Histological Spectrum of Post Covid Debridement Tissues: Salient Histomorphological Features With Respect to Identification Fungal Elements

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ABSTRACT

BACKGROUND: Secondary bacterial and fungal infections in COVID patients have been documented during current pandemic. The present study provides detailed account of histomorphology of debridement tissue received for suspected fungal infections. The primary objective was to determine the morphological characteristics that must be recognized for the identification of fungal hyphae.

METHODS: The detailed histological examination of debridement tissue was performed. Demographic and clinical findings with treatment provided was recorded. Presence or absence of necrosis and lecocytoclasis was noted.

RESULTS: A total of 110 cases of debrided tissues were included in the study. Eosinophilic granular necrosis with lecocytoclasis was observed in 103cases; fungal elements were identified in 89.3% (92/103) of these. Eleven cases where necrosis was observed, strong suspicion of fungus was reported, 6 of them displayed fungus on KOH preparation, 3 on repeat biopsy. However, in 2 of these cases, neither KOH nor repeat biopsies identified the fungus. Mucor with aspergillus was observed in 7 cases and actinomyces in 3. In all these 10 cases dense fungal colonies were evident. In 7 cases careful observation revealed fruiting bodies of aspergillus. Cotton ball appearance of actinomyces was evident. Mucor infection in current disease was so rampant that aseptate ribbon like branching mucor hyphae were evident on H&E sections. Diabetes was significantly associated with fungal infection (97.2%; 70/72; $P < .005$). 90% [19/21] of the patients who were on room air and diagnosed with fungal infection were diabetic.

CONCLUSIONS: Eosinophilic granular necrosis with the presence of neutrophilic debris in a case of suspected fungal disease suggests the presence of fungal elements. This warrants processing of the entire tissue deposited for examination, careful observation, application of fungal stains, and repeat biopsy if clinical suspicion is strong. Moreover, uncontrolled diabetes is more frequently associated with secondary fungal infection in COVID patients as compared to oxygen therapy.

KEYWORDS: Mucor mycosis, debridement tissue, fungal infection, histology, Covid-19

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Introduction

One of the major reverberations of the second wave of COVID 19 in the Indian subcontinent included fungal infections, leading to death and disability in many.[1] The major etiological agent seen was mucor mycosis, followed by aspergillus and candida species.[2] These were commonly referred to as black, white, and yellow fungi. Multiple factors have been implicated to the post covid fungal infections namely injudicious use of steroids, uncontrolled diabetes, use and reuse of oxygen masks, industrial oxygen, innate delta variant nature and others. Some of these have been justified by adequate evidence, while others have been circulated in the media unwisely.[3]

The spike protein of SARS-CoV-2 is the key viral protein responsible for binding of the virus to angiotensin-converting enzyme- 2 (ACE-2) receptor in humans. It confers both infectivity and antigenicity. Multiple versions of SARS-CoV-2 have emerged since its initial emergence in Wuhan province of China. The delta variant was the prominent variant which caused the second and the most devastating wave in India. Mutations in the terminal domain of the Spike protein N, single Spike variations in SARS-CoV-2 have been reported.[4] This led to increased infectivity, transmission, and antibody resistance. Complimented with injudicious use of steroids along with multiple clinical and logistic factors, secondary bacterial and fungal infections conferred poor outcome with delta variant infection.[5]

Fungal infections, especially mucormycosis, are an uncommon histological finding. Mucor mold is ubiquitous. It may be found in soil, manure, rotting produce, even in nose and mucus of healthy individuals. They often are seen as opportunistic infections in immunocompromised and debilitated patients. In our setup, we saw occasional cases in prepandemic times.[6]

Mucor mycosis is an angioinvasive disease caused by mold fungi of the genus Rhizopus, Mucor, Rhizomucor, Cunninghamella, and Absidia of order Mucorales, class Zygomycetes. Rhizopus Oryzae is the most common type and responsible for nearly 60% of Mucor mycosis cases in humans. It also accounts for 90% of the Rhinoorbital Cerebral (ROCM) form.[3,7,8] The mode of contamination occurs by inhalation of fungal spores. Infection with this invasive fungus may be life threatening. In sections stained with hematoxylin and eosin (H&E) they may be visualized as broad aseptate branching fungal hyphae. Angioinvasion and thrombosis are also evident in mucor-infected tissues. Periodic acid shiff (PAS) and Grocott methenamine silver (GMS) are used to stain and identify these fungal elements. It has been postulated that SARS-CoV-2 infection may alter and effect cell mediated immunity, especially CD4+ and CD8+ T cells. T cells are involved in the production of cytokines that damage fungal hyphae, namely interleukin 4, 10, 17 and interferon gamma. This superadded with altered cell-mediated immunity seen in diabetics may contribute in invasive fungal infections seen in delta wave.[9,10]

Surgical debridement tissue samples from patients with suspected fungal infections were received in large numbers during the first months of 2021. In this study, we present a detailed account of the histology of these samples and discuss the salient morphological features for their identification. Highlighting the features that must be recognized while suspecting or diagnosing these fungal infections.

Material and Methods

Ours was a covid-19 L3 facility. The patients were referred from all towns, community centers and even district hospitals. Later we were converted to a fungal management center when secondary fungal infections in patients with COVID started to increase. A robust surgical team consisting of ENT, ophthalmic, oral, and maxillofacial surgeons was formed to complete the procedure.

Standard operating protocol for processing of debridement tissue: To ensure that deadly fungal elements are not missed, standard operating procedures were formed at the laboratory level and it was decided that all tissue would be processed for histology and submitted for fungal identification.[11] Periodic acid shiff (PAS) and Grocott methenamine silver (GMS) stains were also performed as and when necessary. The definitive report was dispatched only after 2 consultant pathologists had interpreted the sections.

In this retrospective observational study, sections obtained from debridement tissues received in histopathology laboratory were recovered from records and reviewed. Collected patient data was de-identified. All the investigators were blinded to the patient identifying details.

The slides were first scanned by third-year resident pathologists (PV, MM, DBD), then by senior residents (SR and DS) followed by consultant pathologists (PA and MK). Objective identification of morphological parameters was performed, namely (1) presence and absence of fungal elements, (2) presence and absence of necrosis, (3) type of necrosis, and (4) vascular invasion and any other morphological characteristics.

After careful review of the histology sections, few elements of patient clinical details were retrieved form records namely history and medication for diabetes mellitus, intervention in form of nebulization or oxygen support and management in terms of intravenous antifungals.

An MS EXCEL spreadsheet was used for data entry; SPSS version 16.0 was used for analysis. The Chi-Square test / Fisher's exact test were used to compare the study variables. For this study, the significance level was set at .05.

Results

Demographic variables

A total of 110 debridement tissue samples were studied. Mean age was 50.16 years which included patients from 25 to 75 years of age. Male: female ratio was 1.97:1. The most common site of fungal infection was the nasal cavity, followed by the maxilla, eyeball, and palate (Table 1). 65.4% (72/110) of the patients were diabetic. Fungal elements were not found in 14 cases. Ninety-four patients received antifungals, mainly intravenous amphotericin and Posaconazole. Diabetes was significantly associated with fungal infection (97.2%; 70/72; $P < .005$). 90% [19/21] of the patients who were on room air and diagnosed with fungal infection were diabetic. In 96 cases, morphological fungal elements were identified. Mucor mycosis was most frequent, dual infection with both aspergillus and mucor was seen in 7 cases and 2 showed actinomyces with mucor hyphae. 66% (6/9) of these patients with dual infection were diabetic and 77% (7/9) received nebulization. No fungal elements that were morphologically consistent with candida were observed in any of the debridement samples studied.

Morphological variables

Necrosis. Significantly large to small focus of necrosis was evident in 96 cases. The necrosis was very peculiar morphologically. It was dense eosinophilic granular enclosing fragmented polymorphs and debris (Figure 1a and b). Associated bone necrosis and pigment deposition were also extensively observed in cases (Figure 1c and d). This necrosis was observed in almost all cases in which fungal elements were present (Figure 1e). In 11 cases where necrosis was seen; and no fungal elements were identified, histological suspicion of fungal infection was reported. Subsequently repeat debridement tissue in 3 out them displayed mucor hyphae. In 6 cases, the KOH smear was positive, which was submitted in microbiology (Table 2). There were only 2 of them that were negative for fungal elements. They were managed conservatively. Associated granulomas

Table 1. Demographic and clinical variables of the studied debridement tissues: n = 110.

S. NO.	PATIENT CHARACTERISTIC	VALUE
1	Age	Mean 50.16 year (25-75)
2	Male:Female ratio	1.97:1 (73:37)
3	Site:	
	1. Nasal Cavity	73
	2. Maxilla	17
	3. Eyeball	8
	4. Palate	7
	5. Maxilla + Palate	6
	6. Brain	1
	7. Endotracheal	1
	8. Endobronchial	1
4	Room Air	21 (19.09%)
5	Nebulization plus oxygen	88 (80%)
6	Diabetes Mellitus (n = 77)	
	1. Present	72
	2. Absent	14
7	No Fungal Elements	14
8	Fingal elements identified	96
	1. Only mucor	83
	2. Mucor on repeat biopsy	3
	3. Mucor + Aspergillus	7
	4. Mucor + Actinomyces	3
9	Treatment	
	1. Death/Lama	14
	2. Anti-fungal given	94
	3. Conservative	2

with entrapped fungal hyphal structures were also evident, although granulomas were morphologically observed in 5 cases only (Figure 2a and b).

Mucor mycosis. Of the 96 fungal infections, 86 (89.58%) were Mucor mycosis alone. In 7 cases, co-infection with aspergillus and in 3 with actinomyces was evident. To our surprise, the fungal elements were readily identifiable in H&E-stained sections as a result of the presence of large numbers of fungal hyphae. Furthermore, PAS and GMS stains merely served to confirm. Large numbers of fungal elements were seen trapped in mucus (Figures 2c to e and 3a).

Morphologically, the fungal hyphae of the mucor were thick, irregular, and branching. They were pauciseptate and right-angle branched. Few broken forms to large hyphae were seen (Figure 2c to e). No septations were evident in many. Fungal hyphae were also seen infiltrating the vascular wall (Figure 4a and b). Fine focus examination could reveal the pale and dark areas within the wall of the fungal structure on H&E. Interestingly, all stages of the fungal life cycle were seen in the same case, and hemosiderin pigment due to red blood cell lysis was evident in many.

Aspergillus. Aspergillosis, when present with mucor in co-infections, had acute angled septate branching hyphal structures (Figure 3b), with intervening blob- or flower-like fruiting bodies (Figure 3c and d). Aspergillus hyphae were thin compared to mucor. They were present in large groups or colonies (Figure 3a).

Actinomyces. Superadded actinomyces with Mucor mycosis were also observed. Cotton ball like structures composed of slender bacteria were seen (Figure 3e), which were well stained with hematoxylin.

Discussion

The prevalence of Mucor mycosis varies from 0.005 to 1.7 per million people worldwide. Its prevalence is approximately 80 times higher (0.14 per 1000) in India when compared to western countries, according to a recent estimate of the year 2019-2020.[7,8] Diabetes mellites remains the leading risk factor associated with Mucor mycosis worldwide, with a global mortality of 46%.[12]

During the second wave in India, March 2021to July 2021; this number increased exponentially. SARS-CoV-2 infection with comorbidities, superimposed with unopposed use of steroids and other immunomodulators, increased the incidence of fungal infections in these patients. It was also seen that use of invasive procedures like intubation, central venous catheters for management predisposed to opportunistic fungal infections.[13,14]

The virologist played an important role in the diagnosis of Covid-19 and the mycologist in the initial identification of fungal hyphae in KOH preparations. However, histology and morphological typing of fungal elements in the debridement tissue was significant. The patients received additional intravenous antifungal therapy based on morphological diagnosis or culture reports.[15,16]

The present study highlights a detailed morphological account of debridement tissue received from suspected fungal infections with the objective of identifying the characteristic features that must be recognized while interpreting these tissues.

Necrosis reflects severe injury to cells, characterized by denaturation of proteins and cellular content leakage. The main cause of necrosis is cellular ischemia. Mucor mycosis is basically an Angio invasive fungus[17] that leads to ischemic necrosis

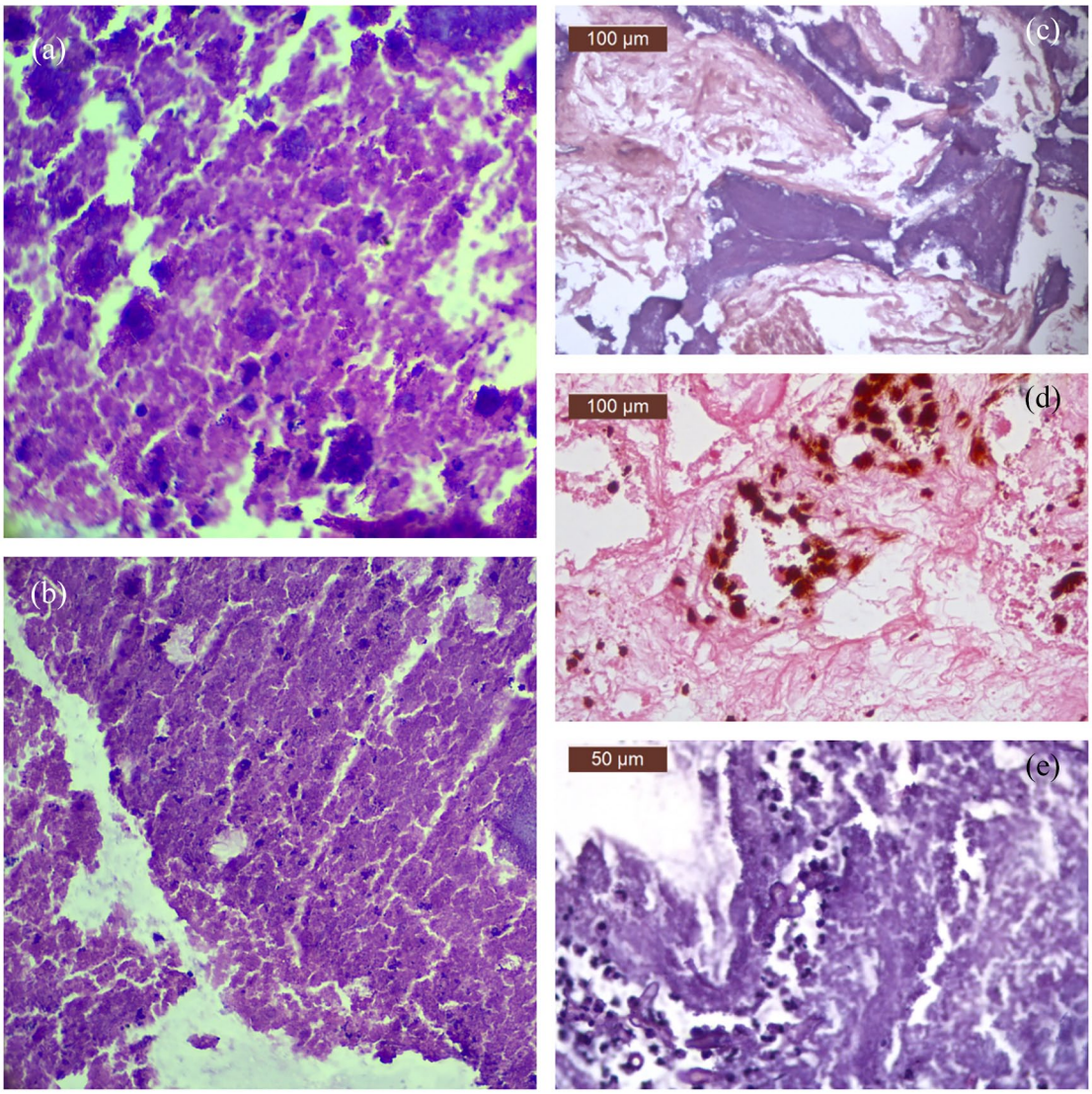

Figure 1. The section shows eosinophilic granular necrosis with an amorphous texture similar to "magic sand" with enclosed neutrophilic debris in (a) (H&E x200) and (b) (H&E x100). Bone necrosis is also observed in (c) and brown pigment is also observed in (d) (H&E x200). Fungal elements entrapped in necrosis are seen to be intermixed with fragmented neutrophils ((e); H&E x400).

Table 2. Histological presence of Necrosis and lecocytoclasis in association with fungal element identification.

FUNGUS ON HPE	NECROSIS AND LECOCYTOCLASIS	
	PRESENT	ABSENT
Present	Mucor = 92 (89.3%)	0
Absent	N = 11 (11.7%) Repeat biopsy positive=3 (27.27%) KOH positive = 6 (54.54%) No further Fungus identified=2 (18.18%)	7 (7.4%)
	103	7

accompanied by acute inflammation. Commonly the ischemic necrosis morphologically shows increased eosinophilia, with glassy homogenous appearance of the necrosed cells.[18] However, necrosis observed in tissues infected with mucor infection was eosinophilic, amorphous, displaying leucocytoclasis. Its texture was similar to "magic sand." The defense of the host against mucor is especially through macrophages and neutrophils. Neutrophils initiate oxidative burst which cause death of

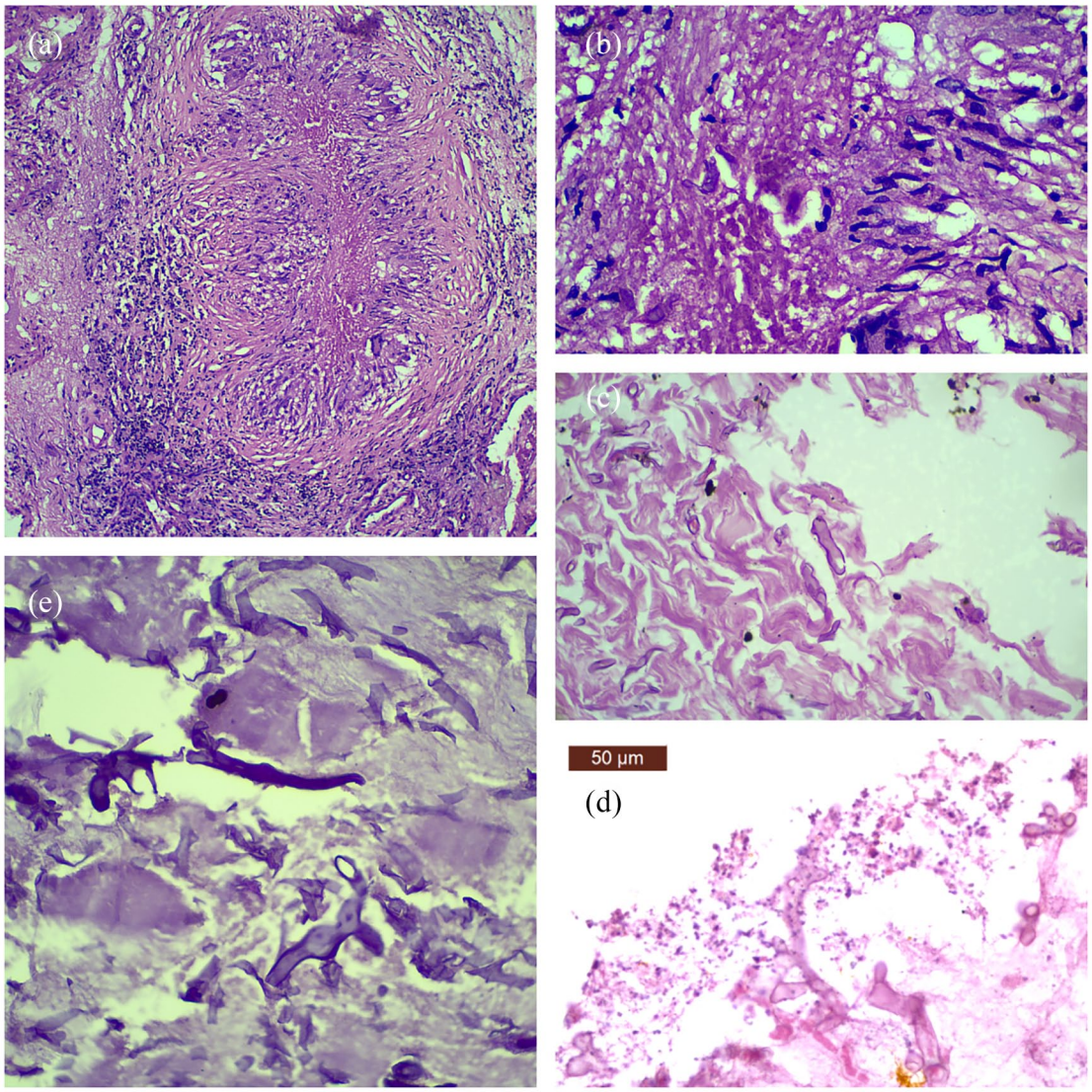

Figure 2. The section shows a well-formed granuloma with central necrosis ((a); H&Ex40) along with giant cells, in higher power view in (b) (H&E x400) trapped fragmented fungal elements are seen. (c-e) (H&E x400) show pauciseptate ribbon-shaped fungal hyphae showing various thicknesses of the wall and branching at the right angle as well. Small blob-like ends are also seen. The fungal elements in this section show background mucus in them.

proliferating hyphae and macrophages inhibit germination of fungal spores in diseased area.[19] Presence of both these inflammatory cells was seen in abundance in debrided tissue.

Interestingly mucor was also seen flourishing in the entrapped mucus of the tissue along with surrounding hemorrhagic areas. Mucor is known to thrive where iron is present and tend to invade the blood vessels. Iron metabolism related genes have been identified to be involved in iron uptake by them. They regulate reductive system via ferric reductase, copper oxidases and high affinity permease. Siderophore permeases and uptake from heme via heme oxygenase are also seen.[3,20]

Morphological identification of fungal elements is vital for any histopathologist who reports on surgical pathology samples. Patients often present with symptoms such as black discharge from the nose, mouth, or gums. It may be accompanied by facial weakness or squinting due to the involvement of cranial nerves. In our study, the patients presented with swelling, black discoloration, pain or discharge from these areas. Usually, these fungus are resistant to multidrug therapy and cause serious life-threatening systemic infection if left untreated. The newer triazoles, posaconazole (POSA) and isavuconazole (ISAV) have been found to be effective therapy in their management.[21]

Another important pertinent morphological feature apart from hyphae being pauci septate and right-angle branching was that fine focus revealed differential density of the thick fungal hyphae which was not seen in either aspergillus or candida. They have been reported to vary in width and may also appear crinkled or folded.[19,22]

In cases where dual infection was observed, the fungal colonies were dense with seemingly intermingling of fungal elements. Clinically and in KOH preparations dual infections are difficult to identify. Histology is diagnostic in this scenario.[23]

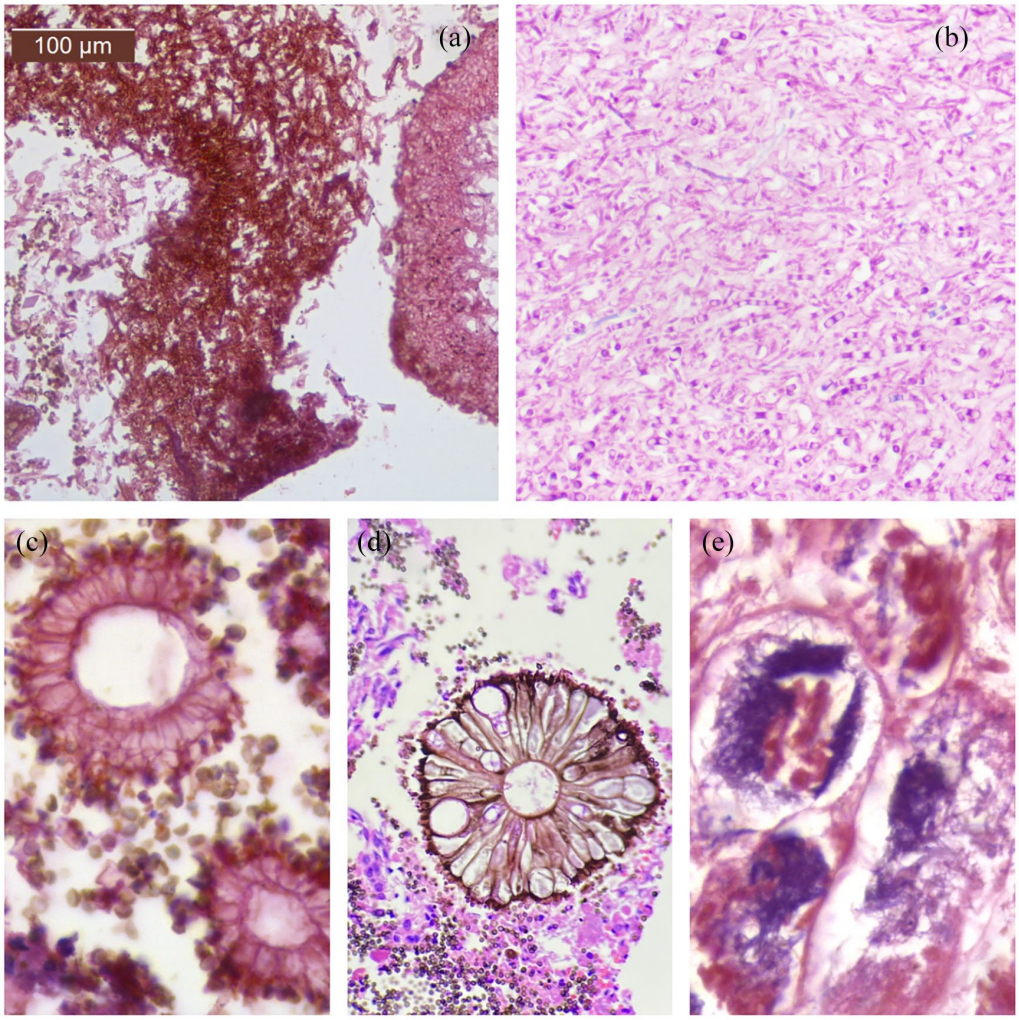

Figure 3. Section (a) shows a dense fungal colony with a large amount of hemosiderin pigment (H&E x200), fungal elements morphologically consistent with aspergillus were observed at higher magnification ((b); H&E x200). The fungal hyphae are acute angle branched with readily appreciable septations. Beautiful fruiting bodies can be seen in (c and d) (H&E x400) with a central core and hyphal arranged in a crown-like manner, and multiple red blood cells are seen in close apposition. Long slender bacteria forming cotton balls are seen in (e) (H&E x400).

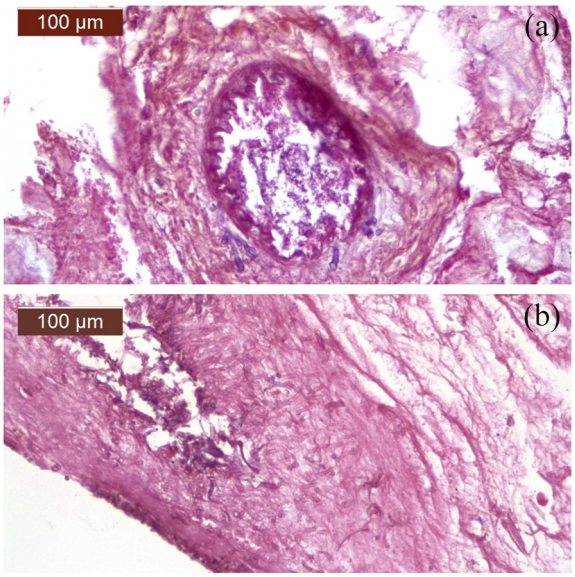

Figure 4. Mucor hyphae are seen infiltrating the vascular walls in (a and b) (H&E x200).

Morphological identification of the 2 separate fungal elements on basis of the described features helped to individualize both. The presence of fruiting bodies on histology was quite diagnostic of aspergillus infection. There are few case reports in literature which report histological features of aspergillus fruiting bodies.[24,25] Fruiting bodies were observed in 5/7 cases with a central core and fungal elements growing out of it (Figure 3c and d). Careful observation and scanning of the sections are needed to find these readily identifiable formations.

Diabetes was seen to be significantly associated with mucor infection in patients who underwent debridement in our study, as compared to patients who underwent intervention or were given oxygen therapy. Patel et al, in their large metacentric study in patients with COVID 19, also reported that accompanying mucor mycosis was seen more frequently in patients with uncontrolled diabetes.[26] Similarly, Jeong et al in their meta-analysis collated the above observations. The plausible reason for this observation is defective innate immunity, cell-mediated immunity, chemotaxis along with the function of polymorphs and macrophages.[27,28]

The strength of the present study is that it provides a detailed account of the morphology of the fungal debridement tissues and supports diabetes being the major cofactor of opportunistic fungal infections in Covid 19 patients. The limitation is that it is a selective study of patients with fungal infections in SARS-CoV-2 patients. The observations cannot be generalized until further investigated. A study that evaluates the morphology of fungal infections in patients who are not infected with SARS-CoV-2 may establish and support our findings.

Conclusion

To conclude, mucormycosis was the most common opportunistic fungal infection seen in cases of Covid-19 in our set-up followed by aspergillus. Eosinophilic granular necrosis with the presence of neutrophilic debris was an important characteristic feature associated with fungal infection. So much so that the presence of this type of necrosis warrants careful observation, embedding of the entire sampled tissue, additional special stains, and repeat biopsy if required. We also describe the salient morphological characteristics for the identification of mucor myosis and aspergillus, along with the histomorphology of the fruiting bodies of aspergillus. Finally, we recommend that while interpreting a case with suspected fungal infection, the morphological identification of fungal elements must be performed with caution. Fine features must be kept in mind, and the entire tissue must be thoroughly examined to avoid misdiagnosis and most importantly "missed diagnoses."

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Author Contributions

Preeti Agarwal: Conceptualization, Methodology, Software, Validation, Formal analysis, Data Curation, Writing—Original Draft, Writing—Review & Editing, Visualization, Project administration. Devanshi B Dubey: Software, Writing—Original Draft. Madhu Kumar: Validation. Pratima verma: Software. Menka Mishra: Software. Shalini Rawat: Software. Damini Singh: Software. Veerendra Verma: Investigation, Resources, Supervision. Ravindra Kumar Garg: Investigation, Resources, Supervision.

Consent for Publication

The manuscript has been reviewed by all authors and they are of the view that this information will be useful to clinicians and pathologists alike. We hereby transfer, assign, or otherwise convey all copyright ownership, including all rights incidental thereto, exclusively to the journal, if such work is published by the journal.

Data Availability

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Statement of Ethics

The work was approved by Institutional Ethics Committee, King George's Medical University. An ethical waiver has been granted, all the information provided in this manuscript has been de-identified and the patients cannot be traced directly. All the investigators were blinded to the patient identifying details.

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