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# Significance of laboratory diagnosis in coronavirus disease associated invasive fungal sinusitis from an otolaryngologist perspective: A retrospective study at a tertiary care nodal centre

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**Abstract**

**Objective:** Rhino-orbital cerebral mucormycosis is an angioinvasive life threatening fungal infection that presents acutely in patients with diabetic ketoacidosis and immunosuppression. The main objective of this study is to evaluate the sensitivity and specificity of various laboratory investigation required for confirmation of coronavirus disease associated invasive fungal sinusitis (CAIFS).

**Methods:** This is a retrospective observational study conducted at a Tertiary care referral centre which included 300 patients with past history of being treated for coronavirus disease with symptoms of invasive fungal sinusitis. The available clinical and laboratory data to diagnose CAIFS was collected and analyzed.

**Results:** Diabetes mellitus was the most common risk factor identified for aggravating of CAIFS. HPE was highly sensitive and specific when compared to other investigations in confirmation of diagnosis of CAIFS.

**Conclusion**: Early diagnosis of CAIFS is important for timely therapeutic intervention, improved survival, and reduced morbidity. If a characteristic black eschar is found on initial diagnostic nasal endoscopy the tissue needs to be sent for KOH fungal mount, culture and histopathological examination. HPE is the most specific and sensitive method to make both a quick initial diagnosis of CAIFS and for initiation of most appropriate anti fungal therapy.

***Keywords***: mucormycosis; invasive fungal rhinosinusitis; coronavirus; histopathology; microbiology.

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## Introduction

The global Coronavirus disease 2019 (Covid-19) pandemic has affected more than 30 million people in India till date and several have succumbed to the disease. As India continues to fight the existing scenario, another imminent threat has emerged as a challenge to India in the form of coronavirus disease associated mucormycosis (CAIFS). Covid-19 is an infection caused by severe acute respira-tory syndrome coronavirus-2 (SARS CoV-2) virus, which principally binds to the angiotensin con-verting enzyme 2 receptors and thus invades the respiratory epithelium. The second stage of this disease is more severe caused by aggravation of systemic inflammation and coagulopathy causing direct damage to the blood vessels. The coagulopathy seen is of complement mediated thrombotic microangiopathies leading to endothelial damage of blood vessels and microvascular thrombosis. Moreover, affected patients likewise show an overexpression of inflammatory cytokines, and im-paired cell mediated immunity thus leading to increased susceptibility to fungal infections.

Mucormycosis (the so called black fungus) is a rare, potentially deadly angioinvasive opportunistic fungal infection and is found to be fatal in 50 to 80% of the cases. It is caused by the by the fungus of the order Mucorales, family Mucoracea including the genera Mucor, Absidia and Rhizopus. It is also known as zygomycosis and phycomycosis. The fungus is ubiquitous in nature and is found in soil and on decaying vegetation. It typically affects immunocompromised individuals. Rhino-orbito cerebral mucormycosis (ROCM) is the most common type. The most recognised risk factors as-cribed to the rise of mucormycosis in Covid-19 patients are uncontrolled diabetes mellitus, acute respiratory distress syndrome (ARDS), those receiving broad-spectrum antibiotics, longer stay of critically ill patients in intensive care units (ICU) especially those who required supportive ventila-tion and excessive use of corticosteroids for immunosuppression.

Early diagnosis and immediate surgical intervention along with institution of appropriate antifungal therapy saves both sight and life of the patient. There are several different methods for the diagno-sis of ROCM, which include red flag signs and symptoms, endoscopic examination, direct micros-copy, culture and histopathological examination (HPE).

The signs, symptoms and radiological findings of invasive fungal sinusitis are nonspecific. Tissue samples sent for direct microscopy by wet mount, microbiological or histopathological examination of the involved tissue or paranasal sinus secretions are diagnostic. Microbiology examination can be done with 10% Potassium Hydroxide (KOH) Mount or by culture on Sabourands Dextrose Agar (SDA). For confirmation of diagnosis of mucormycosis requires either the presence of fungal hy-phae in the involved tissues or growth of the fungus from microbiological cultures [1].

The diagnosis of mucormycosis is more difficult than other fungal infections, and its treatment is challenging because under normal laboratory conditions, as sporulation fails and culture results from the biopsies are often negative due to unviable organism in necrotic tissues [2]. KOH fungal stain is considered as the simplest technique used for diagnosis of fungal pathogens. Histopathological examination is the only definitive means to identify certain uncultivable fungi [3,4]. HPE with special stains such as Grocott-Gomori Methenamine silver nitrate (GMS) or Periodic Acid Schiff (PAS) stain demonstrates the pathognomonic broad, irregular, nonseptate, and rightangle branching fungal hyphae. Differentiation by direct examination may allow amphotericin B treatment and other potentially life saving therapeutic interventions to be initiated. In the past 3 months, we have seen an immense increase in the numbers of cases of CAIFS at our nodal centre. This surge in the number of cases during the second wave could be several peripheral microthrombi from coagulopathy, micro-angiopathy from diabetes mellitus in addition to immunosuppression from corticosteroids all provid-ing an favourable environment for the fungus to grow.

## Material and Methods

### Study design and subjects

The study protocol was approved by the Institutional Research Ethics Committee. This was a retrospective analytical study done on the collected data between April 2021 and June 2021. This study included 300 patients with past history of recently being treated for Covid-19 infection and who presented to our department with signs and symptoms of CAIFS. Patients were characterized as recovered from Covid-19 if they were tested negative on a repeat RT-PCR (reverse transcriptase polymerase chain reaction) or if fourteen days had elapsed since the diagnosis.

Patients were also assessed for the following history before deciding the treatment protocol [1]. History of Covid-19 infection [2]. Diabetes status of the patient (pre-existing or newly diagnosed) [3]. History of steroid use during Covid-19 infection. Patients with a history of radiotherapy, chemo-therapy, osteoradionecrosis of the jaw, granulocytopenic patients or those on other immunomodula-tor drugs were excluded from the study. Patients with non Covid-19 associated invasive fungal si-nusitis were also excluded from the study.

### Clinical examination and intervention

All patients underwent thorough ENT and cranial nerve examination. The patients who presented with one or more of the following symptoms and signs of headache, facial pain, jaw pain or necrosis of the mucosa or the palatal bone, retro-orbital pain, swelling of the eye or proptosis, ptosis and vis-ual disturbances suspicious of CAIFS were all included in the study.

Aside from ascertaining the current Covid-19 status with RT-PCR test, routine blood investigations and high resolution computed tomography of the chest were done for all the patients. DNE was performed to look for necrotic tissue, black eschar or fungal debris i.e., signs of invasive fungal sinusitis (Figure 1). Magnetic resonance imaging (MRI) of the paranasal sinuses (PNS) including orbits and brain were performed for all cases (Figure 1). Patients were taken up for surgical exploration and

debridement. The surgical intervention varied from a simple endoscopic guided surgical debridement to maxillectomy to orbital exenteration depending on the extent of disease. The involved infected tissue was sent for microbiological and HPE to determine and confirm the presence of fungal hyphae (Figures 2,3).

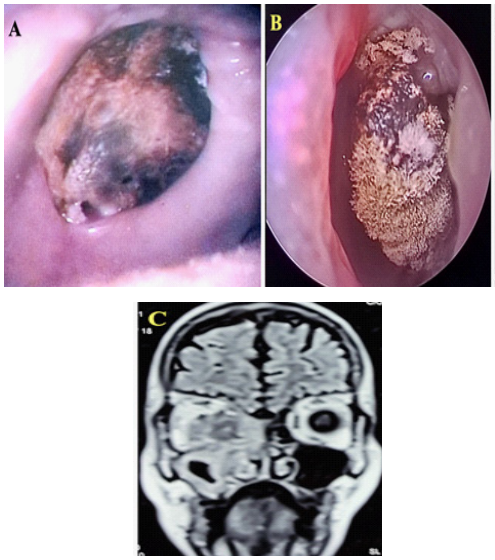


**Figure 1:** (A). Clinical image showing black eschar on the palate. (B). DNE image showing black eschar with fungal spores and (C). MRI image, coronal section showing involvement of the rimaxillary sinus, ethmoid sinus and orbit on the right side.

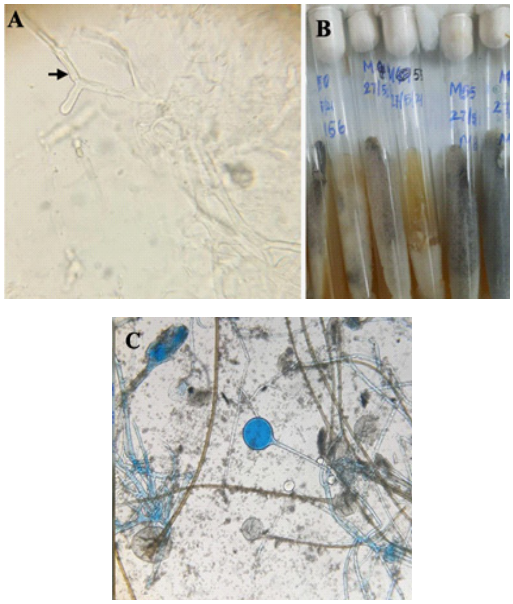


**Figure 2:** A. Microbiological examination showing mucorale species on (A). KOH fungal stain (black arrow), (B). Cottony white to black growth seen on SDA culture and (C). Direct Microscopy image for confirmation of mucor.

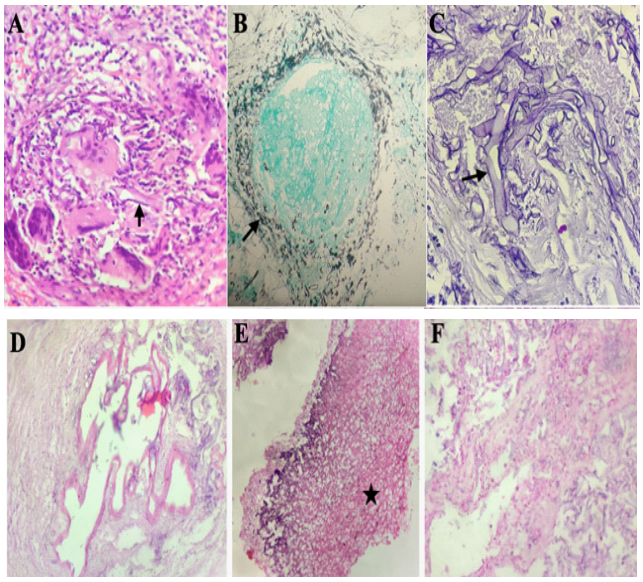


**Figure 3:** Histopathological images showing broad aseptate fungal hyphae characteristic of mucor (black ar-row) on A. Haematoxylin and Eosin Stain demonstrating fungal hyphae with granulomatous reac-tion and neutrophilic infiltration B. GMS stain, C. PAS stain, D,E,F Microscopic image (Haematox-ylin and Eosin Stain, 10X magnification) showing angioinvasion, tissue necrosis and fungal ball (black star).

**Microbiological and histopathological examination**

Microbiological examination was done with KOH wet mount and culture on SDA. KOH fungal stain is used for rapid detection and as a primary screening tool to detect fungal elements. The prin-ciple behind this is when the infected tissue sample is mixed with 10% KOH which is a strong alkali, it softens, digests and clears the tissues such as keratin, surrounding the fungi, so the hyphae and spores of fungi can be seen clearly under the microscope. Mucormyosis appears as aseptate hyphae branching at right angles and aspergillosis appears as septate hyphae with acute angled shaped branching (Figure 2) .

Sabouraud Dextrose Agar (SDA) is used for isolation and cultivation of non-pathogenic and pathogenic species of fungi and yeasts. The principle behind this is the peptone provides the nitrogen and vitamin source required for the growth of organism on SDA. Dextrose provides energy and carbon source. Agar acts as a solidifying agent. Tetracycline and/or chloramphenicol may be added as adjunctive antimicrobials to inhibit the growth of various gram positive and gram negative bacteria. Gentamicin is added to further inhibit the growth of gram-negative bacteria. Sabouraud agar plates when inoculated by streaking or by exposing the medium to ambient air. Mucor appears as fast growing colonies which cover the agar surface with a dense white cottony growth initially later turning to grey or yellowish brown with sporulation (Figure 2).

HPE was done using haematoxylin and eosin, PAS and GMS stains. These stains mainly use formalin-fixed, paraffin-embedded tissue sections. PAS stain demonstrates the presence of carbohydrate compounds such as polysaccharides, mucin, glycogen, and fungal cell wall components. The principle of PAS stain is when the polysaccharides in the fungal cell wall react with the periodic acid it produces an oxidized compound, an aldehyde which is revealed by the red or pink or magenta coloration due to the fixation of the colourless Schiffs fuchsin. During glucose

conversion, the stain appears pink which defines the intra or extracellular mucins (Figure 3). GMS stain is used majorly for the identification of carbohydrates in fungal microorganisms. In comparison to PAS stain, GMS stain has a higher sensitivity for detecting fungi and other polysaccharides rich microorganisms. The principle is, the polysaccharides in the fungal cell wall on interaction with chromatin acid, undergo oxidation to form aldehydes, which is demonstrated by the reduction of the alkaline hexamine silver complex. Grocott's alkaline hexamine-silver solution undergoes reduction to form precipitates of silver ions making the cell wall of the fungi appear black a reaction is known as Argentaffin reaction (Figure 3).

**Postoperative Period**

Depending on the type of fungal organism, liposomal amphotericin therapy was instituted at 5 mg/kg for mucormycosis. Later at the time of discharge patients were started on oral posaconazole 300 mg every 8th hourly on the first day and then continued at once daily dosage for 6 weeks. The patients were followed up after discharge regularly at weekly intervals and were taken up for further surgical debridement if any suspicious lesions were noted.

## Data collection, management and statistical analysis

The data of patients demographic characteristics, risk factors, clinical manifestations and black eschar or infected tissue seen on DNE was collected. Microbiological and HPE reports of all patients with CAIFS were all obtained, collected and analysed. Descriptive statistics for patient characteristics included the mean, standard deviation, median and range for continuous variables, and frequencies and percentages for categorical items was calculated. Fischer exact test of association was perfomed to determine the association between black Eschar, KOH, Culture, and HPE. Sensitivity, specificity, correct classification, Receiver-Operator Characteristic (ROC) curve area were determined using black eschar variable as reference. Data management and statistical analysis was performed by a department appointed statistician using STATA v.11.2 (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845 USA) software.

## Results

Among the 300 patients who were included in the study, 227 (75.6%) were males and 73 (24.33%) were females, with ratio between males to females to be 3.1:1. The age of the patients ranged from 18 years to 80 years with a mean age of 47.14 +/- 10.81 years. Diabetes Mellitus (DM) was the most common risk factor. Majority 287 (95.5%) patients were either newly diagnosed 152 (50.67%) or pre-existing diabetics 133 (44.33%) and 253 (84.33%) received corticosteriods during management of Covid-19 infection. Figure 4 summarises the distribution of symptoms in suspected cases of CAIFS (Figure 4).

Table 1 summarises the distribution of DNE findings, microbiological and histopathological data among 300 CAIFS patients. The charachteristic black eschar was seen in 74.67% of the patients. HPE confirmed mucormycosis was seen in 262 (87.63%) patients, aspergillosis in 11 patients, mixed infection in 4 patients and rest others showed features of chronic sinusitis. Table 2 summarises the association between the classical black eschar seen on DNE to KOH fungal stain, SDA and HPE among 300 CAIFS patients.

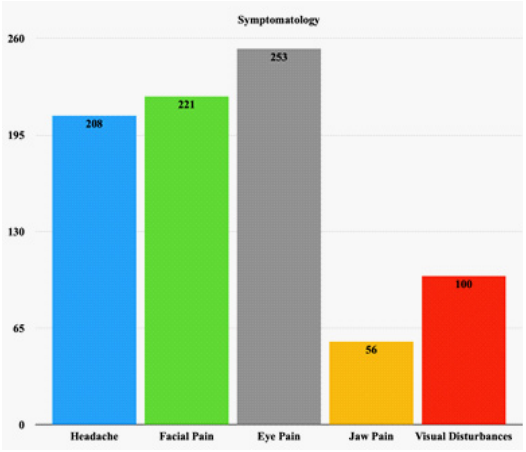


**Figure 4:** Distribution of symptoms among 300 CAIFS cases

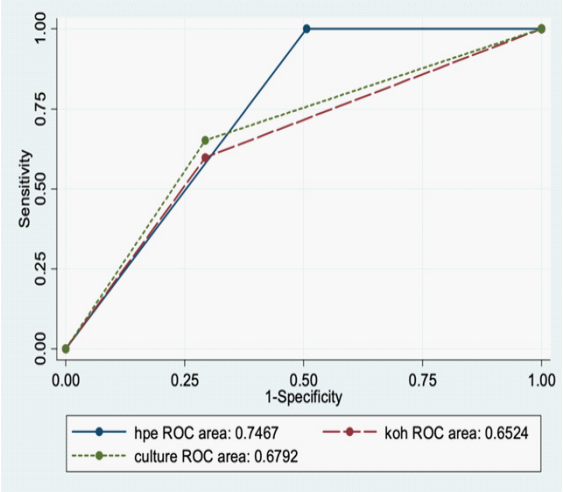


**Figure 5:** Comparison of Area under the curves (AUC) for KOH, Culture and HPE using Black Eschar as reference variable.

**Table 1:** Summary of clinical, microbiological and pathological data among 300 CAIFS patients.

| Variable | Male (n-227) | Female (73) |
|---|---|---|
| DNE -Black Eschar | | |
| Present (n=224/300)(74.67%) | 169 (74.45) | 55 (75.34) |
| Absent (n=76/300) (25.33%) | 58 (25.55) | 18 (24.66) |
| KOH | | |
| Present (n=156/300) (52.00%) | 124/227 (54.63%) | 32/73(43.84%) |
| Absent (n=144/300) (48.00%) | 103/227 (45.37%) | 41/73(56.16%) |
| SDA Culture | | |
| Present (n= 168/300 (56.00%) | 132/227 (58.15%) | 36/73 (49.32%) |
| Absent (n= 132/300 (44.00%) | 95/227 (41.85%) | 37/73 (50.68%) |
| Mucormycosis HPE | | |
| Present (n=262/299 (87.63%) | 200/227 (88.11%) | 62/73 (86.11%) |
| Absent (n=37/299 (12.37%) | 27/227 (11.89%) | 10/73 (13.89%) |
| Necrosis | | |
| Present (n=258/299) (86.29%) | 198 (87.22%) | 60 (83.33%) |
| Absent (n=41/299 (13.71%) | 29 (12.78%) | 12 (16.67%) |
| Neutrophils | | |
| Present (n=253/299)(84.62%) | 192 (84.58%) | 61 (84.72%) |
| Absent (n= 46/299) (15.38%) | 35 (15.42%) | 11 (15.28%) |
| Thrombosis | | |
| Present (n=122/299) (40.80%) | 99 (43.61%) | 23 (31.94%) |
| Absent (n= 177/299) (59.20%) | 128 (56.39%) | 49 (56.39%) |

**Table 2:** Summary on association between black eschar and various laboratory investigations.

| Variable | Laboratory investigation for mucor | |
|---|---|---|
| **Black Eschar** | KOH Positive (156) | KOH Negative (144) |
| Present (n=224) | 134/224 (59.82%) | 90/224 (40.18%) |
| Absent (n = 76) | 22/76 (28.95%) | 54/76 (71.05%) |
| **Black Eschar** | Culture Positive (168) | Culture Negative (132) |
| Present (n=224) | 146/224 (65.18%) | 78/224 (34.82%) |
| Absent (n = 76) | 22/76 (28.95%) | 54/76 (71.05%) |
| **DNE - Black Eschar** | HPE Positive (262) | HPE Negative (37) |
| Present (n=224) | 224/262 (85.50%) | 0/ (0.00) |
| Absent (n = 76) | 38/262 (14.50%) | 37/37 (100.00%) |
| **Necrosis** | HPE Positive (262) | HPE Negative (37) |
| Present (n = 258) | 256/258 (99.22%) | 2/258 (0.78%) |
| Absent (n = 41) | 6/41 (14.63%) | 35/41 (85.37%) |
| **Neutrophils** | HPE Positive (262) | HPE Negative (37) |
| Present (n = 253) | 243/253 (96.05%) | 10/253 (3.95%) |
| Absent (n = 46) | 19/46 (41.30%) | 27/46 (58.70%) |
| **Thrombosis dist** | HPE Positive (262) | HPE Negative (37) |
| Present (n = 122) | 121/122 (99.18%) | 1/122 (0.82%) |
| Absent (n = 176) | 141/177 (79.66%) | 36/177 (20.34%) |
| **Neutrophils, thrombosis and necrosis** | HPE Positive | HPE Negative |
| Present (n = 114) | 113/114 (99.12%) | 1/114 (0.88%) |
| Absent (n = 28) | 1/28 (3.57%) | 27/28 (96.43%) |

The Fisher exact test of association was highly statistically significant for all three variables i.e., between Black Eschar and KOH, Culture, and HPE. Only 299 observations were used for each of the three tests in order to calculate ROC area, and compare them. ROC area under the curve was 0.652 for KOH, 0.679 for SDA Culture and highest for HPE of 0.746. Comparison of ROC areas resulted in Chi-square of 8.81 and p valve of 0.0122 which was statistically significant (Figure 5). Table 3 summarises the sensitivity, specificity of KOH fungal stain, SDA culture and HPE for mucormycosis.

## Discussion

Mucormycosis is a rare, opportunistic, life threatening, angioinvasive fungal infection that affects immunocompromised individuals [5]. The inhaled spores of the fungi get inoculated into the nose and nasopharynx, leading to tissue invasion, thrombosis, and necrosis [6]. This necrosis spreads from the nose, to the PNS, to the orbit, cavernous sinus and then to the intracranial cavity. ROCM is the most common presentation and contributes to about two-thirds of all cases of mucormycosis. Chakrabarti et al. have estimated a prevalence of 0.14 per 1000 cases of diabetics in India, which is about 80 times the prevalence of mucormycosis in developed countries [7]. In the previous reported literature of CAIFS there was a male predilection of 79%.8 The median age of CAIFS in Indian population has been reported to be 45 - 50.7 years [9-11]. In our study the demographic profile was consistent with previous literature with a mean age of 47.14 years and 75.6% male patients with male to female ratio of 3.1:1. DM has been identified as an independent risk factor for mucormycosis [12]. The SARS Covid - 19 virus is said to damage the pancreatic islet cells producing new onset DM or worsening of the already existing DM. This low ph acidic environment presents an additive effect causing an overall increase in free iron levels, providing a fertile environment for the fungus to grow and thrive. A study done by John et al, in a series of 41 cases of Covid-19-associated ROCM, 93% were diabetics [13]. Our data showed similar results, majority 95.6% patients were diabetic. According to the previous published literature, 76.3% of the patients with Covid -19 associat-ed ROCM gave history of use of systemic corticosteroids [12]. Our data revealed that systemic corticosteroids had been used in 84.33% of the patients which was similar to published literature.

Mucormycosis is difficult to diagnose early, and without appropriate treatment, the disease pro-gresses dramatically and may lead to intracranial extension and death. Successful therapy includes rapid control of the underlying disease process, systemic antifungal therapy using liposomal ampho-tericin B and aggressive surgical debridement of the infected tissue. As mucormycosis is highly an-gioinvasive causing extensive necrosis of tissues, contrast-enhanced MRI of the paranasal sinuses, orbit and brain was the preferred imaging modality of choice. With sinus involvement, magnetic resonance imaging (MRI) may demonstrate variable T1 and T2 intensity with lack of enhancement in necrosed devitalised areas. The signs, symptoms, and radiographic findings of mucormycosis are all very nonspecific. DNE allows for quick inspection to look for a black eschar and thus allowing to take biopsy for microbiological and HPE. The classical hallmark of invasive fungal sinusitis is tissue necrosis resulting from angioinvasion and subsequent thrombosis.

Direct Microscopy with KOH fungal stain, SDA culture and HPE are the most commonly done la-boratory investigations for the diagnosis of mucormycosis. The charachteristic hyphae of mucorales organism are typically broad with a diameter of 6–16 µm, ribbon like, irregularly shaped, coenocytic aseptate with branching non dichotomously at right angles. The hyphae may be difficult to observe on an unenhanced KOH wet mount and may not stain well with conventional Gram stain. Members of the order mucorales are identified to the genus or species level according to colonial morphology, microscopic morphology, and growth temperature. To optimize growth, clinical specimens should be inoculated onto appropriate media, such as SDA, and incubated at room temperature at 37°C. Colonies typically appear within 24 to 48 hours unless residual antifungal agents, such as amphotericin B, are present, which can suppress growth.

Histopathology with special stains such as PAS and GMS stain, demonstrates the pathognomonic broad, irregular, nonseptate, and right-angle branching hyphae. These organisms are typically diffi-cult to observe on hematoxyline and eosin stains. On the other hand, PAS and GMS stains may be used for a fully characterized appearance of the organism. In our study HPE is

**Table 3:** Sensitivity and Specificity of Microbiological and Histopathological investigations

| Lab. tests | Sensitivity % | Specificity% | Correctly Classified % | ROC Area | Std. Err. | Asymptotic Normal 95% CI |
|---|---|---|---|---|---|---|
| KOH (n=300) | 59.82 | 71.05 | 62.67 | 0.652 | 0.031 | 0.591 - 0.713 |
| Culture (n=300) | 65.18 | 71.05 | 66.67 | 0.679 | 0.030 | 0.618 - 0.739 |

found to be the most reliable investigation in terms of sensitivity and specificity in the confirmation of diagnosis of mucormycosis. Evidence of angioinvasion, thrombosis, necrosis and neutrophilic infiltration may also be observed on HPE. In immunosuppressed hosts, the hyphal elements will be seen invading the blood vessel wall with abundant necrosis, hemorrhage, and blood vessel thrombosis. Sparse neutrophilic inflammation can also be found in the periphery of the lesion. In immunocompetent hosts hyphae are accompanied by intense granulomatous inflammation with abundant neutrophils, eosinophils, fibrosis, and granulation tissue. In our study 113 patients showed features of thrombosis, neutrophils and necrosis on their HPE slides.

In our study the sensitivity for SDA culture was 65.18% which was almost close to the 70% reported in a study done by Badiee et al [14]. The negative results for specimen culture may be due to initial processing of hyphal elements by grinding, which may damage the large, pauciseptate hyphae and make them nonviable [15,16] or prolonged antifungal pretreatment. In this present study, we found that identification of fungi in histopathological specimens showed strong association with black eschar on DNE with an ROC area of 74.6%. We also calculated the association between black eschar and KOH fungal stain, culture and HPE, which were all highly significant, but the most sensitive and specific test was HPE. The sensitivity of KOH mount observed in our study was 59.82% which was less than that reported by Dass SM et al17 who found the sensitivity to be 83.02%. In our study 87.63% confirmed mucormycosis on HPE which was much more than 63.64% reported in published literature [14]. Distinction by direct examination may allow amphotericin B treatment and other potentially life-saving therapeutic interventions to be initiated. The antifungal drug of choice for mucormycosis is Amphotericin B. In patients with compromised renal functions, isavuconazole and posaconazole have been found to be effective alternatives for treatment.

## Conclusion

Identification of Mucorales organisms to the genus or species level carries valuable epidemiological, therapeutic, and prognostic implications. The red flag signs should be recognized promptly, followed by an expedited diagnosis by DNE, radiological, microbiological and HPE. Antifungal medications should be initiated empirically upon clinical or clinical radiological correlation in symptomatic CAIFS patients. Liposomal amphotericin B is the drug of choice for treatment of CAIFS. HPE is the most reliable and sensitive test for the confirmation of mucormycosis.

## Declarations

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**Declaration of conflicting interests:** The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Availability and data transparency:** Data collected is stored in the institute records folder.

**Ethical approval:** Institute ethical committee approval was taken prior to conducting of the study.

**Consent:** Informed consent was obtained from all the patients to participate in the study and for publication of the article.

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