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RESEARCH

Invasive Fungal Rhinosinusitis: The First Histopathological Study in Vietnam

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Abstract

Background Invasive fungal rhinosinusitis (IRFS) is a rare but highly fatal disease. The two primary groups of pathogens, *Mucorales* and *Aspergillus*, require different treatments and have distinct prognoses.

Purpose This study aimed to analyze the histopathological features of IFRS.

Methods We conducted a retrospective study involving 57 IFRS cases. Demographic and comorbid characteristics were obtained from clinical records. Two pathologists independently examined the histopathological features using H&E, PAS, and GMS-stained slides. Fungal groups were identified with PCR under the guidance of histopathology.

Results The mean age of IFRS was 58.9 ± 13.4. The male-to-female ratio was 1.4:1. 100% of cases had diabetes comorbidity. *Mucorales*, *Aspergillus*, and other fungi were found in 61.4%, 33.3%, and 5.3% of cases, respectively. No *Aspergillus* and *Mucorales* co-infections were detected. Histopathology and PCR results were strongly concordant in classifying pathogens (Cohen's kappa = 84.2%, 95% CI 60.1% – 100%, $p < 0.001$). Mucormycosis exhibited higher rates of extensive necrosis and vascular invasion, and lower rates of pigment and spore presence than the non-Mucormycosis group ($p < 0.001$, $p = 0.01$, $p = 0.02$, $p = 0.03$, respectively). Extensive necrosis and vascular invasion were statistically significantly correlative (OR = 13.03, 95% CI 2.62–64.75, $p = 0.002$).

Conclusions IFRS predominantly affects older adults and males. Histopathology is a reliable method for differentiating between *Mucorales* and *Aspergillus*. When extensive necrosis is detected, it is critical to investigate for vascular invasion carefully. The vascular invasion, degree of necrosis, pigments, and spores are valuable factors for distinguishing fungal agents of IFRS.

Keywords Invasive fungal rhinosinusitis · Histopathology · Mucormycosis · Aspergillosis

Introduction

Invasive fungal rhinosinusitis is now emerging as a global issue. A definitive diagnosis of fungal rhinosinusitis is made based on tissue biopsy and classified as either invasive or non-invasive [1]. IFRS is characterized by the presence of fungal hyphae in the mucosa, submucosa, blood vessels, or bone of the nasal and paranasal sinuses as observed through histopathology [2]. Fungi are not uncommon causes, contributing to approximately 11% of all rhinosinusitis cases

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[3]. Although invasive fungal infections (IFI) are relatively rare, occurring in about 6 cases per 100,000 individuals per year [4], they carry a notably high annual mortality rate, reaching up to 85.2% in those with severe invasive Aspergillosis [5]. The sinuses are one of the frequent sites for invasive fungal infections, second-ranked following after the lungs [6]. The progression of IFRS is often serious, with the potential for invasion into the orbital cavity and brain, leading to death or significant long-term complications [7]. Notably, Mucormycosis, also known as black fungus, typically affects the lungs and the sinuses [8] and is associated with a poor prognosis, with an overall mortality rate of up to 78% in rhinocerebral cases [9].

The two primary groups of pathogens causing IFRS are *Mucorales* and *Aspergillus*, each requiring distinct treatment approaches and exhibiting different prognoses [2]. The diagnosis of IFRS necessitates prompt and precise detection of fungal presence in tissue, together with accurate differentiation between Mucormycosis and Aspergillosis. Diagnostic tests include histopathology, direct microscopy, fungal culture, and polymerase chain reaction (PCR) [10]. PCR analysis of fresh or formalin-fixed, paraffin-embedded (FFPE) tissue samples is highly effective in identifying pathogens due to its considerable specificity. However, the sensitivity of this test varies among different studies [11–13]. Fungal cultures also aid in identifying the fungal species, but this method is time-consuming and has low sensitivity [14, 15]. Direct microscopy is frequently employed as an initial diagnostic approach. However, this test has an even lower sensitivity than fungal cultures [16], due to the smaller fungal quantity involved and the lack of fungal population amplification. Among these laboratory tests, histopathology, which confirms the presence of fungal hyphae in tissue, remains the gold standard for diagnosis [17, 18]. Histopathological testing offers the advantages of high sensitivity and specificity [19], along with reduced time requirements. Additionally, by assessing histopathology, including the location of suspected structures and type of cellular reaction, the pathologist can identify the fungal group responsible for the disease and determine whether it is the true causative agent or merely a contaminant [10]. However, while this method can help distinguish between the two main groups of IFRS agents, it still relies on the pathologist's experience, especially in challenging cases. Consequently, it is essential to compare histopathology results with PCR findings to validate the accuracy of histopathology in identifying pathogenic fungal groups.

IFRS frequently occurs in immunocompromised individuals. Recognized risk factors include poorly controlled diabetes, organ transplantation, hematological malignancies, and HIV infection. Additionally, the outbreak and decline of the Coronavirus disease 2019 (COVID-19) pandemic have led to numerous case reports and studies linking IFRS to this viral infection. Current literature indicates that COVID-19 induces immune system dysregulation [20], coupled with systemic steroid treatment, which predisposes patients to opportunistic pathogens [21]. Despite recent observations of increased IFRS cases following the COVID-19 pandemic [22–24], purely histopathological studies of IFRS are still limited. Therefore, we conducted this study to enhance our understanding of this severe disease.

Materials and Methods

Inclusion and Exclusion Criteria

The retrospective study was conducted on 57 IFRS cases at the Department of Pathology, University Medical Center at Ho Chi Minh City, and the Department of Pathology, University of Medicine and Pharmacy at Ho Chi Minh City, from 01/2021 to 12/2023. All cases included in this study met the criteria of proven invasive fungal disease from The European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium – [EORTC/MSGERC] [25].

Specifically

- Cases with fungi visible in the tissue on H&E (Hematoxylin and Eosin).
- Cases with suspected fungi on H&E and PAS or GMS positive for fungi.
- Cases with suspected fungi on H&E and direct microscopy or culture detecting fungi.
- Cases with suspected fungi on H&E and PCR detecting fungal DNA.

Exclusion criteria for this study included missing information on age or gender, improperly fixed and processed tissue specimens, insufficient tissue for special staining and PCR, and inability to identify the mucosal structures due to excessive necrosis.

PCR Procedure

We performed single-primer PCR assays for two fungal pathogens: *Mucorales* and *Aspergillus*, adhering to the protocols established by the Center for Molecular Biomedicine at the University of Medicine and Pharmacy at Ho Chi Minh City. Initially, pathogens were identified based on morphology using HE, GMS, and PAS stains. Specimens diagnosed with Mucormycosis or Aspergillosis via histopathology

were then subjected to PCR using corresponding specific primers. The PCR products were analyzed through electrophoresis on a 1% agarose gel with a 1Kb DNA ladder as a reference. Electrophoresis results facilitated the identification of *Mucorales* or *Aspergillus* in the samples based on the size of the electrophoretic bands and comparison with control samples. If PCR results were negative, the samples were subsequently tested using primer for the remaining fungal group. In cases where *Mucorales* and *Aspergillus* co-infection, or fungal infections other than these two pathogens, was suspected, sequential PCR was performed with both primers to confirm mono-infection, *Aspergillus* and *Mucorales* co-infection, or other fungal infections. All specimens where fungi were identified on histopathology but yielded negative PCR results for both primers were categorized as infections with other fungal groups. Any negative PCR results were repeated once to minimize the potential for false negatives.

The primers utilized in this study were procured from Integrated DNA Technologies. The primer sequences for amplifying the 18 S rRNA gene region of *Mucorales* were derived from the research of Rickerts et al. [26]. A primer pair was designed to amplify a region of the *Aspergillus* 18 S rRNA gene using the Primer-BLAST tool on the NCBI website. This primer pair was validated using a control sample of *Aspergillus flavus* cultured in the laboratory. The sequences of the two primer pairs are detailed in Table 1.

Demographic, Comorbid, and Histopathological Characteristics

Patient's age, sex, and comorbidity data were collected from clinical records. All cases were stained with H&E, PAS, and GMS, following the protocols of the University of Medicine and Pharmacy at Ho Chi Minh City, based on the literature of Kim Suvarna S et al. [27]. The causative agent was classified as *Aspergillus*, *Mucorales*, *Aspergillus* and *Mucorales* co-infection, or other fungi based on PCR results performed under the guidance of histopathological fungal morphology. Mucormycosis is histopathologically suspected when tissue samples show non-pigmented, broad (5–20 μm), thin-walled, ribbon-like, non-septate or minimally septated, and right-angle branching hyphae [28], while Aspergillosis is characterized by thin (3–12 μm), septate, and acute-angle branching hyphae [29]. The anatomical locations of the fungal invasion were determined by collecting CT scan results, surgical reports, and pathology findings. Histopathological features consisting of mucosal or bone invasion, neutrophil infiltration, eosinophil infiltration, stromal edema, granulomatous inflammation, necrosis, vascular invasion, perineural invasion, the presence of fungal pigments and spores were analyzed on H&E-stained slides by two pathologists to ensure diagnostic accuracy. The thresholds for inflammatory cell infiltration are >1 extravasated neutrophil per high-power field (HPF) [30] and >5 eosinophils per HPF [31], respectively. Granulomatous inflammation is characterized by the aggregate of activated histiocytes, which transform into epithelioid cells or coalesce to form multinucleated giant cells [32]. Vascular invasion is defined as the presence of fungal hyphae within the blood vessel wall or the vessel lumen accompanied by thrombosis [33]. Perineural invasion is the occurrence of fungal hyphae within nerve bundles [33]. The degree of necrosis is classified as absent, mild, or extensive based on the proportion of necrotic tissue area relative to the total tissue sample area. Specifically, this classification is as follows: absent (0%), mild (less than 50%), and extensive (50% or more of the tissue sample affected by necrosis).

Table 1 Primer pair sequences for amplifying the 18 S rRNA gene region of *Mucorales* and *Aspergillus*

Gene regions	Code	Primer sequences (5' → 3')	PCR length (base pairs)
Mucorales			
18 S-rRNA	ANZM1	ATTACCATGAGCAAATCAGA	173
	ANZM3	TCCAAGAATTTCACCTCTAG	
Aspergillus			
18 S-rRNA	18 S-F2	AACGAGGAATGCCTAGTAGG	177
	18 S-R2	CTAAATGACCGGGTTTGACC	

Statistical Analysis

Data were managed and analyzed using STATA 17.0 software. 95% confidence intervals were employed for statistical comparisons, with p-values <0.05 considered statistically significant. Fisher's exact and Chi-squared tests were used for categorical variables, while Student's t-test was employed for continuous variables. The agreement between fungal classification results based on histopathology and PCR was assessed using Cohen's kappa coefficient [34].

Results

Prevalence of Fungal Agents and Comparative Analysis of Histopathology and PCR

In this study, fungal etiologies of IFRS were identified using PCR. *Mucorales* was predominated, accounting for 61.4% (35/57, 95% CI 47.6% – 74%) of cases, followed by *Aspergillus* at 33.3% (19/57, 95% CI 21.4% – 47.1%), and other fungi at 5.3% (3/57, 95% CI 1.1% – 14.6%). No

co-infections involving both *Aspergillus* and *Mucorales* were detected.

Among 38 cases initially suspected to be Mucormycosis based on histopathological examination, 4 cases (4/38, 10.5%) were identified as Aspergillosis through PCR analysis. Conversely, of the 18 cases initially thought to be infected with *Aspergillus* on histopathology, PCR confirmed 1 case (1/18, 5.6%) as *Mucorales* and 3 cases (3/18, 16.7%) as other fungi. Additionally, there was one case of fungal infection presenting with two morphologies on histopathology; however, PCR results indicated that this case was solely infected with *Aspergillus*. The Cohen's kappa coefficient for comparing pathogen identification results between histopathology and PCR is 84.2%, ($p < 0.001$), as shown in Table 2.

Demographics and Comorbidity

In this study, the average age of onset for individuals with IFRS was 58.9 ± 13.4 years, ranging from 19 to 94 years. The most prevalent age group for the disease is 60–69 years, comprising 29.8% of cases (Table 3). The mean age was 58.6 ± 14.8 years for men and 59.3 ± 11.5 years for women. Statistical analysis using a t-test indicated no significant difference in onset age between genders ($p = 0.86$).

The mean age of patients with invasive Aspergillosis was 63.7 ± 14.8 years, while for those with invasive Mucormycosis, it was 56.9 ± 12.3 years. A t-test revealed no statistically significant difference in the mean age between these two groups ($p = 0.08$).

The study comprised 33 male and 24 female patients, corresponding to a male-to-female ratio of 1.4:1. Statistical analysis using the Chi-squared test found no significant association between male gender and Mucormycosis ($p = 0.34$) (Table 4).

Comorbidities were diabetes mellitus and pulmonary tuberculosis, which accounted for 100% and 1.8%, respectively (Table 3).

Histopathological Characteristics

In 42.1% of cases, the fungi invaded multiple anatomical sites. Specifically, invasion occurred in the following proportions: maxillary sinuses (52.6%), sphenoid sinuses (36.8%), nasal cavity (31.6%), ethmoid sinuses (19.3%), orbital cavities (12.3%), frontal sinuses (3.5%), and cerebrum (3.5%). Statistical analysis did not reveal a significant association between invasion of more than one site and Mucormycosis, nor did it indicate significant associations between each site of invasion and this group of fungi (Table 4).

The proportions of mucosal and bone invasion were 70.2% and 29.8%, respectively. Neutrophil and eosinophil infiltration occurred in 98.2% and 50.9% of cases, respectively. Stromal edema and granulomatous inflammation were present in 50.9% and 42.1% of cases, respectively. All cases exhibited necrosis, with extensive necrosis in 56.1% and mild necrosis in the remaining. Vascular and perineural invasion were found in 33.3% and 7% of cases, respectively (Table 3) (Fig. 1). The rate of perineural invasion was significantly lower than that of vascular invasion ($p < 0.001$). Fungal pigments and spores were present in 24.6% and 5.3% of cases, respectively (Table 3).

The percentages of extensive necrosis and vascular invasion in Mucormycosis were statistically significantly higher than in the non-Mucormycosis group ($p < 0.001$, and $p = 0.01$, respectively) (Table 4). In contrast, the non-Mucormycosis group had higher rates of the presence of fungal pigments and spores than Mucormycosis ($p = 0.02$, and $p = 0.03$, respectively) (Table 4). No statistically significant associations were found between bone invasion, neutrophil infiltration, eosinophil infiltration, stromal edema, and granulomatous inflammation with pathogen groups (Table 4). Although the Mucormycosis group exhibited more perineural invasion than the non-Mucormycosis group, this difference was not statistically significant ($p = 0.15$) (Table 4).

These tests were used to compare the two groups Mucormycosis and non-Mucormycosis.)

Most cases of mild necrosis did not exhibit vascular invasion (23 out of 25 cases, or 92%). In contrast, vascular invasion was observed in a significant proportion of cases

Table 2 Comparison of PCR and histopathology results for pathogen classification

		PCR results				Total
		Mucorales	Aspergillus	Co-infection*	Others**	
Histopathological results	*Mucorales*	34	4	0	0	38
	Aspergillus	1	14	0	3	18
	Co-infection*	0	1	0	0	1
	Others**	0	0	0	0	0
Total		35	19	0	3	57

Cohen's Kappa coefficient for comparison was 84.2%, (95% confidence interval: 60.1 – 100%), $p < 0.001$

* *Mucorales* and *Aspergillus* co-infection

** Fungal infections other than *Mucorales* and *Aspergillus*

Table 3 Demographic, comorbid, and histopathological characteristics of IFRS

Characteristics	Summary statistics* ($n=57$)
Age	58.9 ± 13.4 (range: 19–94)
< 20	1 (1.8)
20–29	0 (0)
30–39	3 (5.3)
40–49	10 (17.5)
50–59	16 (28.1)
60–69	17 (29.8)
70–79	8 (14)
≥ 80	2 (3.5)
Male	33 (57.9)
Comorbidity	
Diabetes	57 (100)
Tuberculosis	1 (1.8)
Fungal groups	
Mucormycosis	35 (61.4)
Aspergillosis	19 (33.3)
Others	3 (5.3)
Sites of fungal invasion	
Nasal cavity	18 (31.6)
Maxillary sinuses	30 (52.6)
Ethmoid sinuses	11 (19.3)
Sphenoid sinuses	21 (36.8)
Frontal sinuses	2 (3.5)
Orbital cavities	7 (12.3)
Cerebrum	2 (3.5)
Number of invasive location	
One location	33 (57.9)
More than one location	24 (42.1)
Histopathological features	
Mucosal invasion	40 (70.2)
Bone invasion	17 (29.8)
Neutrophil infiltration	56 (98.2)
Eosinophil infiltration	29 (50.9)
Stromal edema	29 (50.9)
Granulomatous inflammation	24 (42.1)
Necrosis	57 (100)
Vascular invasion	19 (33.3)
Perineural invasion	4 (7)
Fungal pigments	14 (24.6)
Fungal spores	3 (5.3)
Degree of necrosis	
Mild	25 (43.9)
Extensive	32 (56.1)

*N (%) for categorical variables and Mean ± SD for continuous variables

with extensive necrosis (17 out of 32 cases, or 53.1%). The association between extensive necrosis and vascular invasion was statistically significant (OR = 13.03, 95% CI 2.62–64.75, $p=0.002$) (Table 5).

Disscussion

Prevalence of Fungal Agents and Comparative Analysis of Histopathology and PCR

Our study showed that *Mucorales* and *Aspergillus* are the two main fungal groups responsible for IFRS, which aligns with the literature [2]. However, differences in the distribution of fungal groups persist between reports, likely due to variations in sample number and epidemiological characteristics. Fadda GL et al. conducted a study of 17 cases in Italy, which revealed that *Aspergillus fumigatus* was responsible for 41.2%, *Rhizomucor* and *Penicillium* each accounted for 5.9% of cases, and in 47% of patients, the causative agent was not specified [35]. Chaturantabut S et al. conducted PCR on 20 IFRS cases, finding that *Aspergillus* accounted for 45%, *Mucorales* accounted for only 5%, and the remaining fungi accounted for 50% [36]. In contrast, El-Kholy NA et al. investigated 36 patients with post-COVID-19 IFRS and revealed that 77.8% of the cases were infected with *Mucorales*, while 30.6% were infected with *Aspergillus* [7], meaning the rate of co-infection was 8.3%.

The PCR results revealed that 3 out of 57 cases (5.3%) were negative for *Aspergillus* and *Mucorales* primers. Medical literature indicates that certain rare filamentous fungi may exhibit morphological similarities to these two fungal groups, which can complicate the diagnosis. Specifically, *Fusarium* and *Pseudallescheria boydii* are two fungi that require careful differential diagnosis due to their morphology overlapping *Aspergillus*. Invasive fusariosis, caused by *Fusarium species*, is associated with a poor prognosis, with a mortality rate of approximately 56% [37], and can escalate to 60 – 100% in disseminated cases [38]. IFRS caused by *Pseudallescheria boydii* is extremely rare, with only a few cases documented in the literature. This pathogen is often resistant to Amphotericin B, and the mortality rate in patients with immunodeficiency is notably high [39].

Histopathology and PCR results showed strong agreement in identifying pathogenic fungal groups. This underscores that histopathology remains a reliable diagnostic tool for distinguishing Mucormycosis from Aspergillosis. Discrepancies between histopathology and PCR typically arise in cases where the tissue sample exhibits extensive necrosis, which can distort fungal hyphae morphology and complicate the differential diagnosis. This limitation highlights the importance of employing fungal culture and PCR, as these methods can provide more precise identification of the fungal species responsible for the infection.

Table 4 Association between demographic and histopathological characteristics and invasive mucormycosis

Characteristics	Fungal groups*				*p*-value **
	Mucormycosis ($n=35$)	Non-Mucormycosis ($n=22$)			
		Aspergillosis ($n=19$)	Others ($n=3$)	Both ($n=22$)	
Age	56.9 ± 12.3	63.7 ± 14.8	52.3 ± 10.3	62.1 ± 14.6	0.15
Male	22/35 (62.9)	10/19 (52.6)	1/3 (33.3)	11/22 (50)	0.34
Site of fungal invasion					
Nasal sinus	12/35 (34.3)	5/19 (26.3)	1/3 (33.3)	6/22 (27.3)	0.58
Maxillary sinuses	21/35 (60)	8/19 (42.1)	1/3 (33.3)	9/22 (40.9)	0.16
Ethmoid sinuses	8/35 (22.9)	3/19 (15.8)	0/3 (0)	3/22 (13.6)	0.50
Sphenoid sinuses	10/35 (28.6)	10/19 (52.6)	1/3 (33.3)	11/22 (50)	0.10
Frontal sinuses	0/35 (0)	2/19 (10.5)	0/3 (0)	2/22 (9.1)	0.15
Orbital cavities	2/35 (5.7)	5/19 (26.3)	0/3 (0)	5/22 (22.7)	0.10
Cerebrum	2/35 (5.7)	0/19 (0)	0/3 (0)	0/22 (0)	0.52
More than one invasive location	14/35 (40)	10/19 (52.6)	0/3 (0)	10/22 (45.5)	0.69
Histopathological features					
Bone invasion	10/35 (28.6)	5/19 (26.3)	2/3 (66.7)	7/22 (31.8)	0.79
Neutrophil infiltration	34/35 (97.1)	19/19 (100)	3/3 (100)	22/22 (100)	1.00
Eosinophil infiltration	20/35 (57.1)	8/19 (42.1)	1/3 (33.3)	9/22 (40.9)	0.23
Stromal edema	18/35 (51.4)	9/19 (47.4)	2/3 (66.7)	11/22 (50)	0.92
Granulomatous inflammation	15/35 (42.9)	8/19 (42.1)	1/3 (33.3)	9/22 (40.9)	0.89
Extensive necrosis	26/35 (74.3)	5/19 (26.3)	1/3 (33.3)	6/22 (27.3	**< 0.001**
Vascular invasion	16/35 (45.7)	3/19 (15.8)	0/3 (0)	3/22 (13.6)	**0.01**
Perineural invasion	4/35 (11.4)	0/19 (0)	0/3 (0)	0/22 (0)	0.15
Fungal pigments	5/35 (14.3)	7/19 (36.8)	2/3 (66.7)	9/22 (40.9)	**0.02**
Fungal spores	0/35 (0)	3/19 (15.8)	0/3 (0)	3/22 (13.6)	**0.03**

*N (%) for categorical variables and Mean ± SD for continuous variables

** Fisher's exact and Chi-squared tests for categorical variables and Student's t-test for continuous variables

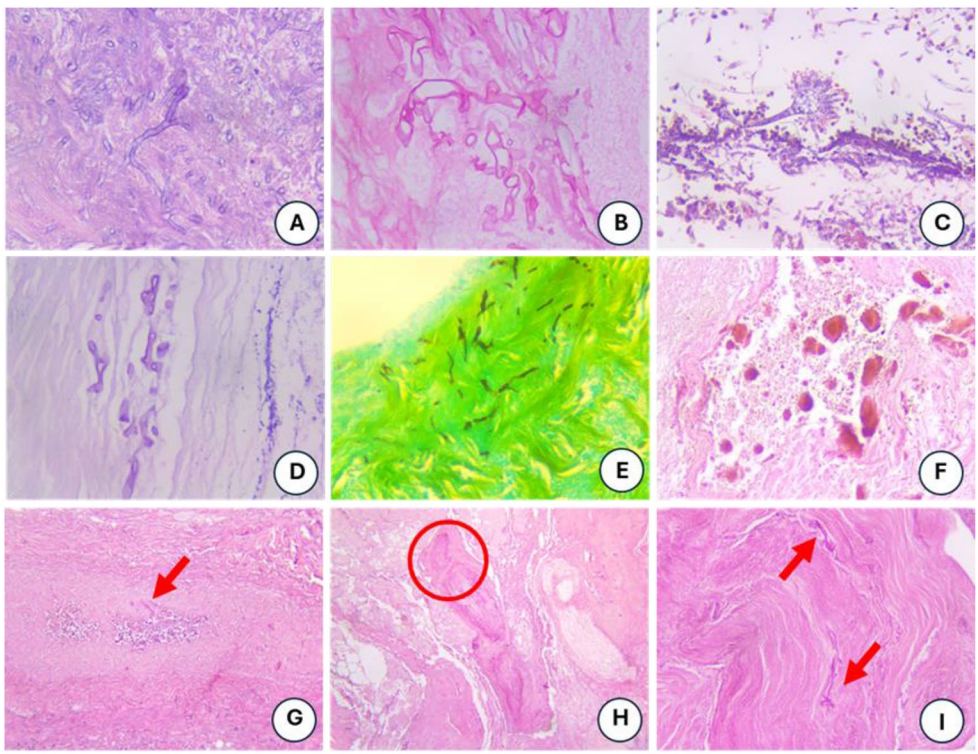

Fig. 1 Microscopic features. (**A**) Narrow, septate, acute-angle branching *Aspergillus* hyphae (H&E, X400). (**B**) Broad, non-septate, ribbon-like, right-angle branching *Mucorales* hyphae (H&E, X400). (**C**) Pigmented conidiophores of *Aspergillus* (H&E, X400). (**D**) PAS-positive *Mucorales* hyphae (PAS, X400). (**E**) GMS-stained *Aspergillus* hyphae (GMS, X400). (**F**) Fungal pigments (H&E, X400). (**G**) Hyphae invade a vascular wall (H&E, X100). (**H**) A degenerative neural bundle within a region of extensively necrotic tissue (H&E, X40). (**I**) Higher magnification of the red-circled field (**H**) shows several hyphae invading the nerve bundle (H&E, X100). Red arrows indicate the fungal hyphae

Table 5 Correlation between extensive necrosis and vascular invasion in IFRS

Degree of tissue necrosis	Vascular invasion		*p*-value*	OR (95% CI)
	Present	Absent		
Extensive	17/32 (53.1%)	15/32 (46.9%)	0.002	13.03 (2.62–64.75)
Mild	2/25 (8%)	23/25 (92%)		

* Fisher's exact test

Demographics and Comorbidity

Our study found that IFRS was more prevalent among men and older adults, commonly over 40 years, which aligns with previous research [19, 23, 40–42]. In addition to older age and male gender, several immunocompromising conditions are associated with invasive fungal disease, including uncontrolled diabetes, solid organ transplantation, hematopoietic stem cell transplantation, HIV infection, tuberculosis, cytomegalovirus infection, and more recently, COVID-19 [43]. A recent review of Kurokawa M et al. indicated that 36 – 40% of Mucormycosis and 21 – 34% of Aspergillosis cases were associated with diabetes, with an observed increase in incidence following the COVID-19 pandemic [1]. In contrast, our study reported a significantly higher rate of concurrent diabetes, with 100% of our cases presenting with this condition. In addition, only one case of co-infection with pulmonary tuberculosis was reported, and no instances of immunodeficiency from other causes were observed, possibly due to the limited number that was not representative. For these immunocompromised individuals, prompt diagnosis and accurate identification of the causative agents are crucial, as antifungal treatments can lead to severe complications. Amphotericin B, a key antifungal medication with proven effectiveness, must be used judiciously due to its potential for significant toxicities, particularly in patients with multiple underlying life-threatening conditions [44].

Histopathological Characteristics

Based on the existing literature, IFRS is classified into three primary types: acute IFRS, chronic IFRS, and chronic granulomatous IFRS; which is mainly based on the time of disease onset and clinical course [2]. However, in our study, these data were not collected, which precluded precise classification of IFRS types. Nevertheless, we suggest that most cases in our study likely fall into the acute IFRS category. This suggestion is supported by the predominant histopathological features indicative of acute inflammation: 98.2% of cases exhibited neutrophil infiltration, 100% showed necrosis (with 56.1% displaying extensive necrosis), 50.9% had stromal edema, and 33.3% demonstrated vascular invasion. Furthermore, the significant granulomatous reaction observed in 42.1% of cases suggests that a substantial proportion of patients may have been hospitalized due to acute exacerbations of underlying chronic invasive fungal disease. Further clinical-histopathological studies are needed to address the limitations of this study.

Due to significant differences in treatment approaches, we find it essential to investigate the IFRS's histopathological characteristics and to compare the features of Mucormycosis and the non-Mucormycosis group. Despite this, there is a notable lack of research comparing the histopathological features of different invasive fungal pathogens in the nasal and paranasal sinuses. Although some known unfavorable factors are poorly controlled diabetes and brain involvement [45], the discrepancy in prognosis between Mucormycosis and Aspergillosis continues to be a subject of debate [45–49].

The literature indicated that orbital invasion is more likely to occur in Mucormycosis than in Aspergillosis [1]. In contrast, our results showed that *Aspergillus* exhibited a higher incidence of orbital involvement than *Mucorales*; however, this difference was not statistically significant. The rate of fungal cerebral invasion in our study was significantly lower compared to the 55% observed in the sinonasal Mucormycosis patients in the review of Roden et al. [50]. These differences may be attributed to the limited sample number of our study.

Although both *Aspergillus* and *Mucorales* fungi can invade blood vessels, *Mucorales* are especially noted for their pronounced affinity for arterial invasion, a key element in the pathogenesis of IFRS [2]. *Mucorales* invade and proliferate along vessel walls, which leads to disruption of the tunica media. The fungal hyphae subsequently grow within the vessel lumen, causing thrombus formation and coagulative necrosis. After the vascular invasion, *Mucorales* can rapidly breach the carotid artery, spread intracranially, and involve other areas, potentially leading to rapid mortality, particularly in acute IFRS cases [1, 2]. This mechanism explains the correlation between necrosis and vascular invasion observed in our study. The statistically significant association between the degree of necrosis and vascular invasion suggests that careful examination for vascular invasion is particularly crucial in cases of extensive necrosis.

Our data reveal a higher incidence of perineural invasion in the Mucormycosis group (11.4%) compared to the non-Mucormycosis group (0%). However, this difference was not statistically significant ($p = 0.15$). Mucormycosis has

been documented in the literature as having a propensity to invade the perineural [1], and cranial nerve presentations are considered to be hallmarks of IFRS caused by *Mucorales* in contrast to *Aspergillus* [47]. These results highlight the need for further research with larger sample sizes to elucidate the correlation between this histopathological factor and different fungal agents.

In addition to the typical morphological features of hyphae, vascular and perineural invasion may serve as potential differentiators between Mucormycosis and the others. Although histopathological factors are theoretically important in the pathogenesis of IFRS, no established histopathological-based prognostic system currently exists. Our study reveals that, while identifying hyphal morphology and detecting vascular and perineural invasion are crucial for diagnosis, these tasks are challenging due to extensive necrosis in all IFRS cases involved in our study. Severe necrosis often distorts and enlarges hyphae, obscuring the distinguishing features of *Mucorales* and *Aspergillus* and making it difficult to identify vascular and neural structures. The sinus mucosa, characterized by its abundant thick-walled, dilated blood vessels [51], facilitates the identification of vascular structures compared to nerve bundles. This may contribute to the observed higher incidence of vascular invasion than perineural invasion. We hypothesize that the transition zone between normal and necrotic tissue, where ischemic necrosis has recently occurred, may be the most appropriate site for sampling to identify the histopathological features of IFRS. However, this hypothesis requires confirmation through further studies.

In comparison with other studies, our findings have several similarities and differences. A remarkable study by Crist H et al. [52] concluded that the presence of fungi within necrotic tissue is the key to diagnosing acute IFRS in frozen biopsy specimens. This finding is particularly relevant to our work, as all cases in our study exhibited both fungal hyphae and coagulative necrosis on histopathology. Regarding invasive Mucormycosis, during and following the COVID-19 pandemic, the emergence of this disease has raised considerable concern among Indian researchers. Recent influential studies by Sree Lakshmi I et al. [53], Ganesan N et al. [33], Mani S et al. [54], and Keerthika R et al. [55] have underscored the increasing incidence of invasive Mucormycosis and have provided valuable insights into its clinical and histopathological characteristics. However, these studies share the limitation of not utilizing molecular techniques and lacking comparative analyses between Mucormycosis and Aspergillosis, which could enhance diagnostic clarity. When we compare our findings to those studies, it is evident that necrosis is a prominent feature of invasive sinonasal Mucormycosis, typically observed in over 60% of cases. Vascular invasion also plays a critical role, occurring in 24 – 71.7% of cases. Conversely, perineural invasion is less frequent, ranging from 8.3 to 16%. Notably, our study found a higher incidence of granulomatous inflammation associated with Mucormycosis, reported at 42.9%, compared to 11 – 23.3% in other studies. In contrast to the diversity of studies on invasive Mucormycosis, there is currently a lack of published research on the histopathology of invasive sinonasal Aspergillosis.

The statistically significant correlation between fungal pigments and *Aspergillus* is a noteworthy finding. Despite their great potential for fundamental biology and pharmaceutical development [56], the role of fungal pigments in IFRS has not yet been thoroughly investigated. Fungal pigments can be categorized into four principal groups: carotenoids, melanins, polyketides, and azaphilones. The majority of these pigments are synthesized by four genera: *Aspergillus*, *Penicillium*, *Paecilomyces*, and *Monascus* [56]. Notably, the primary pathogenic species within *Aspergillus* genus are capable of producing a diverse spectrum of pigments, including yellow asperenone, yellow-green neoaspergillic acid, yellow to orange-brown questin, red ferriaspergillin, orange-red flavioline, reddish-brown viomellein, brown eurorubrin, purple viopurpurin, black aspergillin, and dark brown-black melanin [57]. In contrast, only a limited number of *Mucorales* species are known to synthesize pigments; among them, *Mucor circinelloides* is notable for producing yellow-orange β-carotene [57]. These insights are consistent with our results. The broad spectrum of pigmentation and their association with fungal groups necessitate a thorough evaluation of this histopathological factor during the diagnosis of IFRS.

An interesting correlation between the presence of fungal spores and *Aspergillus* was identified. According to the literature, conidia of *Aspergillus* may be found in the infected space is exposed to air, whereas sporangiospores of *Mucorales* are typically absent under such conditions [58]. Our study is consistent with the findings reported in the literature. Therefore, it can be concluded that fungal spores are absent within the tissue and are only present on air-exposed surfaces. Although sporulation and conidiation are not easily distinguishable through histopathology compared to fungal culture, the presence of spores in a biopsy specimen can be a significant marker for fungal infection. Additionally, the morphological characteristics of fungal reproductive units may aid in differentiating between several common fungal species.

Strengths, Limitations, and Future Opportunities of this Study

Our study provides insights that can enhance the diagnosis of IFRS. However, it has certain objective limitations

that warrant discussion. Firstly, our analysis was restricted to FFPE samples, and we did not gather additional clinical information beyond age, gender, and comorbidities. Further research should involve essential clinical factors not yet addressed in our study, including disease duration, symptoms, imaging findings, treatment, and outcome. Additionally, utilizing electrophoresis to detect PCR products presents another restriction. While electrophoresis is a valid technique for analyzing PCR results [59], it does not provide specific DNA sequence information. Given the scope and budget constraints, we did not pursue species identification. In the future, we aim to conduct additional studies on IFRS that utilize sequencing to accurately identify pathogenic fungal species, thereby elucidating the etiology of this disease. Another restriction to consider is that, due to the diversity in color and chemical nature, fungal pigments were not specifically classified in our research. Accurately identifying these pigments requires advanced techniques, commonly including infrared spectroscopy, spectroscopy or ultraviolet-visible spectrophotometry, nuclear-resonance spectroscopy, and mass spectrometry [60]. We hope that future studies will involve larger sample sizes and employ advanced techniques to investigate the role of specific fungal pigments in diagnosing IFRS. Last but not least, as previously mentioned, establishing a histopathologically-based prognostic system, determining the relationship between perineural invasion and fungal pathogens, and investigating the correlation between sampling sites and histopathological factors are also issues that we aim to address in the future.

Conclusion

IFRS predominantly affects older adults and males. Histopathology is a reliable method for differentiating between Mucormycosis and Aspergillosis. In addition to hyphal morphological features, the vascular invasion, degree of necrosis, and presence of fungal pigments and spores are valuable for distinguishing IFRS's two primary fungal agents. When extensive necrosis is observed, it is crucial to carefully investigate for vascular invasion, as there exists a significant correlation between these two characteristics. Further studies with larger sample sizes and more clinical factors should be conducted to develop a histopathologically-based prognostic system, confirm the association between perineural invasion and fungal groups, investigate the relationship between the sampling site and histopathological features, accurately identify pathogenic fungal species, and explore the role of each specific fungal pigment in diagnosing IFRS.

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Author Contributions GHT and KAL were responsible for the conceptualization, formal analysis, solicitation of funding, design of research methods, resource management, and the writing and editing of the initial draft. GHT and VAH served as the research supervisor. GHT, KAL, and BAL were involved in data management. All authors contributed to the investigation and approved the final manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Institutional Review Board of Biomedical Research at the University of Medicine and Pharmacy at Ho Chi Minh City (IRB number 807/HĐĐĐ-ĐHYD on September 22, 2023) and performed following the principles of the Declaration of Helsinki.

Consent for Publication For this type of study consent for publication is not required.

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Conflict of Interest The authors declare that they have no potential conflicts of interest.

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