosis methods, including culture, KOH, and MALDI-ToF-MS. Multiple studies in India have reported varying numbers of mucormycosis cases, ranging from 27 to 382 [26-28,34].

According to the Centers for Disease Control and Prevention (CDC), samples of respiratory fluid are collected from individuals suspected of having pulmonary mucormycosis for examination [35-37]. In this study, the majority of specimens collected were BAL, followed by sputum, CSF, necrotised tissue, nasal swabs, and tracheal aspirates. Utilising KOH wet mounts enhances visualisation through direct microscopy, and the characteristics of fungal hyphae can aid in early diagnosis [1]. Diabetes was the predominant risk factor or co-morbidity observed in this study, consistent with findings from other studies conducted in India [8,9,11,26]. Mucorales causing mucormycosis were preliminarily identified by the presence of distinctive broad or narrow pauciseptate or aseptate ribbon-like hyphae with acute or right-angled branching [19]. Mucorales grew rapidly when cultured at 25°C and 37°C, displaying white, pale gray, or brown cottony-fluffy colony morphology within 24-72 hours [20]. The MALDI-ToF-MS method was used to identify the fungal agents of mucormycosis from the culture-positive samples.

In this study, MALDI-TOF/MS was used for the detection of Mucorales. Out of 36 non duplicate mucorales, 31 were identified as "*Rhizopus oryzae* complex" using MALDI-TOF/MS. LPCB microscopy, on the other hand, was found to be effective in detecting *Rhizopus homothallicus* and *Rhizopus microsporus,* making MALDI-TOF identification unnecessary. Another study conducted in India focused on the epidemiology and diagnosis of mucormycosis, revealing that species of *Rhizopus*, *Lichtheimia*, *Mucor*, *Apophysomyces*, and *Saksenaea* complex were the main causative factors [8,26]. The study also reported the detection of new emerging species using MALDI-TOF-MS. While MALDI-TOF-MS is considered sophisticated for diagnosing mucormycosis, culture-based identification coupled with micrometry-aided LPCB microscopy was found to be a more feasible and relevant method [2].

A separate study evaluated non mucorales as causative agents of mucormycosis, reporting *Aspergillus* spp., *Fusarium* spp., *Pseudallescheria* spp., *Scedosporium* spp., and *Pseudomonas aeruginosa* as the main agents. These findings were consistent with the present study, which also identified other non mucorales fungi in culture. The study on the applications of MALDI-TOF-MS in clinical microbiology demonstrated its rapid, accurate, and easy identification of cultures. Despite this, the study emphasised the importance of combining culture-based identification with micrometry-aided LPCB microscopy for accurate detection of mucormycosis cases and their causative agents, as confirmed by MALDI-TOF-MS.

The study acknowledged the strength of the large sample size, allowing for effective analysis, as well as the comparison between KOH examination, culture, and MALDI-TOF-MS techniques to determine the accuracy of identifying agents causing mucormycosis. However, the study recommended further research, specifically a phase II study focusing on PCR specificity and sequencing, to augment early identification and confirmation of Mucorales.

Limitation(s)

Limitations of the study included the inability of KOH microscopy to identify mucorales at the genus or species level. Positive cultures from non sterile sites required clinical and radiological data to establish a probable diagnosis, while the low sensitivity of culture posed a challenge, potentially leading to false-negative results. The study also highlighted the need to expand commercially available databases of MALDI-TOF-MS and gather more validation data. PCR for mucormycosis was not conducted due to it not being part of routine diagnostic methods or national guidelines.

CONCLUSION(S)

In conclusion, simple KOH microscopy, conventional culture, and micrometry-aided LPCB microscopy can detect Mucorales. MALDI-TOF-MS can be used for rapid detection, confirmation, and epidemiological purposes, although treatment does not differ among various species causing mucormycosis.

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