

Laboratory approach for detection of non-invasive fungal rhinosinusitis: A case-control study

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Abstract

Objective: Noninvasive fungal rhinosinusitis (NFRS) is a problematic clinical condition due to its resistant to traditional medical treatment. Surgical debridement is the basis for its treatment. Mycology laboratory investigation can help to solve this issue. This case control study was designed to investigate the frequency of NFRS in our locality and identify fungal species incriminated in this clinical condition at the Otorhinolaryngology department, Zagazig University Hospitals. The study evaluated the role of microscopic examination, antigen detection and PCR in comparison to culture methods used in diagnosis of fungal agents.

Materials: Sinus materials were collected from 78 cases which have been clinically and radiologically confirmed with NFRS over the period June 2013 to September 2015. A control group 78 subjects were included with healthy sinuses from whom nasal smears were obtained. Samples were processed in the Mycology Laboratory, and all specimens were examined microscopically in 10% KOH preparations. Lactophenol cotton blue slide preparations were used to detect microscopic structures of hyphae and conidia. PCR amplification of the extracted DNA was performed using fungal universal primers for amplification of 28 S rDNA genes.

Results: Microscopic examinations revealed hyphae and fruiting bodies in 37 (47.4%) of the cases. Fungal culture detected 36 patients infected with NFRS. *Aspergillus fumigatus* was the most frequently isolate from fungal rhinosinusitis (52.7 %) of the cases, followed by *Penicillium spp.* (22.2%). PCR amplification exhibits the same sensitivity and specificity as those demonstrated by microscopic examination (100% and 97.3%,

respectively). ELISA of *Aspergillus* galactomannan (GM) antigen lacked sensitivity (58.3%), while specificity was high (100%).

Conclusion: This study demonstrates that proper mycological investigation using direct microscopic examination and culture are necessary to confirm presence of noninvasive fungal rhinosinusitis in patients.

Key words: *Rhinosinusitis; fungus; KOH; galactomannan; PCR*

Introduction

Non-invasive fungal rhinosinusitis (NFRS) presented with sinus fungal ball is a common disorders affecting immunocompetent individuals of all age groups. The incidence of NFRS has increased significantly in many countries possibly due to air pollution, allergy, and climate changes [1,2]. Sinus fungal ball is associated clinically by nasal congestion, purulent or bloody discharge, headache, and impaired smell sensation, which are non-conclusive for diagnosis, and may be misdiagnosed as soft-tissue tumors [3-5]. Available studies indicate that *Aspergillus spp.* is the most common etiological agents for NFRS [6]. Surgical removal of the affected sinus mucosa is the mainstay for treatment of almost all cases with re-establishing the drainage from the affected sinus. There is no need for antifungal therapy, except in cases of allergic association or complication with bone erosion [7,8]. Microbiology Laboratory can help in confirmation of the diagnosis and verification of the etiological fungal agent [9].

Mycology culture is still considered the gold standard despite of being an old traditional diagnostic technique. Culture practice should not be ignored, even with delay in fungal identification [10, 11]. Non-molecular markers are available alternative

tools supporting the diagnosis of fungal infection in combination with clinical, radiologic, and other microbiological criteria [12].

PCR is a useful method that detects viable and nonviable fungal pathogens and includes a variety of protocols. Some protocols use pan fungal primer PCR assays, whereas single species detection can be carried out using species specific primers [13]. Mycology laboratory investigation followed by surgical debridement would prevent usage of unnecessary antifungal therapy in NFRS.

According to our best knowledge, data about incidence and etiology of NFRS in Egypt is not satisfactory. This study was planned to determine the frequency of NFRS in our locality at Zagazig University Hospitals, using microscopic examination, antigen detection and PCR as compared to fungal culture.

Materials and Methods

Setting and study design

A case-control study was carried out in Otorhinolaryngology Department, Zagazig University Hospi-

tals in association with the Mycology Lab, Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, over a period June 2013 to September 2015. An informed written consent was obtained from all investigated subjects as well as the approval of the Institutional Board Review of Zagazig University Hospitals.

Subjects and clinical Samples

A total of 78 clinical samples were collected from clinical case group with suspected NFRS. Paranasal sinus tissue specimens were collected with the following inclusion criteria; CT radiological findings as soft tissue masses with partial or complete opacification of a sinus with hyper dense areas (mottling) without bone invasion, intraoperative characteristic muddy discharge with nasal polyps. Exclusion criteria include the following: Any immunodeficiency condition to rule out invasive or opportunistic fungal infection, no previous treatment with oral, parenteral corticosteroids or antifungal agents. The second (control) group included 78 subjects with apparently healthy sinus (patients who were admitted for septoplasty and/or rhinoplasty).

Specimens Collection

Paranasal sinus tissue specimens were collected from the clinical case group in the operating room [14]. A povidoniodine solution was used for antiseptics of nostrils [5]. Regarding control group, nasal smears were obtained and examined with light microscopy and fungal cultivation.

Surgical specimens of sinus mass were obtained in sterile screw-capped containers containing Sabouraud's dextrose broth. Clinical data was collected and analyzed. Specimens were processed in safety cabinet of the Mycology Lab.

Microscopic Examination and Cultivation

All surgically excised specimens and nasal smears placed in Sabouraud's dextrose broth were centrifuged with glass beads. A portion of the pellet was

subjected to 10% potassium hydroxide (KOH) wet mount preparation and was examined by 40 X objectives for the presence of fungal elements [15].

The remaining portion of the pellet was inoculated on two plates of Sabouraud's dextrose agar (SDA) containing gentamicin (20 mg/liter) and chloramphenicol (50 mg/liter) and Sabouraud's brain heart infusion agar (SBAHI) [16]. One plate from both media was incubated at 37°C, and the other one at the room temperature. Culture plates were observed every two days for fungal growth and up to 6 weeks before considering them negative. Growth was recognized by gross examination of aerial mycelium, color and growth rate. Slide culture technique was performed with Lactophenol cotton blue mount, to reveal fungal structure of the mycelium, conidial types and hyphae which are characteristics for each species [16].

Fungal antigen detection

Aspergillus Galactomannan (GM) was examined in supernatant of the processed tissue specimens with Platelia Aspergillus kit (England, UK) according to the manufacture instructions. Briefly, it is a sandwich immunoenzymatic technique depends on detection of Aspergillus Galactomannan using rat monoclonal antibody EBA2.

DNA extraction and PCR

A loopful of fresh tissue was suspended in 200 µl of sterile water, and DNA extraction was performed by QIAmp tissue DNA extraction kit (QIAGEN, United Kingdom) according to the procedure described by the manufacture.

PCR of DNA extract was performed using universal fungal primers for the 28S rDNA forward primer [5'-GTG AAA TTG TTG AAA GGG AA-3'] and reverse [5'-GAC TCC TTG GTC CGT GTT-3'] (Ebersdorf, Germany) as recommended [17]. PCR amplifications reaction was carried out in 25-µl reaction volumes with a Biometra thermocycler (Biometra, Germany). All PCR runs included a positive control with DNA

of *A. fumigatus*, and two negative controls with PCR buffer. Cycling conditions were as follows: initial denaturation at 95°C for 10 min followed by 49 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min followed by a final extension at 72°C for 10 min. Amplification products were separated and analyzed by electrophoresis in a 2% agarose gel with ethidium bromide stain. PCR products (260 bp) in length were interpreted as an evidence of fungal DNA.

Statistical analysis

Collected Data were tabulated and analyzed using IBM SPSS version19, and the results of two groups were compared. Regarding case group, symptoms of patients with positive and negative fungal cultivation were compared. Validity tests and Chi-square test were applied in statistical analysis.

Results

Out of 78 cases enrolled in this study, 36 cases (46.1%) were confirmed to be associated with NFRS using culture media. No mycological evidence of FRS was found in the remaining 42 patients. The frequency of positive sinus NFRS was 18 (50%) in both males and females, and was more frequent in middle age patients with Mean \pm SD of 33.3 \pm 9.4 years.

Culture method has diagnosed 36 cases of NFRS. *Aspergillus fumigatus* was the most frequent fungal species isolated from FRS cases with statistically significant difference ($P < 0.001$). It was recovered from 19 (52.7 %) of NFRS cases, followed by *Penicillium spp.* in 8 (22.2%). Only 3 (8.3%) positive fungal cultures were recovered from the healthy control group (**Table 1**). Microscopic examination by 10% KOH revealed the presence of hyphae in 33 (42.3%) of the cases), hyphae with fruiting bodies and conidia in 4 cases (5.1%) of samples.

Antigen ELISA revealed the presence of *Aspergillus* galactomannan in 21/78 (26.9 %) in association with chronic sinusitis cases. Of these 18 cases with were positive for *Aspergillus fumigatus* culture. Three false positive ELISA cases were identified as *Scopulariopsis* by culture. Negative assay results were noticed in 15 cases of positive fungal culture. The remaining 42 cases were negative by both Antigen ELISA and fungal cultures.

PCR amplification **was validated first by the presence of fungal 28 S rDNA of 260 bp using positive control strain of Aspergillus fumigatus**. Positive fungal DNA was demonstrated in 37/78 (47.4%) of clinically suspected cases, of these **36/78 (46.2%) were confirmed by positive fungal cultures**. *Aspergillus* antigen detection method showed high specificity and the lowest sensitivity (58.3%). Performance comparison of the four used techniques for diagnosis of FRS is shown in **Table 3**.

Table 1. Fungal species isolates from non-invasive rhinosinusitis patients and healthy control

Fungus species	Case group Total no.36(%)	Healthy control Total no. (%)	P value
<i>Aspergillus fumigatus</i>	19 (52.7)	1(33.3)	<0.001
<i>Penicillium spp.</i>	8 (22.2)	2(66.7)	0.007
<i>Scopulariopsis spp.</i>	3 (8.3)	0(0.0)	0.014
<i>Aspergillus niger</i>	2 (5.5)	0(0.0)	0.046
<i>Alternaria spp.</i>	2 (5.5)	0(0.0)	0.046
<i>Cladosporium spp.</i>	1 (2.7)	0(0.0)	0.157
<i>Aurobasidium spp.</i>	1 (2.7)	0(0.0)	0.157
Total	36 (100.0)	3(100.0)	

Discussion

The incidence of Non-invasive fungal rhinosinusitis (NFRS) increased markedly over the past three decades due both to increased fungal pollution in the environment and improved diagnostic modalities [8,18, 19]. Different prevalence rates of fungal species causing rhinosinusitis are related to the geographic and seasonal variations as have been reported in India and Germany [20,21]. To our best knowledge, NFRS is not being extensively discussed as a separate clinical entity in Arabian countries. A combined phenotypic and molecular methods are currently the best approach for fungal identification, especially for moulds [9].

This study revealed that aspergillosis was diagnosed in more than half of cases (58.2%), with *Aspergillus fumigatus* being the most predominant isolate. Several studies from India and other countries found NFRS mostly associated with aspergillosis (63.1% and 70%), respectively [8,22]. Whereas, *Aspergillus flavus* followed by *Aspergillus fumigatus* are being the most common etiological agents of NFRS, and were responsible for 76.19% and 88% of cases in two separate studies in India [23, 24].

This study detected that *Penicillium spp.* accounted for 22.2% of the positive cases. This result is similar to a study done in Crete/Greece where

28.1 % of NFRS cases caused by *Penicillium spp.* [25], while *Cladosporium spp.* was less detected (one case) in our study and other studies in Greece and Iran [25,26]. Additionally, our study found only 2 (5.5%) cases of *Alternaria spp.*, whereas this fungus was recovered from 75% and 9.7 of the positive cases in Iran and Greece, respectively [25,27]. The study of Buzina et al. [28] in Germany, reported that *Cladosporium spp.*, *Alternaria spp.* and *Aureobasidium pullulans* showed a significantly higher occurrence during late summer and early autumn.

In this study, scopoularis was recovered from 3 cases (8.3%), and both candidiasis and zygomycosis were not recovered from any patient in contrast to reported studies [25].

Chatterjee and Chakrabarti reported that epidemiology of fungal rhinosinusitis is still not satisfactory, and its classification is still confusing, each of the clinicopathological variants of FRS is associated with exclusive geographical and host related risk factors, and different fungal etiological agents [29].

All cases included in this study were diagnosed to have NFRS, and these were mostly occurred in immunocompetent patients. Additionally, the most prevalent clinical presentation associated with NFRS was nasal obstruction (100%) (**Table 2**). This result is in agreement with other researchers who reported the same result [22].

Table 2. Clinical presentations of non-invasive rhinosinusitis case group in association with culture results

	Culture results		P value
	Positive cases Total no. 36(%)	Negative cases Total no.42(%)	
Nasal obstruction	36 (100)	23 (54.7)	0.017
Nasal discharge	24 (66.6)	32 (76.1)	0.131
Facial pain	24 (66.6)	38 (90.4)	0.012
Headache	18 (50)	34 (80.9)	0.002
Rhinorrhoea	6 (16.6)	26 (61.9)	<0.001
Epiphora	6 (16.6)	33 (78.5)	<0.001
Snoring	6 (16.6)	27 (64.2)	<0.001

Table 3. Comparison of microscopic examination, antigen detection, and PCR methods used for diagnosis of 78 fungal rhinosinusitis cases in relation to performing culture

	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy	K
Microscopic examination	100%	97.6%	97.3%	100%	98.7%	0.974
Antigen detection	58.3%	100%	100%	73.7%	80.8%	0.601
PCR	100%	97.6%	97.3%	100%	98.7%	0.974

This study confirmed the highest sensitivity of microscopic examination (100%), and specificity of 97.6%, compared to the study of Lacroix and his colleagues who reported sensitivity and specificity of 73% and 100%; respectively [30]. In addition, microscopy revealed an extra positive case over the culture diagnosis, and the same observation was documented by the study of Broglie et al. [31]. However, a difference between microscopy and culture results could be achieved by using multiple culture media for detection of fungal-like mucin [32,33].

The results of our fungal detection by PCR showed 100% sensitivity and 97.6% specificity as compared with standard culture techniques used. Similar results were reported by Polzehl et al. [21], since their study demonstrated that 61/77 of sinus lavage samples were positive for *Aspergillus spp.* in both culture and *Aspergillus*-specific nested PCR.

The present study showed that galactomannan (GM) antigen ELISA test has 58.3% sensitivity, 100% specificity and 80.8% accuracy, while a study of Kostamo et al. [34] showed that all FRS positive samples were negative by GM antigen ELISA test, and this test gave false positive results in five samples, that were negative by both culture method and PCR. Therefore, we could conclude that GM ELISA is not reliable in diagnosing *Aspergillus* infection of the paranasal sinuses.

It has been reported that there is an association between sinusitis and elevated mould-specific IgG-levels for *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aureobasidium pullulans* [32,34], and it has also been demonstrated that cross-reactivity exist between cell wall components of *Scopulariopsis brevicaulis* and *Aspergillus spp.* [35]. This study also confirm that galactomannan antigen test is not specific for detection filamentous fungi and can't differentiate between *Aspergillus*, *Penicillium* and *Scopulariopsis*. In conclusion, *Aspergillus fumigatus* was the most frequent species isolated from NFRS cases, and both direct microscopic examination and PCR method have nearly the same sensitivity and specificity in detection the fungal agents. Surgical debridement was mandatory to remove fungal elements.

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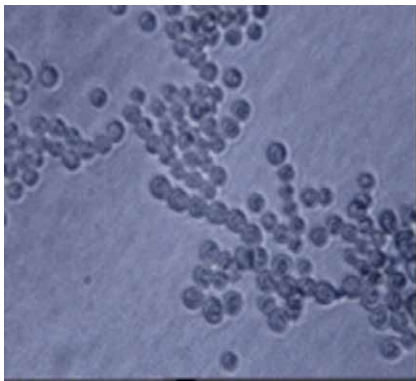


Figure 1. Microscopic appearance (X40) of transparency tape preparation showing growth of *Scopulariopsis*.



Figure 2. Corn meal agar showing the seven days growth of *Scopulariopsis* colonies.

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