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Original Article

Comparing immunological and molecular tests with conventional methods in diagnosis of acute invasive fungal rhinosinusitis

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Abstract

Introduction: This study compared immunological and molecular tests with conventional methods in the diagnosis of acute invasive fungal rhinosinusitis.

Methodology: All tissue samples from maxillary sinuses were cultured and stained using periodic acid-Schiff. Two blood samples were cultured by bedside inoculation into BACTEC medium. Diagnostic tests were used: enzyme-linked immunosorbent assay for detection of galactomannan and mannan in serum samples, real-time PCR assays for *Aspergillus* spp. and *Candida* spp., and nested PCR for the *Mucoraceae* family.

Results: Among 31 immunocompromised patients, based on host factors, clinical, radiological and mycological findings, 18 patients were diagnosed with documented infection. By direct smear examination, 9 patients (50%) had positive results for *Mucoraceae* family, but only 5 samples showed growth in the culture (55.5%). Nested PCR results for species in this family were negative in all serum samples from patients. In addition, 9 patients had positive direct microscopic findings, of which 7 specimens produced positive growth for *Aspergillus flavus* (77.7%). The galactomannan test was positive in 6/9 (66.6%), and *Aspergillus* PCR were positive in 6 patients (66.6%). The isolated agent was *C. albicans* in one patient. The mannan antigen test to detect *Candida* was negative. None of the blood cultures was positive for fungal infection.

Conclusions: The efficient method to diagnose fungal rhinosinusitis was direct microscopic examination of tissue samples. Immunological and molecular methods, which are available for some important fungi, can help clinicians with the diagnosis and management of infections in patients in critical condition when tissue sampling is not available.

Key words: fungal rhinosinusitis; galactomannan Ag test; real-time PCR; *Mucoraceae*; *Aspergillus* spp.

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Introduction

Invasive fungal rhinosinusitis (IFR), is a rare, life-threatening infection, especially in immunocompromised patients. It is largely attributable to *Aspergillus* and *Mucoraceae* species [1], but other kinds of fungi, such as *Candida albicans*, can also act as the etiologic agent [2]. Distinguishing between IFR is important because the treatment and prognosis depend on the correct identification of the causal agent. Paranasal sinus and orbit infection can be either non-invasive (chronic fungal rhinosinusitis, allergic rhinosinusitis, or mycetoma), which is usually seen in immunocompetent individuals, or invasive, especially acute forms, which are usually seen in immunocompromised patients. The latter type involves the risk of infection spreading from the sinuses into the orbit and brain by causing bone erosion, and to multiple organs by hematogeneous spread [3]. IFR is commonly distinguished from allergic fungal sinusitis by nasal obstruction, one-sided facial pain, and inability to move the eyes.

IFR is difficult to diagnose because the physical findings and ambiguous symptoms are non-specific, and radiological features such as bone erosion and tissue destruction become detectable only in advanced stages, with computed tomography [4,5]. Rapid orbital and intracranial spread and a delay in diagnosis and treatment can lead to high mortality rates ranging from 50% to 100% in immunocompromised patients [1,6,7]. Moreover, patients who do not recover from neutropenia might have a poor prognosis irrespective of adjuvant therapeutic measures [6]. Current diagnostic methods are based on tissue biopsy culture or histology – invasive procedures that may not

feasible in some immunocompromised patients with pancytopenia, but have the greatest diagnostic significance.

The present study was designed to compare non-invasive methods such as blood culture and sandwich enzyme-linked immunosorbent assay (ELISA) used to detect galactomannan (GM) and mannan (MN) antigens, and polymerase chain reaction (PCR) in serum samples, with conventional methods including histopathological examination and culture of sinus mucosa tissue samples in the diagnosis of IFR.

Methodology

This prospective study was conducted from May 2011 to December 2012, and 31 consecutive high-risk patients were included. The inclusion criteria for patients were receiving corticosteroid therapy for organ transplantation or having hematologic disorders or diabetes mellitus, along with radiological and clinical signs and symptoms of IFR according to their medical records. Exclusion criteria were chronic rhinosinusitis or allergic rhinitis in patients. As a part of the patients' treatment procedures, tissue biopsy of patients transferred to Prof. Alborzi Clinical Microbiology Research Center, Shiraz, Iran, were examined for the presence of fungus and were evaluated by mycological, immunological, and molecular diagnostic methods. Moreover, in accordance with EORC/MSG criteria for the diagnosis of opportunistic invasive fungal infections [8], this type of infection in the patients was diagnosed based on the positive tissue culture or the presence of tissue invasion by the fungus on histopathologic examination of a biopsy specimen.

Tissue samples from maxillary sinuses were collected during standard surgical treatment and were divided into two parts, one for routine culture methods and PCR in normal saline and the other in formalin for histopathological examination. All tissue samples were cultured on Sabouraud dextrose agar (Merck, Darmstadt, Germany) with chloramphenicol (Merck,Darmstadt, Germany). Each sample was cultured in three plates and the result was considered positive only when all plates showed growth of similar fungi. The plates were incubated at room temperature for 14 days and the species was identified based on its macroscopic and microscopic features. For histopathological examination, periodic acid-Schiff and haematoxylin/eosin staining was done on tissue sections. Two blood samples were cultured by bedside inoculation onto BACTEC medium (Becton-Dickinson, Sparks, USA). Serum samples were used for MN and GM Ag ELISA, and 3 to 5 milliliters of serum from each patient were stored at -20°C for the PCR assay.

Platelia *Aspergillus* enzyme immunoassay (EIA) (Bio-Rad Laboratories, Marnes-la-Coquette, France) was used to measure *Aspergillus* GM levels, and *Candida* MN antigen was measured with the Platelia*Candida* Ag assay (Bio-Rad Laboratories). These tests are based on the detection of fungal antigen in the serum by immunoenzymatic sandwich microplate assay (sandwich ELISA). All kits were used according to the manufacturer's instructions. Optical absorbance of the samples and controls was determined with a microplate spectrophotometer equipped with 450 nm and 620 nm filters (Thermo Labsystems Multiskan Ascent ELISA, Helsinki, Finland). Sera with an optical density index of ≥ 0.5 were considered positive for *Aspergillus* GM, and samples with a MN concentration ≥ 0.5 ng/mL were considered positive (according to the manufacturer's guidelines).

To extract DNA from the serum, QIAmp DNA Minikits (Qiagen, Hilden, Germany) were used in accordance with the manufacturer's recommendations. The conditions for fungal amplification primers and probes, thermal cycling for all *Candida* spp. and *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*,and *C. krusei*, and fluorogenic probes were as reported by Shin and colleagues [9]. The probes and primers used to identify all *Aspergillus* species were those previously described by Kami and colleagues [10]. To avoid contamination, all samples were handled under sterile conditions in a laminar flow cabinet.

In the first stage, the blood samples were tested with the *Aspergillus* probe and a universal *Candida* species probe. If the result for *Candida* species was positive, testing continued to identify the individual *Candida* species. All primers and TaqMan probes were from Metabion (Martinsried, Germany). TaqMan universal PCR master mix (Roche, Branchburg, USA) and 0.2 µmol/L of each primer and species probe were used for sample analysis. In addition, the Gene Amp 7500 sequence detection system (Applied Biosystems, Foster City, USA) was used with the following thermal cycling conditions for amplification: heating at 94°C for 10 minutes, followed by a two-stage temperature profile of 30 seconds at 95°C and 90 seconds at 60°C for 40 cycles [11]. To determine the sensitivity of the real-time PCR assay, 1,000 copies/well of each *A. flavus* and *C. albicans* DNA were serially diluted and measured. Furthermore, a

nested-PCR assay as described by Rickerts *et al*. [12] was used to detect DNA from members of the order *Mucoraceae*. The primers were designed to amplify 18S rDNA of fungi belonging to this order. These primers were divided into two groups: ZM1 and ZM2 for amplification of a 407–408 bp fragment (for the first round of PCR) and ZM1 and ZM3 for amplification of a 176–177 bp fragment (for the second round of PCR). Serial dilutions of cloned DNA were used to determine the lower detection limit of the nested PCR assay.

Since the study was descriptive, statistical analysis was done for only data collection and for some frequency data; SPSS Statistics software for Windows, version 15.0 was used. Regarding ethical considerations, written informed consent was obtained from the adults and children's guardians. Meanwhile, the research conformed to the Helsinki Declaration, and the ethics committee at Professor Alborzi Clinical Microbiology Research Center, Shiraz University of Medical sciences, Iran approved the study.

Results

A total of 31 patients were included in the study. Their median age was 35.3 years (range, 11–75 years), and 16 patients were males. Eighteen patients (58.1%) were diagnosed with documented IFR caused by 19 different species (two fungi were isolated from one patient) on the basis of host factors and clinical, radiological, and mycological findings. The median age of the patients with IFR was 27.8 years. Of these patients, 10 (55.5%) had hematologic disorders and 8 (44.5%) had diabetes. The female-to-male ratio was 8:10. The characteristics of these patients with their corresponding laboratory findings are presented in Table 1.

The clinical symptoms and signs of IFR were fever, facial pain or pain over the affected sinus, purulent nasal discharge, decreased or absent sense of smell, erythema, and edema of the nasal mucosa. The radiologic features included maxillary rhinosinusitis with expanding bone mass erosion (axial computed tomography of the sinuses), invasion in maxillary rhinosinusitis, pneumocephalus, necrosis in the nasal cavity, hypo-attenuating mucosal thickening, bone destruction in the sinus walls, and soft-tissue attenuation in the lumen of the involved paranasal sinus and nasal cavity.

According to EORTC/MSG criteria [8], together with the clinical and radiological signs and symptoms in this study, direct microscopic pathological examination of the biopsy samples seemed to be the best method of diagnosis for IFR; therefore, microscopic examination was considered the gold standard test.

Direct microscopic examinations with histopathological evidence of tissue invasion by fungal hyphae in bone, sinus mucosa, or blood vessels were positive in 18 patients. Nine patients had positive results for *Mucoraceae* organisms (non-septate hyphae), but only 5/9 samples produced growth in the culture (55.5%). Nested PCR results for species in this family were negative in all serum samples from patients.

Table 1. Characteristics of patients with documented invasive fungal rhinosinusitis.

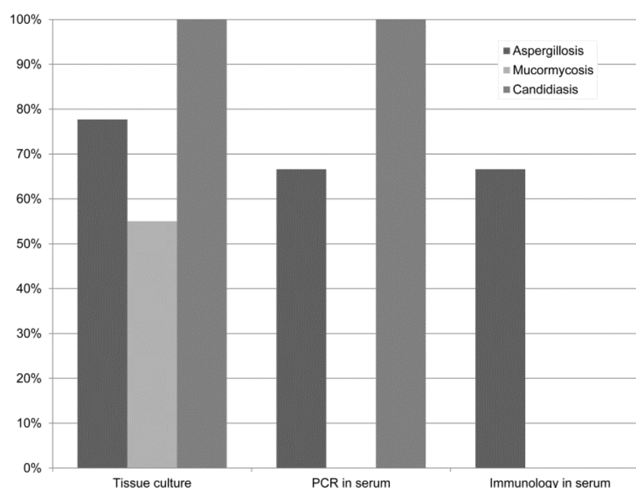
No	Sex/age (years)	Background	Microscopic examination	Culture	Galacto-mannan	Mannan	PCR
1	M/24	Bone marrow transplant	Septate hyphae	*A. flavus*	0.2	0	+
2	M/51	Diabetes	Non-septate hyphae	Negative	0.16	0	0
3	F/19	Thalassemia major	Non-septate hyphae	Negative	0.05	0	0
4	M/31	Diabetes	Non-septate and Septate hyphae	*Rhizopus* sp.*	2.5	0	0
5	M/19	ALL-L2	Septate hyphae	*A. flavus*	0.4	0	0
6	F/44	Diabetes	Pseudohyphae and blastoconidia	*C. albicans*	0.2	0	0
7	F/50	Diabetes	Non-septate hyphae	No growth	0.3	0	0
8	F/75	Diabetes	Non-septate hyphae	*Rhizopus* sp.	0.25	0	0
9	F/68	Diabetes	Non-septate hyphae	*Mucor* sp.	0.34	0	0
10	F/17	ALL	Septate hyphae	*A. flavus*	1.4	0	0
11	F/27	ALL	Septate hyphae	*A. flavus*	4.7	0	+
12	M/13	NHL	Septate hyphae	*A. flavus*	2.3	0	+
13	M/12	AML	Septate hyphae	*A. flavus*	0.55	0	+
14	M/66	Diabetes	Non-septate hyphae	No growth	0.18	0	0
15	M	AML	Non-septate hyphae	*Mucor* sp.	0.16	0	0
16	F/11	AML	Septate hyphae	*A. flavus*	0.3	0.25	+
17	M/66	Diabetes	Non-septate hyphae	*Mucor* sp.	0.18	0	_
18	M/12	AML	Septate hyphae	No growth	0.55	0	+

A: *Aspergillus*; C: *Candida*; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; PCR: polymerase chain reaction; *Direct smear examination identified septate and non-septate hyphae. It was difficult to isolate species with septate hyphae because of overgrowth by *Rhizopus*sp.

In addition, 9 patients were positive by direct microscopic examination (septate hyphae), 7 specimens produced positive growth for *A. flavus* (77.7%), the GM test was positive in 6 of the 9 patients (66.6%), and *Aspergillus* PCR was positive in 6 patients (66.6%) with documented aspergillosis.

Direct microscopy examination in one patient disclosed septate and non-septate hyphae; however, only *Rhizopus* grew in the culture because of the rapid growth of this fungus, which probably inhibited the growth of septate hyphae fungi. The isolated agent was *C. albicans* in one patient. The MN test to detect *Candida* was negative. None of the blood cultures were positive for fungal infection. A comparison of culture, immunological, and molecular tests in the diagnosis of IFR are presented in Figure 1.

Figure 1. Comparing tissue culture, molecular, and immunological tests in the diagnosis of acute invasive fungal rhinosinusitis



Discussion

Fungal rhinosinusitis is a serious infection that can be acute or chronic according to clinical, pathological, and radiological presentation [7,13], with a reported mortality rate of up to 100% [7]. The highest rate of infection was found in 10/18 of our patients with hematologic disorders (55.5%), which is consistent with other reports that lymphoma, acute myeloid leukaemia, and myeloma were the most common hematological diagnoses in patients with IFR [14,15]. Nucci *et al*. reported that the rate of invasive fungal infections in these patients was as high as 30.5% [16]. In this study, all the patients enrolled had acute presentation of rhinosinusitis, given their immunocompromised status and clinical and radiologic criteria. To avoid a protracted clinical course or fatal outcome, early diagnosis and initiation of appropriate therapy are essential.

Diagnosis of IFR requires adequate quantities of sinus contents and biopsy specimens from the diseased and healthy mucosa and bone adjacent to the areas of frank necrosis [1]. One of the strengths of the current study was our use of these specimens to diagnose IFR. The panel of diagnostic procedures we tested showed that direct smear (histopathology or potassium hydroxide) was the most sensitive method of diagnosis in our high-risk patients with clinical signs and symptoms. As previously reported, frozen sections are important in the initial stage of diagnosis [7]. Therefore, obtaining appropriate samples during surgery and subsequent processing for histopathologic or microscopic examination to detect fungal elements are important steps in the management of IFR. Unfortunately, in patients with neutropenia or in poor general condition, sampling is not feasible, and non-invasive methods are recommended instead.

In general, blood culture is not suitable for the diagnosis of fungal infections; in this study, none of the patients with proven IFR had positive blood culture results, and the tissue cultures were respectively positive in 55.5% (5/9) and (7/9) 77.7% of cases of mucormycosis and aspergillosis. It should be recalled, however, that sensitivity is related to the infective species. For example, some fungi in the order *Mucoracea* are difficult to cultivate. In a review of 929 cases of mucormycosis reported between 1940 and 2003, only 50% were culture positive [17]. In the present study, the prevalence of *A. flavus* was 38.8%; another study in South east Asia found a prevalence of 44% [15], while in Europe, this percentage was reported to be higher (61.5%) [18]. Because resistance to antifungal agents has been reported in many studies [19,20], one of the advantages of culture is that it helps clinicians to select the best antifungal agents for treatment.

The detection of *Aspergillus* GM antigen can serve as early evidence of invasive aspergillosis. Different results have been reported regarding the validity of this test in the diagnosis of fungal rhinosinusitis. According to Kostamo *et al*., the GM ELISA is not reliable for diagnosing *Aspergillus* infections of the paranasal sinuses [21]. Chen *et al*. reported a sensitivity of about 64% and a specificity of 60% for the GM test in sera from patients with invasive *Aspergillus* rhinosinusitis [15]. In the present study, the GM test was positive in 66.6% (6/9 cases) of the patients with documented aspergillosis. Methods to test for GM Ag and other immunological methods may

aid the diagnosis of IFR; however, they also have their limitations. For example, there are many reports of false-positive results with the GM test in patients treated with beta-lactams, amoxicillin-clavulanate, piperacillin-tazobactam; also, in patients infected with fungi other than *Aspergillus*,the GM test may yield false-positive results in serum samples [22-26]. The mannan antigen test is used to detect systemic candidiasis [27]. The sensitivity and specificity of the MN test were reported to be 90.9% and 46.2%, respectively [28]. Only one patient in the present study was diagnosed with *Candida* IFR on the basis of the culture results, whereas the results of the mannan antigen test were negative for all patients.

In previous studies, PCR has been found to be able to detect fungal infections [29,30]. Kostamo *et al*. reported that in comparison with the GM ELISA, PCR might have the additional merit of allowing a diagnosis of *A. fumigatus* in paranasal sinus infections to be made sooner [21]. In the present study, *Aspergillus* PCR assay was positive in 66.6% (6/9 patients) of patients suffering from *Aspergillus* rhinosinusitis.

After candidiasis and aspergillosis, mucormycosis is the third-most prevalent infection [27] in immunocompromised patients. Unfortunately, there is no serological method for the diagnosis of this infection, and none of the serum samples in the present study had positive results for molecular diagnosis of mucormycosis in patients with a positive direct smear from tissue samples. Therefore, no non-invasive method is currently available for the diagnosis of rhinosinusitis by members of the family *Mucoraceae*.

Conclusions

Early diagnosis is essential for the successful treatment of IFR. Our study suggests that using specific PCR targeting several species of fungi and immunological methods to screen high-risk patients could be helpful for clinicians to manage the infection efficiently. Non-invasive methods may help the diagnosis of IFR, but direct microscopic examination of tissue samples can be considered the most efficient way to diagnose such infections. GM, MN, and PCR methods are recommended for immunocompromised patients, in whom tissue sampling is not possible and among whom the mortality rate from IFR is high. However, for patients in critical condition, a combination of immunological and molecular methods can help clinicians to manage the infection efficiently.

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