Microbial Pathogenesis 159 (2021) 105125

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

# Acute invasive fungal rhinosinusitis: Molecular identification and update in management of frozen section biopsy

Sina Shamsaei [a], Mehraban Falahati [a,*], Shirin Farahyar [a,b], Omid Raiesi [c], Leila Haghighi [a], Hamed Eraghiye Farahani [d], Asghar Akhavan [e], Alireza Shamsaie [f], Mohammad Yarahmadi [g], Mahyar Keymaram [a]

[a] Department of Medical Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[b] Microbial Biotechnology Research Center, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[c] Department of Parasitology, School of Allied Medical Sciences, Ilam University of Medical Sciences, Ilam, Iran
[d] Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[e] Department of Otorhinolaryngology, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran
[f] Department of Pathology, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran
[g] Department of Medical Parasitology and Mycology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran

ARTICLE INFO

*Keywords:*
AIFRS
Frozen-section biopsy
FFPE
Sensitivity
Specificity
GMS

ABSTRACT

The clinical diagnosis of Acute Invasive Fungal Rhinosinusitis (AIFRS) is technically difficult because it presents with non-exclusive and nonspecific clinical symptoms. Laboratory confirmation (usually via histopathologic techniques such as formalin-fixed paraffin-embedded (FFPE)) is necessary but it is time-consuming, despite the urgent need for timely diagnosis of AIFRS for effective management. This study aimed to investigate the sensitivity and specificity of the GMS frozen-section biopsy in the diagnosis of AIFRS and compare the same with that of different tissue staining methods to provide valid decision-grounds that may guide clinicians in prompt diagnosis of acute fungal invasive rhinosinusitis. A cross-sectional study was conducted in the Medical Mycology Laboratory, Faculty of Medicine, Iran University of Medical Sciences between 2018 and 2020 on 200 patients with suspected AIFRS referred to Baqiyatallah and Imam Khomeini Hospital, Tehran. All patients were subjected to diagnostic nasal endoscopy and computed tomography (CT) scan of paranasal sinuses. Magnetic resonance imaging (MRI) was done in cases of suspected intracranial extension. After screening by routine mycological examination, the diagnosis was confirmed using complementary molecular methods. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the frozen-section biopsy were also compared with FFPE. Of the 200 suspect patients, 47 cases (23.5%) met the criteria for AIFRS. Species of the genus *Aspergillus* were the predominant 27 (57.4%) followed by *Mucorales* species 10 (21.3%), and *Fusarium* spp 3 (6.4%). Also, 3 cases (6.4%) of co-infection due to *Aspergillus/Rhizopus* were reported. The accuracy, sensitivity, specificity, PPV, and NPV of frozen section assessments were 99.5%, 97.9%, 100%, 100% and 99.3%, respectively. For GMS frozen-section alone, sensitivity, specificity, NPV, and PPV was 100%. Overall, the calculated accuracy of FFPE was 98.5%, sensitivity was 94%, specificity was 100%, PPV was 100%, and NPV was 98.1%. Examination of the frozen-section biopsy is a highly predictive tool for a rapid and effective diagnosis of patients with suspected AIFRS. We observed that GMS frozen-section is a fast and reliable exam to confirm the diagnosis of fungal invasion, with good accuracy, sensitivity, and specificity compared to the gold-standard FFPE biopsy.

*Abbreviations:* AIFRS, Acute Invasive Fungal Rhinosinusitis; FFPE, Formalin-fixed paraffin-embedded; PPV, positive predictive value,; NPV, negative predictive value; ENT, Ears, Nose and Throat; MRI, Magnetic resonance imaging; CT, computed tomography; H&E, hematoxylin and eosin; PAS, periodic acid–Schiff; GMS, Gomori Methenamine-Silver.

* Corresponding author. Department of Medical Parasitology and Mycology, School of Medicine, Iran University of Medical Science, Tehran, Iran.
*E-mail address:* Drfalahati1397@gmail.com (M. Falahati).

https://doi.org/10.1016/j.micpath.2021.105125
Received 27 May 2021; Received in revised form 10 July 2021; Accepted 4 August 2021
Available online 8 August 2021
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## 1. Introduction

Acute invasive fungal rhinosinusitis (AIFRS) is a rare and fulminant infection, usually defined by the presence of fungal hyphae within the sinonasal mucosa, submucosa, vasculature, or bone, within one month of the appearance of sinusitis symptoms. *Mucor* and *Aspergillus* spp are the predominant isolates recovered from AIFRS patients with a combined mortality rate between 50 and 80% in immunocompromised patients. Both *Aspergillus* spp. and *Mucor* spp. can be angioinvasive, and *Mucor* spp. appears more aggressive with frequent neurovascular and orbital invasion. Infections with multiple fungal species have been reported in patients with AIFRS. Commonly reported co-infections involve *Mucor* spp, *Rhizopus* spp. and *Aspergillus* spp including rare fungi such as black fungi, *Fusarium* spp, and *Candida* spp. Overall, increased incidence of invasive fungal infections parallels an increase in risk factors such as malignancy, diabetes, AIDS, protracted hospital stay, neutropenia, prolonged and indiscriminate use of corticosteroids and broad-spectrum antibiotics. A study involving 800 patients with AIFRS showed that 40% had hematological malignancy, 50% were diabetic, and the rest were patients with other immunosuppressive conditions [1–5]. (see Figs. 1 and 2)

Usually, the infections start with the inhalation of fungal spores, whereupon reaching the airways, the fungi invade the mucosal lining including the contiguous adjacent neurovascular structures. In the early stages, there may be subtle mucosal changes, such as a pale or edematous nasal mucosa. As the disease progresses, the neurovascular invasion causes thrombosis with local and/or distant ischemia leading to necrosis. This facilitates spread outside the infected sinus cavity into bones and surrounding tissues [6].

Early diagnosis is the key to the successful management of AIFRS and requires prompt recognition of clinical symptoms, especially in immunocompromised patients, followed by intranasal biopsy and histopathological analysis. However, the diagnosis of AIFRS is technically challenging because it presents with non-exclusive and nonspecific clinical symptoms; there is no pathognomonic symptom for AIFRS. Facial pain, facial edema, nasal obstruction, and fever occur in 50–65% of patients [3]. Cases of AIFRS require both nasal endoscopy and CT scan are required. MRI increases the accuracy of the diagnosis and assesses the degree of spread. Recently, histopathological analysis of frozen-section from suspected patients becomes a preferred diagnostic process due to its rapid turnaround. Formalin-fixed paraffin-embedded (FFPE) histopathological examinations, including hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Gomori Methenamine-Silver (GMS) staining are the most reliable test to confirm fungi invasion within tissues. However, FFPE processing is time-consuming, deterring the indispensable need for speedy diagnosis of AIFRS. Some researchers advocated the inclusion of frozen-section biopsy in the evaluation of patients at risk to hasten the diagnosis of AIFRS. Most of these studies proved frozen-section biopsy analysis to have moderate to higher sensitivity and specificity during surgery when compared with FFPE histopathology [7–9]. This study aimed to investigate the sensitivity and specificity of the GMS frozen-section biopsy in the diagnosis of AIFRS and compare the same with that of different tissue staining methods to provide valid decision-grounds that may guide clinicians in prompt diagnosis of acute invasive rhinosinusitis. It is hoped that the findings might complement the adequate and successful management of AIFRS.

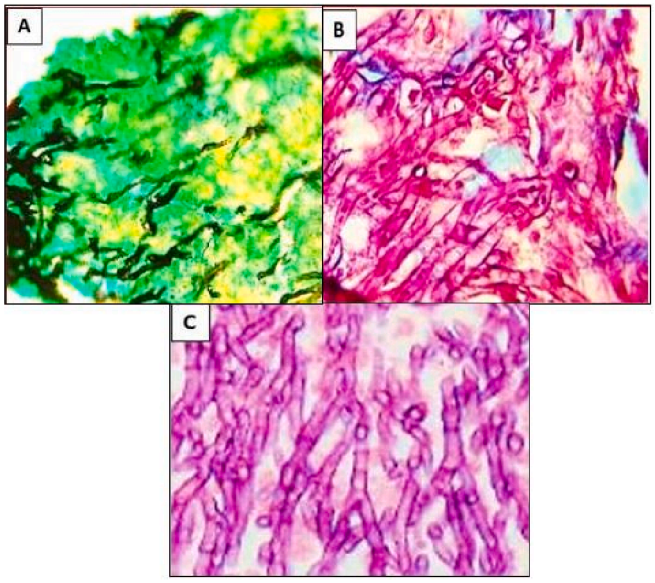


**Fig. 1.** Frozen section biopsy: GMS (A), PAS (B) and H&E (C) stain showing an invasion of fungal forms into the submucosa.

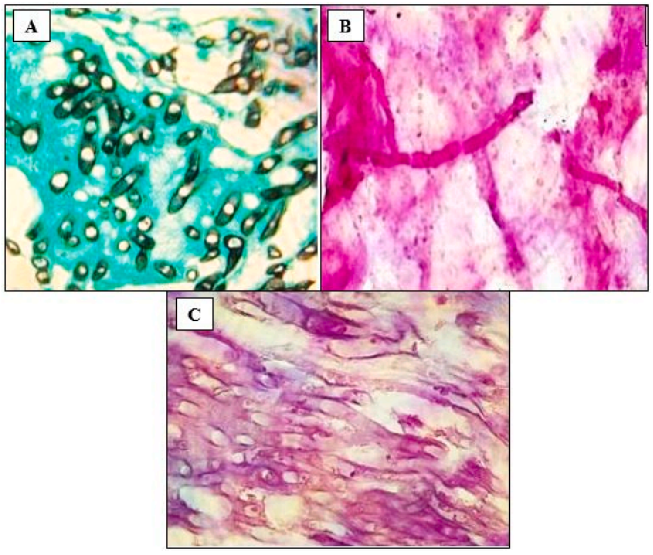


**Fig. 2.** Formalin-fixed paraffin-embedded (FFPE) biopsy: GMS (A), PAS (B) and H&E (C) stain showing an invasion of fungal forms into the submucosa.

## 2. Materials and methods

### 2.1. Study design and population

This cross-sectional study was conducted at the Medical Mycology Laboratory, Faculty of Medicine, Iran University of Medical Sciences, Iran, between 2018 and 2020. It involved 200 patients with suspected AIFRS referred to Baqiyatallah and Imam Khomeini Hospital, Tehran. We collected samples from different anatomic sites such as paranasal sinuses, orbit, and hard palate along with the patient's information such as age, gender, the location of the lesion, the duration of the sickness, medical and drug history from the patient's records. The inclusion criteria for the subjects were confirmed diagnosis of AIFRS according to the guidelines of the *European Organization for Cancer Research and Treatment* (i.e., clinical, microbiological, and histological evidence of invasive fungal infection) [10,11]. We excluded patients who did not have a microbiologically and histologically proven diagnosis of AIFRS. Any immunocompromised patient with persistent fever of unknown origin (48 h or more, not responding to antibiotic therapy) or sinonasal symptom - nasal obstruction, nasal crusts, facial edema, rhinorrhea, epistaxis, orbital swelling, and palatal ulceration - were immediately referred to ENT surgeons. [Table 1].

**Table 1**
Inclusion and exclusion criteria in patients with acute invasive fungal rhinosinusitis.

| Acute invasive fungal rhinosinusitis | |
|---|---|
| Inclusion criteria | Exclusion criteria |
| Histopathological evidence of fungal invasion of the mucosa, submucosa, blood vessels or paranasal sinus bones | Patients without a positive clinical, microbiological, and histological evidence of invasive fungal infection |
| Rhinosinusitis confirmed on the imaging exam | Inadequate imaging by failure to cover the entirety of the craniofacial region |
| Necrotic tissue with minimal infiltration of inflammatory cells | _ |
| A clinical time course of ≤4 weeks<br>age ≥18 years | A clinical time course of >4 weeks<br>age <18 years |
| Every immunocompromised patient with persistent fever of unknown origin (48 h or more, not responding to antibiotic therapy). | Immunocompetent patients |
| Every immunocompromised patient with sinonasal symptom – rhinorrhea, nasal obstruction, epistaxis, crusting; and/or facial edema. | Every immunocompetent patient without sinonasal symptom |
| Every immunocompromised patient with bony erosion, with sinonasal mass, or any orbital involvement on CT imaging | _ |
| Every immunocompromised patient with intracranial extension (intracranial abscess, cavernous sinus thrombosis) on MRI | _ |

### 2.2. ENT evaluation

All patients were subjected to diagnostic nasal endoscopy and CT scan of paranasal sinuses. CT scan was able to diagnose bony erosion or any orbital involvement while MRI was done in cases of suspected intracranial extension. The endoscopic findings diagnostic for AIFRS included mucosal ischemia, plain necrotic areas with a blackish or grayish color, crusting, and absence of bleeding upon scraping, especially in the region of the middle turbinate, ethmoid, or septum. The diagnostic findings upon Sinus CT scan were unilateral sinus opacification, increased density of peri-antral fat, bony erosion, and involvement of extra sinus tissues.

### 2.3. Mycological studies

#### 2.3.1. Direct examination

We directly examined all samples using potassium hydroxide (KOH) wet preparation by placing a small portion of the samples on microscopic slides containing a few drops of 10% KOH solution and visualized under a light microscope searching for fungal elements like mycelium (with or without cell-wall), spore, yeast (without or without bud), pseudohyphae, etc. by a professional mycologist. KOH preparation is used to clear clinical material in order that fungal elements can be seen more easily.

#### 2.3.2. Culture technique

To cultivate the fungal agents, we inoculated brain-heart infusion agar (BHIA) and Sabouraud's dextrose agar containing chloramphenicol (SC) with the sample under sterile conditions. We incubated the inoculated plates at 30° Celsius and examined weekly for fungal growth, until a maximum of four weeks. At this stage, we could partially determine the genus and the species of the fungus.

#### 2.3.3. PCR & sequencing

PCR technique was used to confirm the identity of isolates from culture (Culture/PCR). Some culture-negative specimens were also directly subjected to PCR methods (FFPE/Tissue/PCR). We used specific primers including ITS1, ITS4, β-tubulin, and elongation factors for the amplification. We designed three pairs of forward and reverse primers based on the sequences of translation elongation factor-1, ITS rDNA, and tubulin genes for *Fusarium* sp., *Aspergillus* sp., and *Mucorales* sp., respectively. PCR was conducted in 25 μL of the reaction mixture, comprising 7 μL Master Mix containing $MgCl_2$, dNTPs, 1 μL of every primer (10 pmol), reaction buffer, and 1 μL of gDNA. Amplification was done in an ABI PRISM 2720 (Applied Biosystems, Foster City, USA) thermocycler using the primers ITS1 (5′ CCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) for ITS; primers Bt2α (5′ GGTAACCAAATCGGTGCTGCTTTC 3′) and Bt2b (5′ ACCCTCAGTGTAGTGACCCTTGGC 3′) for β-tubulin; and EF1 (5′ ATGGGTAAGGARGACAAGAC 3′) and EF2 (5′ GGARGTACCAGTSATCATGTT 3′) for elongation factors [12]. The amplification process was carried out according to PCR programs selected for each primer. The PCR products were subjected to DNA sequencing with the same primers. We analyzed the obtained sequences in the GenBank database using NCBI BLAST search tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and determined the fungal identities by comparing with the highest matches in DNA databases [13].

#### 2.3.4. Histopathological examination

Immediately upon collection of the samples, two separate specimens were sent to the Pathology Laboratory. The first was submitted for frozen-section biopsy, following a standardized protocol. Briefly, the specimen was immersed in the Tissue-Tek medium in Freezing Microtome at −28 °C. Histological sections of 4–5 μm were obtained; stained with H&E, PAS, and GMS; and evaluated for the presence of fungi under a light microscope. The other specimen was fixed in 10% buffered formalin, processed by an automatic tissue processor, and evaluated using FFPE analysis. The specimen embedded in paraffin was sectioned (4–5 μm), stained with H&E, PAS, and GMS, and analyzed for evidence of fungal mucosal invasion (about 5–7 days) [7].

The biopsy specimens stained with H&E, PAS, and GMS for frozen-section analysis and FFPE examination were reviewed and compared by the study pathologists. The reviewing pathologists were blinded to which was the frozen-section or FFPE slides. Sensitivity and specificity for the two methods were calculated, compared, and recorded. The current study was conducted after obtaining informed consent, according to the Declaration of Helsinki, and the approval of the Institutional Ethics Committee.

## 3. Results

Of the 200 suspected patients, 47 cases (23.5%) met the criteria for AIFRS. Out of 47 inpatients studied, 28 were men (59.6%), and 19 women (40.4%). The mean age of the patients was 41.5 ± 11.4 years, with the majority of the patients (36.1%) in the group of at least 50 years.

The distribution of the sources of clinical samples in this study includes nasal sinus (35 cases; 74.4%), orbit (5 cases; 10.6%), palates (5 cases; 10.6%), and intracranial lesion (2 cases; 4.25%). Molds were the predominant fungi 43 (91.5%) recovered followed by yeasts 4 (8.5%). The yeast species identified were *Candida* species 3 (75%) and non-*Candida* yeasts 1 (25%). Species of the genus *Aspergillus* were the most common molds 27 (57.4%) –recovered predominantly from males - followed by *Mucorales* species 10 (21.3%), and *Fusarium* spp 3 (6.4%). Besides, three cases (6.4%) of co-infection due to *Aspergillus/Rhizopus* were detected (two cases of *Aspergillus flavus/Rhizopus arrhizus,* and one case of *Aspergillus fumigatus/Rhizopus arrhizus*). [Table 2].

Most patients infected with *Aspergillus* spp were in the age group of 41–50 years and the most common species of *Aspergillus* detected was *Aspergillus flavus*. In addition, 10 patients (21.3%) were infected with *Mucorales* spp; *Rhizopus arrhizus* was the only agent (10 cases; 100%) of mucormycosis observed with diabetes and malignancy being the most common underlying disease. Most patients infected with *Mucorales* spp were predominantly males in the age group of 31–40 years. Three patients were also infected by *Fusarium* spp (2 cases of *Fusarium oxysporum*

**Table 2**
Distribution of isolated fungi from patients with AIFRS.

| Number of patients | 47 |
|---|---|
| Organism | no (%) |
| ***Aspergillus flavus*** | 22 (46.8) |
| ***Aspergillus fumigatus*** | 3 (6.4) |
| ***Aspergillus tubingensis*** | 1 (2.1) |
| ***Aspergillus niger*** | 1 (2.1) |
| ***Candida albicans*** | 1 (2.1) |
| ***Candida glabrata*** | 1 (2.1) |
| ***Candida tropicalis*** | 1 (2.1) |
| ***Fusarium oxysporum*** | 2 (4.2) |
| ***Fusarium moniliforme*** | 1 (2.1) |
| ***Rhizopus arrhizus*** | 10 (21.3) |
| ***Rhizopus arrhizus/Aspergillus*** | 3 (6.4) |
| ***Trichosporon asahii*** | 1 (2.1) |

and one case of *F. moniliforme*).

Table 3 shows the clinical and radiographic characteristics of the patients diagnosed with AIFRS.

The prevalent underlying diseases among the patients identified with AIFRS were acute myeloblastic leukemia (18 cases; 38.3%) and diabetes mellitus (10 cases; 21.3%). Whereas nasal crusts (78.7%), nasal obstruction (65.9%), and chronic headache (57.4%) were the major clinical presentations. The most prominent CT scan findings were mucosal thickening (91.4%) and sino-nasal bone erosion (29.7%), commonly involving bilateral multiple sinuses [Table 3].

The validity of frozen-section biopsy in the diagnosis of AIFRS using different histologic stains is shown in Table 4. GMS-stained frozen-section correctly diagnosed all 47 (100%) AIFRS confirmed cases. Although H&E-stained frozen-section accurately identified 45 (95.7%) of the 47 confirmed patients, besides the two (4.25%) false-negative diagnoses, PAS frozen-section correctly diagnosed one of the two H&E-stained false-negative slides. Moreover, on FFPE analysis, H&E-stained paraffin-section correctly detected 43 (91.4%) of the 47 AIFRS cases; with 4 (8.51%) false-negative cases that were correctly diagnosed by GMS-stained paraffin-section examination. [Table 5].

**Table 3**
The major presenting symptoms in patients with AIFRS.

| Number of patients | 47 |
|---|---|
| Characteristic | no (%) |
| Age at the time of diagnosis-years* | 41.5 ± 11.4 |
| Sex | no |
| Male | 28 (59.6) |
| Female | 19 (40.4) |
| Certainty of diagnosis | |
| Proven | 47 (23.5) |
| Probable | 153 (76.5) |
| Underlying cause of immunosuppression | |
| Acute lymphoblastic leukemia | 7 (14.9) |
| Acute myeloblastic leukemia | 18 (38.3) |
| Chronic myeloblastic leukemia | 4 (8.5) |
| Diabetes Mellitus | 10 (21.3) |
| Liver transplantation | 2 (4.2) |
| HIV | 3 (6.3) |
| Tuberculosis | 3 (6.3) |
| Signs and symptoms | |
| Nasal obstruction | 31 (65.9) |
| Nasal crusts | 37 (78.7) |
| Rhinorrhea | 26 (55.3) |
| Fever | 20 (42.5) |
| Facial edema | 18 (38.2) |
| Palatal ulceration | 5 (10.6) |
| Epistaxis | 13 (27.6) |
| Headache | 27 (57.4) |
| Extension | |
| Sinunasal | 35 (74.4) |
| Orbit | 5 (10.6) |
| Intracranial | 2 (4.25) |
| Palatal | 5 (10.6) |
| Paranasal sinus computed tomographic findings | |
| Mucous thickening | 43 (91.4) |
| Hard palate erosion | 5 (10.6) |
| Sinonasal bone erosion | 14 (29.7) |

**Table 4**
Accuracy of H&E, PAS, and GMS staining in the diagnosis of AIFRS on Frozen Section biopsy.

| Characteristic | No. of Cases (N = 200) | Final Diagnosis, No. | H&E Frozen-Section Diagnosis, No. (%) | PAS Frozen-Section Diagnosis, No. (%) | GMS Frozen-Section Diagnosis, No. (%) |
|---|---|---|---|---|---|
| Proven | 47 | (+) 47 | (+) 45 (95.7) | (+) 46 (97.8) | (+) 47 (100) |
| | | (−) 0 | (−) 2 (4.25) | (−) 1 (2.12) | (−) 0 (0) |
| Probable | 153 | (+) 0 | (+) 0 (0) | (+) 0 (0) | (+) 0 (0) |
| | | (−) 153 | (−) 153 (100) | (−) 153 (100) | (−) 153 (100) |

## 4. Discussion

Early and precise diagnosis of AIFRS is important to allow timely commencement of antifungal treatment and to reduce unnecessary mortality. Although traditional approaches such as direct microscopic examination, histopathological evaluation, and culture are still the gold standard, the diagnosis of AIFRS is generally difficult because of the weak validity of some of these tests [13,14]. Conventional mycologic techniques, utilizing phenotypic characteristics for fungal species identification are laborious and time-consuming and usually limited by some unpredictable culture outcomes that are influenced by variable environmental conditions such as temperature, humidity, and time of cultivation. However, molecular techniques such as nucleic acid sequencing are uncomparably faster, more objective, and specific than the traditional phenotypic methods; they can be designed to discriminate between similar fungi that fail to produce specific morphological characteristics [12].

In the present study, 74.4%, 65.9%, 100%, and 100% of proven patients were positive for fungi by microscopic, culture, histopathology examination, and PCR-sequencing methods, respectively [Table 6].

However, some patients positive on microscopic examination were culture-negative. Given the significant morbidity and mortality associated with IFS, the development of multiple methods for obtaining a

**Table 5**
Accuracy of H&E, PAS, and GMS staining in the diagnosis of AIFRS on Paraffin -Section biopsy.

| Characteristic | No. of Cases (N = 200) | Final Diagnosis, No. | H&E Paraffin-Section Diagnosis, No. (%) | PAS Paraffin -Section Diagnosis, No. (%) | GMS Paraffin -Section Diagnosis, No. (%) |
|---|---|---|---|---|---|
| Proven | 47 | (+) 47 | (+) 43 (91.4) | (+) 43 (91.4) | (+) 46 (97.8) |
| | | (−) 0 | (−) 4 (8.51) | (−) 4 (8.51) | (−) 1 (2.12) |
| Probable | 153 | (+) 0 | (+) 0 (0) | (+) 0 (0) | (+) 0 (0) |
| | | (−)153 | (−) 153 (100) | (−) 153 (100) | (−) 153 (100) |

**Table 6**
The comparison of direct examination, culture, histopathology, and sequencing methods.

| Characteristic | No. of Cases (N = 200) | KOH* | Culture | Histopathology | PCR & Sequencing |
|---|---|---|---|---|---|
| | | No. (%) | No. (%) | No. (%) | No. (%) |
| Proven | 47 | (+) 35 (74.4) | (+) 31 (65.9) | (+) 47 (100) | (+) 47 (100) |
| | | (−) 12 (25.6) | (−) 16 (34.1) | (−) 0 (0) | (−) 0 (0) |
| Probable | 153 | (+) 0 | (+) 0 (0) | (+) 0 (0) | (+) 0 (0) |
| | | (−) 153 | (−) 153 (100) | (−) 153 (100) | (−) 153 (100) |

timely and accurate diagnosis of this pathology is crucial [15].

Although some studies reported that frozen biopsy method showed higher sensitivity and specificity than Formalin-fixed paraffin-embedded methods in the diagnosis of acute invasive fungal rhinosinusitis, false-negative results from H&E and PAS stained frozen section biopsies have nevertheless been reported in other studies [3,8,15–18]. In this study, in addition to comparing FFPE and frozen biopsy approaches, we investigated the effectiveness of the GMS frozen-section procedure in the diagnosis of AIFRS. Our results showed that this staining method can eliminate false negatives and therefore can be used as an effective staining technique to diagnose AIFRS. In summary, we have shown that GMS frozen-section biopsy is a highly predictive tool for a rapid and effective diagnosis of patients with suspected AIFRS. However, further studies in this field are required.

Microbiological cultures, although useful for mycological speciation, are less sensitive [19]. Furthermore, we used molecular methods to confirm the identity of some isolates that were not detectable using routine methods.

Our data proved that the PCR-sequencing methods and histologic diagnosis are more sensitive than unenhanced sinus CT scan, and conventional microbiological methods in the diagnosis of AIFRS. These results corroborate the findings of Alejandro's study conducted in Mexico [20].

In the current study, we observed a high prevalence of *Aspergillus* spp infection, as per the finding of a similar study in Mexico [20], Miami [21,22], and Pennsylvania [23]. However, it does not agree with the results of some studies from other countries like California [24] and India [25] that reported a high prevalence of *Mucorales* spp.

Our result shows that among fungi recovered, 4 (8.5%) were yeasts, of which 3(6.4%) belonged to *Candida* spp, and 1(2.1%) represents other yeast. Similar to our findings, *C. albicans* represents 5% and other yeasts 6% of the isolates recovered from patients suffering from AFRS, as reported by Montome et al. [23].

In our study, the predominant underlying diseases among the patients with AIFRS were hematologic malignancies in 29 cases (61.7%) and diabetes mellitus in 10 cases (21.3%), like other study [21,22]. Also, our data obtained from CT scans showed mucosal thickening, sinonasal bone erosion, and hard palate erosion in 91.4%, 29.7%, and 10.6% of the patients. Similar findings were reported by Alejandro et al. where hematologic malignancies constitute 77.7% of the underlying conditions and mucosal thickening (94.4%), sinonasal bone erosion(16.7%), and palate erosion (16.7%) were found on paranasal CT scan evaluation [20].

According to the results of this study, the rate of false-negative diagnosis of AIFRS using H&E, PAS, and GMS stain frozen-section biopsy was 4.3%, 2.1%, and 0%, respectively. These findings did not concede those reported by other studies ranging from 12.5 to 27.3% [16–18]. In addition, the rate of false-negative diagnosis with H&E, PAS, and GMS stained paraffin-section biopsy was 8.51%, 6.4%, and 2.1%, respectively. In the study conducted by Hennessy et al., 34 out of 41 AIFRS-confirmed biopsies were diagnosed using frozen-section. Of the 7 that were not, 5 were identified by the addition of the PASF-fs stain, reducing the failure rate from 17% to 5% [26]. In a 12- years study involving 20 IFS patients, frozen-section was found to have a sensitivity of 84%, a specificity of 100%, PPV of 100%, and NPV of 72% for the rapid diagnosis of AIIFS [19]. In addition, in the study conducted by Alkhateb et al., frozen sections had a sensitivity of 88.5%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 90.6% [27]. In this study, the sensitivity and specificity of the frozen-section biopsy, as well as FFPE, were evaluated and compared. For GMS-stained frozen-section, sensitivity, specificity, NPV, and PPV was 100%. Whereas for FFPE, the calculated accuracy was 98.5%, sensitivity was 94%, specificity was 100%, PPV was 100%, and NPV was 98.1%. We observed that frozen-section biopsy is a fast and reliable exam to confirm the diagnosis of fungal invasion compared to the gold-standard FFPE biopsy.

We acknowledge that a limitation of this study is the small number of cases available for analysis. These limitations are derived from the fact that AIFRS is rare, and although this is one of the largest cohorts of AIFRS patients reported, the power of statistical analysis is limited. Besides, some of the disadvantages of frozen tissue samples are the rapid deterioration rate of the samples once it is in room temperature. The sample will need to be frozen as soon as the sample is collected. Formalin-fixed paraffin-embedded tissue can be stored long term at room temperature whereas frozen tissue can only be stored for up to one year at −80 °C. Moreover, Ice crystal formation may negatively affect tissue structure.

## 5. Conclusion

Early detection etiology of AIFRS and their associated risk factors can improve the therapeutic outcome and decrease the mortality rates among patients. We have shown that frozen-section biopsy is a highly predictive tool for a rapid and effective diagnosis of patients with suspected AIFRS. *Aspergillus* spp remains the predominant cause of AIFRS. Our data showed the molecular methods and histologic diagnosis, especially GMS-stained frozen-section proved to be more sensitive than unenhanced sinus CT scans, and conventional microbiological methods. We observed that GMS frozen-section biopsy is a fast and reliable exam to confirm the diagnosis of fungal invasion, with good accuracy, sensitivity, and specificity when compared to the gold-standard FFPE biopsy.

## Financial disclosure

The current study was conducted in accordance with the Declaration of Helsinki and with approval from the Institutional Ethics Committee. The work was approved by the Research Council of Iran University of Medical Science; the project funding number is IR. IUMS.FMD. REC.1399.392. Informed consent was obtained from all individual participants included in the study.

## CRediT authorship contribution statement

**Sina Shamsaei:** Conceptualization, Formal analysis, Collecting samples and laboratory work, Statistical analysis, Study concept and design, Critical revision of the manuscript for important intellectual content, . **Mehraban Falahati:** Conceptualization, Study concept and design, . **Shirin Farahyar:** Formal analysis, Analysis and interpretation of data, Statistical analysis. **Omid Raiesi:** Writing – original draft, Collecting samples and laboratory work, Drafting of the manuscript, Critical revision of the manuscript for important intellectual content. **Leila Haghighi:** Collecting samples and laboratory work. **Hamed Eraghiye Farahani:** Collecting samples and laboratory work. **Asghar Akhavan:** Formal analysis, Collecting samples and laboratory work, Analysis and interpretation of data. **Alireza Shamsaie:** Formal analysis, Collecting samples and laboratory work, Analysis and interpretation of

data. **Mohammad Yarahmadi:** Collecting samples and laboratory work. **Mahyar Keymaram:** Collecting samples and laboratory work.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgment

We acknowledge the financial support of Iran University of Medical Sciences to this study, our thanks and gratitude. Sincere gratitude of all professors and students of parasitology and mycology at the School of Medicine in Iran University of Medical Sciences, Imam Khomeini Hospital and Baqiyatallah Hospital.

## References

[1] O. Raiesi, H. Shabandoust, P. Dehghan, S. Shamsaei, A. Soleimani, Fungal infection in foot diabetic patients, Journal of Basic Research in Medical Sciences 5 (2018) 47–51.
[2] H. Khalandi, L. Masoori, S. Farahyar, A.A. Delbandi, O. Raiesi, A. Farzanegan, et al., Antifungal activity of capric acid, nystatin, and fluconazole and their in vitro interactions against Candida isolates from neonatal oral thrush, Assay Drug Dev. Technol. 18 (2020) 195–201.
[3] J.R. Craig, Updates in management of acute invasive fungal rhinosinusitis, Curr. Opin. Otolaryngol. Head Neck Surg. 27 (2019) 29–36.
[4] O. Raiesi, M. Siavash, F. Mohammadi, J. Chabavizadeh, B. Mahaki, M. Maherolnaghsh, et al., Frequency of cutaneous fungal infections and azole resistance of the isolates in patients with diabetes mellitus, Adv. Biomed. Res. 6 (2017).
[5] O. Raiesi, H. Shabandoust, M. Getso, V. Raissi, A.A. Rezaei, Candida Auris: a New Emerging Fungal Monster, vol. 14, Archives of Clinical Infectious Diseases, 2019.
[6] P.G. Deutsch, J. Whittaker, S. Prasad, Invasive and non-invasive fungal rhinosinusitis—a review and update of the evidence, Medicina 55 (2019) 319.
[7] M.L. Silveira, W.T. Anselmo-Lima, F.M. Faria, D.L. Queiroz, R.L. Nogueira, M. G. Leite, et al., Impact of early detection of acute invasive fungal rhinosinusitis in immunocompromised patients, BMC Infect. Dis. 19 (2019) 310.
[8] M.T. Ghadiali, N.A. Deckard, U. Farooq, F. Astor, P. Robinson, R.R. Casiano, Frozen-section biopsy analysis for acute invasive fungal rhinosinusitis, Otolaryngology-Head Neck Surg. (Tokyo) 136 (2007) 714–719.
[9] A.E. Süslü, O. Öğretmenoğlu, N. Süslü, Ö.T. Yücel, T.M. Önerci, Acute invasive fungal rhinosinusitis: our experience with 19 patients, Eur. Arch. Oto-Rhino-Laryngol. 266 (2009) 77.
[10] B. De Pauw, T.J. Walsh, J.P. Donnelly, D.A. Stevens, J.E. Edwards, T. Calandra, et al., Revised definitions of invasive fungal disease from the European organization for Research and treatment of cancer/invasive fungal infections cooperative group and the national institute of allergy and infectious diseases mycoses study group (EORTC/MSG) consensus group, Clin. Infect. Dis. : an official publication of the Infectious Diseases Society of America 46 (2008) 1813–1821.
[11] J.P. Donnelly, S.C. Chen, C.A. Kauffman, W.J. Steinbach, J.W. Baddley, P. E. Verweij, et al., Revision and update of the consensus definitions of invasive fungal disease from the European organization for Research and treatment of cancer and the mycoses study group education and Research consortium, Clin. Infect. Dis. 71 (2019) 1367–1376.
[12] O. Raiesi, S.J. Hashemi, M.M. Ardehali, K. Ahmadikia, M.I. Getso, F. Pakdel, et al., Molecular identification and clinical features of fungal rhinosinusitis: a 3-year experience with 108 patients, Microb. Pathog. 158 (2021) 105018.
[13] Z.B. Boroujeni, S. Shamsaei, M. Yarahmadi, M.I. Getso, A. Salimi-Khorashad, L. Haghighi, et al., Distribution of invasive fungal infections: molecular epidemiology, etiology, clinical conditions, diagnosis and risk factors: a 3-year experience with 490 patients under intensive care, Microb. Pathog. (2020) 104616.
[14] O. Raiesi, S.J. Hashemi, P. Ardi, M. Mohammadi Ardehali, V. Raissi, S. Shamsaei, et al., First report of chronic invasive fungal rhinosinusitis in patient with ovarian cancer caused by didymella pedeiae, and successful treatment with voriconazole: a case report, Current Medical Mycology (2021).
[15] I.J. Fernandez, F.M. Crocetta, M. Dematte, P. Farneti, M. Stanzani, R.E. Lewis, et al., Acute invasive fungal rhinosinusitis in immunocompromised patients: role of an early diagnosis, Otolaryngology-Head Neck Surg. (Tokyo) 159 (2018) 386–393.
[16] P. Papagiannopoulos, D.M. Lin, S. Al-Khudari, K. Rajan, S. Reddy, P. Gattuso, et al., Utility of intraoperative frozen sections in surgical decision making for acute invasive fungal rhinosinusitis, in: International Forum of Allergy & Rhinology, Wiley Online Library, 2017, pp. 502–507.
[17] C.C. Melancon, J.D. Clinger, The use of frozen section in the early diagnosis of acute invasive fungal sinusitis, Otolaryngology-Head Neck Surg. (Tokyo) 157 (2017) 314–319.
[18] H. Crist, M. Hennessy, J. Hodos, J. McGinn, B. White, S. Payne, et al., Acute invasive fungal rhinosinusitis: frozen section histomorphology and diagnosis with PAS stain, Head and neck pathology 13 (2019) 318–326.
[19] T.A. Schuman, J.H. Nguyen, J.C. Yelverton, J.A. Almenara, C.N. Powers, The sensitivity and specificity of touch preparation for rapid diagnosis of invasive fungal sinusitis: a pilot study, Ear Nose Throat J. 97 (2018) E32–E36.
[20] A. Alejandro, C. de la Torre González, M. Edgar, V. Perla, Factors associated with all-cause mortality in pediatric invasive fungal rhinosinusitis, Int. J. Pediatr. Otorhinolaryngol. 129 (2020) 109734.
[21] F.C. Valera, T. do Lago, E. Tamashiro, C.C. Yassuda, F. Silveira, W.T. Anselmo-Lima, Prognosis of acute invasive fungal rhinosinusitis related to underlying disease, Int. J. Infect. Dis. 15 (2011) e841–e844.
[22] Z. Borjian Boroujeni, S. Shamsaei, M. Yarahmadi, M.I. Getso, A. Salimi Khorashad, L. Haghighi, et al., Distribution of invasive fungal infections: molecular epidemiology, etiology, clinical conditions, diagnosis and risk factors: a 3-year experience with 490 patients under intensive care, Microb. Pathog. (2020) 104616.
[23] K.T. Montone, V.A. Livolsi, M.D. Feldman, J. Palmer, A.G. Chiu, D.C. Lanza, et al., Fungal rhinosinusitis: a retrospective microbiologic and pathologic review of 400 patients at a single university medical center, International journal of otolaryngology 2012 (2012).
[24] G. Vengerovich, K.A. Echanique, K.W. Park, C. Wells, J.D. Suh, J.T. Lee, et al., Retrospective analysis of patients with acute invasive fungal rhinosinusitis in a single tertiary academic medical center: a 10-year experience, American Journal of Rhinology & Allergy 34 (2020) 324–330.
[25] R. Shanbag, N.R. Rajan, A. Kumar, Acute invasive fungal rhinosinusitis: our 2 year experience and outcome analysis, Eur. Arch. Oto-Rhino-Laryngol. 276 (2019) 1081–1087.
[26] M. Hennessy, J. McGinn, B. White, S. Payne, J.I. Warrick, H. Crist, Frozen section as a rapid and accurate method for diagnosing acute invasive fungal rhinosinusitis, Otolaryngology-Head Neck Surg. (Tokyo) 159 (2018) 576–580.
[27] R. Alkhateb, P.D. Menon, H. Tariq, S. Hackman, A. Nazarullah, D.D. Mais, Accuracy of Intraoperative Frozen Section in Detection of Acute Invasive Fungal Rhinosinusitis, Archives of Pathology & Laboratory Medicine, 2020.