

An investigation on non-invasive fungal sinusitis; Molecular identification of etiologic agents

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Background: Fungal sinusitis is increasing worldwide in the past two decades. It is divided into two types including invasive and noninvasive. Noninvasive types contain allergic fungal sinusitis (AFS) and fungus ball. AFS is a hypersensitivity reaction to fungal allergens in the mucosa of the sinonasal tract in atopic individuals. The fungus ball is a different type of noninvasive fungal rhinosinusitis which is delineated as an accumulation of debris and fungal elements inside a paranasal sinus. Fungal sinusitis caused by various fungi such as *Aspergillus* species, *Penicillium*, *Mucor*, *Rhizopus*, and phaeohyphomycetes. The aim of the present study is to identify fungal species isolated from noninvasive fungal sinusitis by molecular methods. **Materials and Methods:** During 2015–2016, a total of 100 suspected patients were examined for fungal sinusitis. Functional endoscopic sinus surgery was performed using the Messerklinger technique. Clinical samples were identified by phenotypic and molecular methods. Polymerase chain reaction (PCR) sequencing of ITS1-5.8S-ITS2 region and PCR-restriction fragment length polymorphism with *MspI* restriction enzyme was performed for molecular identification of molds and yeasts, respectively. **Results:** Twenty-seven out of 100 suspected cases (27%) had fungal sinusitis. Nasal congestion (59%) and headache (19%) were the most common clinical signs among patients. Fifteen patients (55.5%) were male and 12 patients (44.5%) were female. *Aspergillus flavus* was the most prevalent fungal species (26%), followed by *Penicillium chrysogenum* (18.5%) and *Candida glabrata* species complex (15%). **Conclusion:** Since clinical manifestations, computed tomography scan, endoscopy, and histopathological findings are very nonspecific in AFS and fungus ball; therefore, molecular investigations are compulsory for precise identification of etiologic agents and appropriate management of these fungal infections.

Key words: Fungal agents, molecular identification, non-invasive, sinusitis

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INTRODUCTION

Allergic fungal sinusitis (AFS) is a hypersensitivity reaction to fungal allergens including surface antigens and fungal metabolites in the mucosa of the sinonasal tract in atopic individuals. It was first reported by Katzenstein *et al.* in 1983.^[1] Clinical manifestations present with different signs containing nasal congestion, facial deformity, diplopia, osteolytic impairment, and entanglement of the skull base.^[2] The incidence of disease is unknown and seems account for up to 10% of surgical cases;^[3] however, mixed bacterial and fungal infections represent 13%–28.5% of all maxillary

sinusitis cases.^[4] The diagnosis of AFS is supported by the presence of thickened allergic mucin that is coarsely evident at surgery and a positive histopathological staining for fungal hyphae.^[5] Fungus ball is a type of noninvasive chronic fungal sinusitis without thickened allergic mucin. It is composed of fungal hypha, fibrin, inflammatory cell, shapeless debris, and mucous in pulmonary cavities. It usually appears in the maxillary and sphenoid sinuses.^[4] Fungus ball is commonly found in older individuals and females.^[6,7] A variety of fungal agents such as yeasts, dematiaceous fungi, and hyaline fungi have been involved in fungal sinusitis.^[8,9] The aim of the present study is to identify the etiologic agents of fungal sinusitis isolated from AFS and fungus ball using molecular methods.

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MATERIALS AND METHODS

Clinical samples

During December 2015 to December 2016, a total of 100 cases referred to the two university hospitals (Alzahra and Kashani Hospitals) of Isfahan University of Medical Sciences, Isfahan, Iran, due to the acute or chronic sinusitis. All patients were nominated for computed tomography (CT) scan of the paranasal sinuses (PNS). Bony erosion, inspissated mucus in sinuses that cause heterogeneous soft tissue density and calcification were clinical symptoms among patients. Functional endoscopic sinus surgery was performed using the Messerklinger technique by means of Karl Storz Endoskope (Germany). The Messerklinger technique is a mainly diagnostic endoscopic concept presenting that the maxillary and frontal sinuses are subordinate cavities. It can be used for various scopes of indications, excluding nasal polyposis. Sufficient experience and training are needed for the surgical approach.^[10] All samples were sent to the medical mycology laboratory in serum saline for direct microscopy and culture. Potassium hydroxide 20% and Sabouraud Dextrose Agar with chloramphenicol (BD, USA) were used for direct microscopic examination and culture, respectively. Periodic acid-Schiff (PAS) (Sigma-Aldrich, USA) was applied for histopathological staining.

Molecular identification

DNA extraction

DNA of fungi was extracted using glass beads (Sigma-Aldrich, USA) and boiling method.^[11] Briefly, a bit of fresh colonies were suspended in 50 µl of double distilled water and simmered for 10 min, afterward centrifuged for 5 min at 5000 rpm, and supernatant was separated for polymerase chain reaction (PCR). ITS1-5.8S-ITS2 rDNA region of fungi was amplified using ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers.^[12]

Polymerase chain reaction

The PCR cycling was performed as follows: initial denaturation phase at 95°C for 5 min, followed by 32 cycles of denaturation at 94°C for 35 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, with a final extension phase at 72°C for 7 min. PCR products were purified by QIAquick PCR Purification Kit (Qiagen, USA). Five microliters of each amplicon was run onto 1.5% agarose gel, electrophoresed in TBE buffer (90 mM boric acid, 2 mM 127 ethylenediaminetetraacetic acid, and 90 mM Tris) at 10 V/cm for 90 min, and stained with 0.5 µg/ml ethidium bromide (Sigma-Aldrich, USA).

Sequencing

Purified PCR products were employed for cycle sequencing reactions in forward direction (Bioneer, Korea). Sequence

products were analyzed with Chromas Software 2.4 (<http://www.chromas.software.informer.com/2.4/>) and evaluated using of NCBI BLAST searches against fungal sequences existing in DNA databases (<http://www.blast.ncbi.nlm.nih.gov/Blast.cgi>).

Restriction fragment length polymorphism

All clinical yeasts were identified by restriction fragment length polymorphism patterns of PCR products.^[13,14] *MspI* (*HpaII*) restriction enzyme (Fermentas, Vilnius, Lithuania) was used in this step of identification.

RESULTS

Fungal sinusitis was diagnosed for 27 out of 100 suspected cases (27%). The most patients were house keeper (41%) [Table 1]. Nasal congestion ($n = 16$; 59%) and headache ($n = 5$; 19%) were the most common clinical signs among patients [Table 1]. Fifteen patients (55.5%) were male and 12 patients (44.5%) were female. Polyps (33%), diabetes mellitus (30%), and hypothyroidism (15%) were the most risk factors among patients. Age range of patients was between 8 and 88 years, with a mean age of 46 years. *Aspergillus flavus* was the most prevalent fungal species (26%), followed by *Penicillium chrysogenum* (18.5%) and *Candida glabrata* species complex (15%). Table 1 summarizes characteristics of patients of the present study.

DISCUSSION

Fungal sinusitis is increasing worldwide in the past two decades. It is divided into two types including invasive and non-invasive. Invasive fungal sinusitis (IFS) represents acute, chronic, and granulomatous forms of infection. Many fungi are implicated containing: Zygomycetes (usually in diabetic patients), *Aspergillus* species (in neutropenic patients), *Mucor* spp., *Rhizopus* spp., *Rhizomucor* spp., and *Lichtheimia* spp. Noninvasive types contain AFS and fungus ball.^[9] AFS is pathologically and clinically a specific form of rhinosinusitis in atopic immunocompetent people. It is most frequent among young individuals; the mean age of occurrence is about 21 years, whereas the mean age of patients in the present study was 46 years. Many studies reported that the male-to-female sex ratio is same;^[15,16] however, in the present investigation, the sex ratio of infected individuals was nearly the same (15/12). Hypersensitivity reaction in the mucosa of the sinonasal tract, tissue edema, and reproduction of organisms intensifies inflammation that cause polyposis and chronic rhinosinusitis;^[16] here, we found nine patients (33%) with polyposis in this study, too. This condition occurs with a wide variety of fungi such as *Aspergillus* species, *Penicillium*, *Mucor*, *Rhizopus*, and many phaeohyphomycetes.^[17] Patients with AFS usually show signs of chronic sinusitis and allergic rhinitis containing

Table 1: Details of patients with allergic fungal sinusitis and fungus ball in the present study

Number	Age	Sex	Types of surgery	City	Occupation	Symptoms	Predisposing factors	Fungal species
1	41	Male	ESS	Isfahan	Dentist	Nasal congestion	Polypectomy	<i>C. glabrata</i> species complex
2	60	Female	BNPS	Isfahan	Housekeeper	Shortness of breath	Atopic syndrome	<i>Ulocladium</i> sp.
3	57	Male	ESS	Isfahan	Self-employed	Purulent exudate	Hypothyroidism	<i>C. glabrata</i> species complex
4	68	Male	ESS	Borkhar county	Employee	Gray patch on palate	Diabetes mellitus + blood pressure	<i>A. fumigatus</i>
5	38	Male	NPS	Isfahan	Self-employed	Nasal congestion	Fatty liver Postextraction sepsis	<i>A. flavus</i>
6	33	Male	NPS	Isfahan	Stone cutter	Nasal congestion	Asthma	<i>P. polonicum</i>
7	16	Male	MFSS	Isfahan	Student	Nasal congestion	Polypectomy	<i>A. flavus</i>
8	20	Male	ESS	Isfahan	Self-employed	Nasal congestion + headache	Splenectomy + facial trauma	<i>A. flavus</i>
9	68	Male	ESS	Golpayegan	Retired	Bulge and facial pain	Diabetes mellitus + breast cancer	<i>A. fumigatus</i>
10	52	Female	ESS	Isfahan	Housekeeper	Nasal congestion + headache	Hypothyroidism	<i>C. macrocarpum</i>
11	65	Female	ESS	Isfahan	Self-employed	Deviated septum	Diabetes mellitus + hypothyroidism	<i>A. flavus</i>
12	28	Female	NPS	Isfahan	Housekeeper	Nasal congestion	Polypectomy	<i>P. chrysogenum</i>
13	28	Male	ESS	Isfahan	Self-employed	Nasal congestion	Polypectomy	<i>C. glabrata</i> species complex
14	36	Female	BNPS	Najafabad	Housekeeper	Bilateral polyps + headache	Polypectomy	<i>A. alternata</i>
15	82	Male	MTNCS	Fereydunshahr	Farmer	Nasal congestion	Nasal tumor	<i>C. glabrata</i> species complex
16	52	Male	MESS	khomeini shahr	Self-employed	Nasal congestion	Diabetes mellitus + polypectomy	<i>A. malorum</i>
17	28	Female	NSDS	Isfahan	Housekeeper	Deviated septum	Polypectomy	<i>P. chrysogenum</i>
18	64	Female	ESS	Isfahan	Housekeeper	Headache + catarrhal inflammation	Rhinoplasty surgery	<i>P. chrysogenum</i>
19	8	Male	ESS	Mobarakeh	Student	Nasal congestion + eyesore	Polypectomy	<i>P. chrysogenum</i>
20	41	Female	ESS	Falavarjan	Housekeeper	Polyps + nasal congestion	Diabetes mellitus + hypothyroidism + atopic syndrome	<i>A. parvisclerotigenus</i>
21	37	Male	BNPS	Lenjan	Self-employed	Right polyps	Polypectomy	<i>P. chrysogenum</i>
22	72	Female	ESS	Isfahan	Housekeeper	Diplopia + ptosis	Diabetes mellitus	<i>G. candidum</i>
23	27	Male	ESS	Khuzestan	Worker	Nasal congestion	Allergic rhinitis	<i>A. flavus</i>
24	52	Female	MESS	Isfahan	Housekeeper	Nasal congestion	-	<i>G. candidum</i>
25	52	Female	MESS	Isfahan	Housekeeper	Nasal congestion	-	<i>A. flavus</i>
26	53	Male	ESS	Isfahan	Self-employed	Nasal congestion + severe headache	Diabetes mellitus + blood pressure	<i>A. flavus</i>
27	54	Female	NPS	Fereydan	Housekeeper	Polyps	Diabetes mellitus+asthma	<i>B. neergaardii</i>

ESS = Endoscopic sinus surgery; BNPS = Bilateral nasal polyps surgery; NPS = Nasal polyps surgery; MFSS = Maxillary and frontal sinuses surgery; MTNCS = Malignant tumors of the nasal cavity surgery; MESS = Maxillary and ethmoid sinuses surgery; NSDS = Nasal septum deviation surgery; *C. glabrata* = *Candida glabrata*; *A. fumigatus* = *Aspergillus fumigatus*; *A. flavus* = *Aspergillus flavus*; *P. polonicum* = *Penicillium polonicum*; *C. macrocarpum* = *Cladosporium macrocarpum*; *P. chrysogenum* = *Penicillium chrysogenum*; *C. glabrata* = *Candida glabrata*; *B. neergaardii* = *Bipolaris neergaardii*; *G. candidum* = *Geotrichum candidum*; *A. parvisclerotigenus* = *Aspergillus parvisclerotigenus*; *A. malorum* = *Alternaria malorum*; *A. alternata* = *Alternaria alternata*

purulent rhinorrhea, nasal obstruction, bony erosion, postnasal drainage, and headache. Pain is a rare sign; however, seven patients (26%) had made a complaint of headache, eyesore, and facial pain, in the present survey. Mucin in the AFS is plentiful, thick, firm, and adhesive. It has better appearance leading to the nonspecific radiographic findings of AFS on CT scans, i.e. heterogeneous areas within the PNS that are seen due to the collection of heavy metals such as manganese, iron, accompanied by calcium crystals

in the inspissated mucin.^[18] All PNS and nasal cavity are usually involved, with the maxillary and ethmoid sinuses being the most common.^[15] In the present investigation, 15% of cases were diagnosed with the involvement of maxillary and ethmoid sinuses. In terms of histology, AFS has a typical appearance of “tree rings” or “tide lines” of mucin interchanging with inflammatory fragments, which reveals as alternating blue and pink lines. The debris is composed of aggregated neutrophils, eosinophils, and deteriorated

eosinophils forming Charcot–Leyden crystals that are bipyramidal, refractile, and needle shaped.^[3] Identification of fungal elements is complicated on hematoxylin and eosin staining and may be seen with PAS and Grocott's methenamine silver. For this reason, all clinical samples in this study were stained with PAS, and fungal hyphae were diagnosed clearly. If fungal elements are not seen using of culture and staining, the entity of AFS may be diagnosed as eosinophilic mucin rhinosinusitis.^[19] Treatment of AFS is efficient with debridement of involved tissue, complete evacuation of inspissated mucus, and polypectomy. Table 1 show the different types of surgery were performed for each patient in the present study. Postoperative steroids' therapy is usually recommended to decrease allergy, inflammation, and sensitization for long-term control, but recurrences are usual.^[15,16,20] All patients are usually treated with intranasal steroid sprays, similar to the patients of this investigation. AFS is treated much differently than IFS and must be marked with discerning histopathologic features of IFS that is inflammatory cells within the tissue instead of free-floating in mucin, presence of fungal elements within the vascular spaces and tissues.^[16,21] The fungus ball (mycetoma) is a different type of noninvasive fungal rhinosinusitis which is delineated as an accumulation of debris and fungal elements inside a PNS. Fungus ball of the PNS is characterized by a mass of fungal elements and fruiting heads of fungi and minimal host immune responses. It is always located in the maxillary sinus and sphenoid sinus, respectively.^[22] Various fungal species have been isolated from fungus ball such as *Aspergillus*, *Alternaria*, *Mucor*, *Penicillium*, and *Bipolaris*.^[22,23] Some risk factors have been suggested for fungus ball such as diabetes mellitus,^[24] previous surgery on the sinonasal area,^[25] estrogens,^[26] and living in a rural area.^[25] In the present study, we recognized a patient with fungus ball (patient no. 24) [Table 1]; however, she did not live in rural regions, and also she did not have any predisposing factor. The etiologic agent of fungus ball was *Geotrichum candidum*, which is yeast-like fungus with worldwide distribution. The pathogenicity of *G. candidum* in humans has not been obviously determined.^[27] To the best of our knowledge, this is the first case of fungus ball owing to *G. candidum* reported in Iran. *A. flavus* was the most common species in the present study, whereas Marglani and Shaikh^[9] reported *Aspergillus terreus* and *Aspergillus fumigatus* as the etiologic agent of AFS by phenotypic methods. Cavanna *et al.*^[28] isolated a dematiaceous fungus as the *Curvularia lunata* from an immunocompetent male with allergic fungal rhinosinusitis. Similar to the present study, the fungus was identified by sequencing of internal transcribed spacer regions of fungal ribosomal DNA. Gupta *et al.*^[29] reported a case of chronic rhinosinusitis with the involvement of orbit by *Exserohilum rostratum* in an immunocompetent child.

In opposition to the present study, the fungus was only identified on the basis of morphological features such as direct microscopy, culture, and histopathology. Azar *et al.*^[30] reported the frequency of various fungi using histological assays and culture from biopsy samples of patients with rhinosinusitis, in Khorramabad. *Candida albicans*, *A. flavus*, and *A. fumigatus* were the most common fungal species in their study. Nazeri *et al.*^[31] showed *A. flavus* as the most prevalent causative agent of allergic fungal rhinosinusitis, chronic invasive type, and fungus ball. They also isolated a few infrequent fungal species such as *Fusarium proliferatum* and *Schizophyllum commune* from clinical samples based on PCR sequencing. Phenotypic methods were used in Jahromi and Khaksar in a retrospective investigation.^[32] They reported *A. flavus* and *C. albicans* as predominant fungal species isolated from PNS.

CONCLUSION

Gene sequencing can be helpful for identification of etiologic agents of AFS and fungus ball and may be functional for discriminating of phaeohyphomycetes from other filamentous fungi, especially if nonsporulating molds that are fast growing at 37°C; fails to identify on general media with actidione. Since clinical manifestations, CT scan, endoscopy, and histopathological reactions are very nonspecific in AFS and fungus ball; therefore, molecular investigations are compulsory for precise identification of etiologic agents and appropriate management of these fungal infections.

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Conflicts of interest

There are no conflicts of interest.

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