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Role of Fungal Species in the Etiology of Nasal Polyposis

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ABSTRACT

Introduction: To investigate fungal species in nasal polyps (NP) by microscopy, fungal culture, fungal DNA isolation, and sequencing.

Methods: Twenty-four patients who applied to our outpatient clinic with complaints of chronic sinusitis and were found to have bilateral NP on clinical examination were included in our study. A control group was formed from 20 patients without NP who underwent septoplasty and endoscopic concha bullosa resection in our clinic. Samples from the participants were subjected to the same microbiological evaluations and the two groups were compared.

Results: The mean age of the patients included in our study was 38.14±14.13 years (range from: 17 to 80). Nine of the participants were female and 35 were male. Direct microscopy and fungal culture positivity rates did not significantly differ between the groups ($p>0.05$). A significant ($p<0.05$) lower rate of microorganisms was detected in tissue cultures obtained from the nasal polyp group. Polymerase chain reaction (PCR) techniques were unable to identify the fungal species in any of the positive fungal cultures. By sequencing, fungal species emerged at similar rates in both groups ($p>0.05$).

Conclusion: We concluded that fungal colonization is not more frequent in patients with NP than in the normal population. We did not observe the superiority of PCR-based sequencing over conventional fungal isolation techniques. However, larger series using molecular methods are needed.

Keywords: Nasal polyps, fungi, sequence analysis

Introduction

Nasal polyps (NP) are benign growths arising from the mucous layer of the nasal cavity and paranasal sinuses due to chronic inflammation. Its prevalence in the general population has been reported as 1-4% (1). They can occur in conditions such as chronic sinusitis, allergies, asthma, and aspirin intolerance. NP can cause symptoms such as nasal congestion, facial pain, nasal discharge, and loss of smell. Treatment can usually be done with methods such as corticosteroid sprays, antihistamines, and surgery (2).

Although the underlying mechanisms in chronic rhinosinusitis with NP (CRSwNP) are not fully defined, treatment modalities that will control the type 2 inflammatory response are being studied (3). Increased exposure to pathogenic microorganisms or allergen defects in the sinonasal epithelial barrier and the state of the individual's immune system play an important role in the pathogenesis of the disease (4). Bacteria play a role in the etiopathogenesis of chronic inflammatory diseases, but the role of fungi is controversial (5). Although the role of fungi in CRS is unknown, their detection has increased significantly in recent years.

In this study, we aimed to detect fungal cues by microscopy, culture, polymerase chain reaction (PCR), and sequencing in patients with NP and to evaluate the importance of fungi in pathogenesis by comparing them with healthy individuals.

Methods

Ethical Approval

The study was approved at the University of Health Sciences Turkey, İstanbul Training and Research Hospital Institutional Review Board (approval number: 64, date: 11.02.2022). All procedures were carried out

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in accordance with the ethical standards specified in the Declaration of Helsinki. An informed consent form was obtained from all patients.

Patients and Study Design

Twenty-four patients who applied to our outpatient clinic with complaints of nasal congestion, nasal discharge, and loss of smell and were found to have bilateral NP on clinical examination were included in our study. The following groups were excluded from the study: a) other causes of nasal obstruction such as septum deviation, inferior turbinate hypertrophy, b) patients with a history of previous nasal surgery, c) patients who have used intranasal or oral corticosteroid therapy for allergic rhinitis in the past 6 months, d) patients who used antibiotics with the diagnosis of chronic sinusitis in the past 6 months, e) patients with a diagnosis of chronic inflammatory disease or drug use, f) immunosuppressed patients.

A control group was formed from 20 patients without NP who underwent septoplasty and endoscopic concha bullosa resection in our clinic. Nasal polyp samples were taken from the study group in the local surgery room, and middle turbinate mucosa samples were taken from the control group by endoscopic concha bullosa resection under general anesthesia. For direct microscopic examination, fungal culture, and fungal PCR studies, using a sterile aseptic technique, two 2-3 mm^3 nasal tissue samples taken from each patient were placed directly into sterile sample containers containing 1 mL of saline and sent to the microbiology laboratory. The first tissue samples were planted for direct microscopic examination, aerobic culture, and fungal culture. Fungal PCR and DNA sequencing tests were performed on the second tissue samples.

Direct Microscopic Examination

Tissue biopsy specimens, of which direct preparations were prepared by suspending with 10% potassium hydroxide solution, were kept for 20 min and then examined with a light microscope for the presence of fungal hyphae, spores, and yeasts with 10X and 40X objectives (Figure 1).

Fungal and Aerobic Culture

Fungal culture: Biopsy samples were inoculated on thioglycolate broth and two separate sabouraud dextrose agar (SDA) (BioMérieux, Lyon, France) media under sterile conditions for fungal isolation. One inoculated SDA was incubated at 26-30 °C and the other at 35-37 °C conditions for 21 days to monitor fungal growth. Gram-stained preparations were prepared from suspicious colonies. Fungal colonies containing yeast or hyphae were evaluated for colony and conidial morphologies and defined at the species level.

Aerobic culture: After incubation of the biopsy samples inoculated in thioglycolate broth for 18-24 hours at 37 °C, passages were taken into 5% sheep blood agar, chocolate agar, and EMB agar media and incubated for 48 h in terms of bacterial growth. Colony morphology and Gram staining characteristics of the cultures with growth were determined and pre-identification was made at the species level using conventional methods. Then, species-level identification was performed using Vitek 2 GN and GP cards in the VITEK® 2.0 Compact (BioMérieux, Lyon, France) automatic ID/AST system for bacterial typing.

Fungal DNA Isolation, PCR and Sequencing

Biopsy samples stored at -80 °C were thawed and taken into a sterile Petri dish and cut into small pieces with the help of a scalpel. 500 µL of Buffer ATL (Qiagene) and proteinase K (50 ng/mL) were added and incubated at 56 °C for 1 h. After homogenization, nucleic acid isolation was performed with the ZymoBiomics® DNA Miniprep Kit (Zymo, CA, U.S.), designed to purify DNA from various sample inputs for microbiome or metagenome analysis according to the manufacturer's instructions (https://doi.org/10.1371/journal.pone.0241732).

The same procedure was used for strains isolated from the culture. Following nucleic acid isolation, fungal DNA was investigated by real-time PCR. The D1-D2 domain of 28S ribosomal RNA was targeted with NL1 (5´-GCA TAT CAA TAA GCG GAG GAA AAG-3´) and NL4 (5´-GGT CCG TGT TTC AAG ACG G-3´) (Figure 2). Positive bands were purified with NucleoSpin® Gel and the PCR Cleanup procedure (Machery-Nagel,

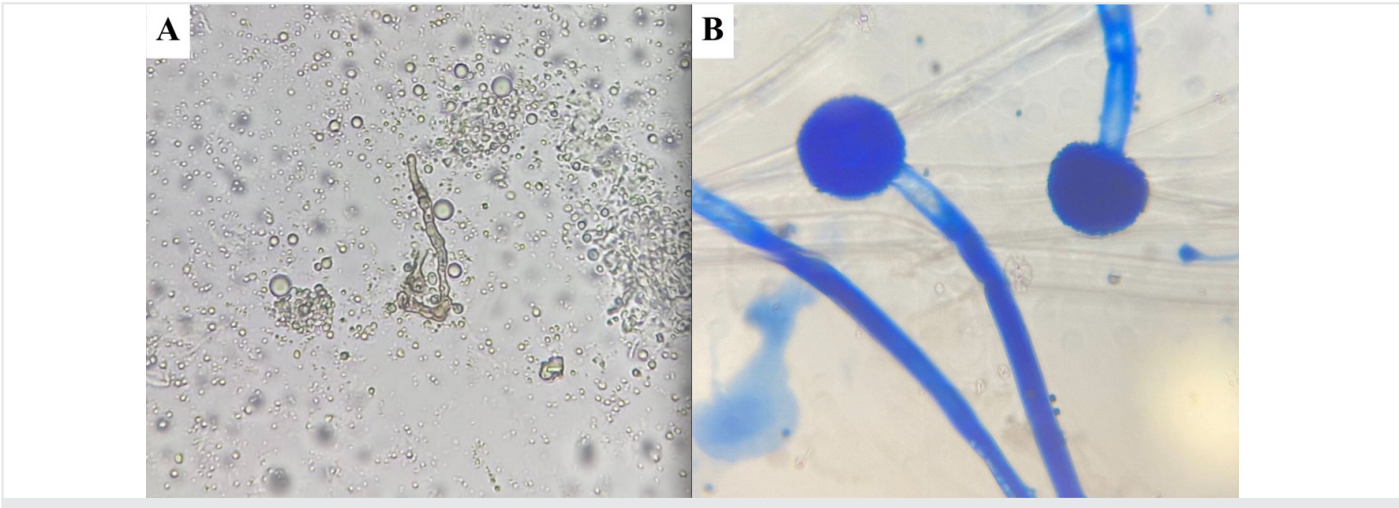

Figure 1. (A) Fungal hyphae visualized in tissue biopsy specimens suspended in 15% potassium hydroxide by direct microscopy. (B) Aspergillus spp. visualized in tissue biopsy specimens suspended in lactophenol cotton blue by direct microscopy

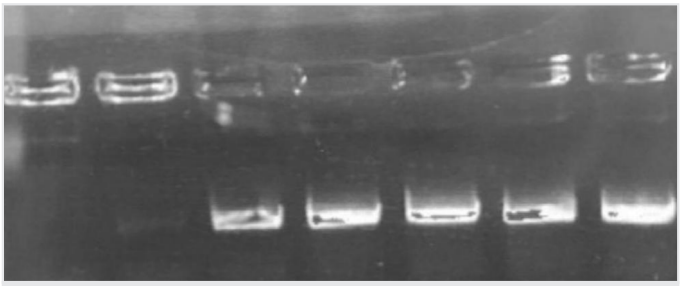

Figure 2. 28s rRNA region of approximately 600 bp amplified with NL1-4 primers (1,2 negative controls, 3 positive control and 4,5,6,7 patient samples)

Cologne, Germany). Bidirectional sequence analysis was performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Massachusetts, U.S.) (http://genomedicine.com/content/5/7/63). Baseline search was performed with sequencing analysis software and consensus sequences were edited with SeqMan software. (Dnastar, Inc., Winconsin, U.S.) For typing of the strains, the most overlapping types were determined by comparison with the sequences defined by blast search.

Statistical Analysis

The IBM SPSS 28.0 package program (SPSS Inc.; Chicago, IL, USA) was used in the analysis. Mean and standard deviation values were used in descriptive statistics of the data. The sample t-test was used in the analysis of independent quantitative data, and the chi-square test was used in the analysis of independent qualitative data. A P-value of <0.05 was considered statistically significant.

Results

The mean age of the patients was 38.14±14.13 years (range from: 17 to 80). Given the possible effect of gender on the findings, there were similar numbers of women in both groups that allow for a fair/valid comparison. Nine of the participants were female and 35 were male.

The age of the patients in the polyp group was significantly higher ($p<0.05$). Gender distribution was similar between the groups ($p>0.05$). The direct microscopy fungus positivity rate did not significantly differ between the groups ($p>0.05$). A significant ($p<0.05$) lower rate of microorganisms was detected in tissue cultures obtained from the nasal polyp group. The fungal culture positivity rate did not significantly differ between the groups ($p>0.05$). PCR techniques were unable to identify fungal species in any of the positive fungal cultures, and by sequencing, fungal species emerged at similar rates in both groups (Table 1). In summary, parallel results were obtained using microscopy, fungal culture, and sequencing.

Discussion

CRS is defined in the European Position Paper on Rhinosinusitis and NP 2012 guidelines as inflammation of the nasal cavity lasting more than 12 weeks with at least two symptoms of nasal congestion, rhinorrhea, facial pain, and hyposmia (6). The phenotype is determined by nasal endoscopy with (CRSwNP) or without polyps (CRSsNP) and the diagnosis is confirmed by paranasal sinus CT. NP are expected to be seen bilaterally in tomography, and when unilateral polyps are seen in adults, care should be taken in terms of malignancy (4).

The pathophysiological process progressing from chronic sinonasal inflammation to nasal polyposis is still not fully defined. It is thought that problems in the sinonasal epithelial barrier may result in prolonged exposure to inhaled pathogens or antigens, thereby increasing chronic inflammation. In CRSwNP, the resistance of sinonasal epithelium is weak and the barrier is defective, but the reason for this is still unclear (7).

It is well known that the upper respiratory tract or paranasal sinuses of patients with NP are often chronically colonized with fungi and bacteria (8). In 2009, the International Society of Human and Animal Mycology classified fungal rhinosinusitis as invasive and non-invasive subtypes, and it was emphasized that invasive types occur in immunosuppressed patients (9). In immunocompetent individuals, non-invasive fungal rhinosinusitis phenotypes present as local fungal colonization, fungus ball, and allergic fungal rhinosinusitis (AFRS). Patients with CRSwNP have predominantly eosinophilic mucin without the fungal invasion. As defined by the Bent-Kuhn criteria, patients with AFRS have a much more intense nasal discharge than patients with NP (10). In addition, Lund-Mackay scores calculated from paranasal sinus CT examination are higher and sinus opacification is more common in patients with AFRS (11).

In 1999, Ponikau et al. (12) in their article investigating the incidence of AFRS found 96% (202 of 210) positivity in fungal culture made from nasal secretions in patients with CRS. With these data presented, they concluded that almost all patients with CRS with or without polyps had positive AFRS diagnostic criteria. In a similar study, Lebowitz et al. (13) isolated fungi in 56% of the samples taken from patients who had undergone endoscopic sinus surgery for CRS. However, negative clinical experiences recently have called into question the role of fungi in the etiology of CRSwNP. In our study, although fungal culture positivity was more common in patients with NP than in those without CRS, no significant difference was found.

PCR-based technologies including sequencing were introduced in the mid-1990s and are considered more effective than conventional methods in detecting fungi (14). While investigating the presence of fungus in the sinus mucosa, Rao et al. (15) in a study that compared PCR with conventional methods detected fungal DNA in 6.5% of patients in whom fungus could not be detected by conventional methods. In our study, no fungal species could be detected in positive fungal cultures and the control group using PCR techniques. This may be due to the absence of fungal DNA in the control samples or the presence of fungal DNA in our sample below the DNA detection limit of the PCR kit.

Today, sequencing is a frequently used alternative for the detection and identification of fungi (16). The sequencing kit used in this study targeted 28S rRNA. Targeting the 18s ribosomal RNA gene in sequencing is not specific to fungi and may also indicate the eukaryotic contamination. Therefore, the internally replicated spacer region, which is more specific to fungi, is used as an alternative target, and this method is the most likely to identify fungi (17). Zhao et al. (17) in their study of 64 patients with CRS concluded that this method cannot be a universal determinant of sinus disease pathogenesis in all CRS patients.

Table 1. Statistical comparison of the groups by each technique individually

Mean ± SD/(n, %)		Control group	Nasal polyp group	p	
		Mean ± SD/(n, %)			
Age		31.55±9.145	43.63±15.345	0.004	t
Gender	Female	4 (20.0%)	5 (20.8%)	0.946	x^2
	Male	16 (80.0%)	19 (79.2%)		
Microscopy					
Negative		19 (95.0%)	21 (87.5%)	0.614	x^2
Positive		1 (5.0%)	3 (12.5%)		
Gram-positive cocci		1 (5.0%)	1 (4.2%)		
Fungal hyphae		0 (0.0%)	2 (8.3%)		
Tissue culture					
Negative		2 (10.0%)	13 (54.2%)	**0.002**	x^2
Positive		18 (90.0%)	11 (45.8%)		
Aspergillus spp.		0 (0.0%)	1 (4.2%)		
Citrobacter koserii		1 (5.0%)	0 (0.0%)		
Diphtheroid bacilli		4 (20.0%)	0 (0.0%)		
Escherichia coli		0 (0.0%)	1 (4.2%)		
Klebsiella pneumoniae		0 (0.0%)	1 (4.2%)		
CoNS		6 (30.0%)	2 (8.3%)		
Leuconostoc mesenteroides		1 (5.0%)	0 (0.0%)		
Alpha hemolytic streptococci		0(0.0%)	1 (4.2%)		
Penicillium spp.		1 (5.0%)	2 (8.3%)		
Staphylococcus aureus		1 (5.0%)	0 (0.0%)		
Staphylococcus aureus MS		1 (5.0%)	2 (8.3%)		
Staphylococcus epidermidis		1 (5.0%)	0 (0.0%)		
Staphylococcus hominis MR		0 (0.0%)	1 (4.2%)		
Staphylococcus hominis MS		1 (5.0%)	0 (0.0%)		
Staphylococcus lugdunensis		1 (5.0%)	0 (0.0%)		
Fungal culture					
Negative		19 (95.0%)	21 (87.5%)	0.614	x^2
Positive		1 (5.0%)	3 (12.5%)		
Aspergillus spp.		0 (0.0%)	1 (4.2%)		
Penicillium spp.		1 (5.0%)	2 (8.3%)		
PCR	(-)	20 (100.0%)	24 (100.0%)	1.000	x^2
	(+)	0 (0.0%)	0 (0.0%)		
Sequencing					
Negative		19 (95.0%)	21 (87.5%)	0.614	x^2
Positive		1 (5.0%)	3 (12.5%)		
Aspergillus sydovii		0 (0.0%)	1 (4.2%)		
Penicillium griseoroseum		1 (5.0%)	2 (8.3%)		

t: T-test, x^2: Chi-square test, CoNS: Coagulase-negative staphylococci, MS: Methicillin-sensitive, MR: Methicillin-resistant

In a study published by Aydil et al. (18) in 2007, they evaluated microscopy and PCR as more sensitive than fungal culture. Unlike this study, we obtained similar results in microscopy, fungal culture, and sequencing. In a 2012 study by Montone et al. (19) on 400 patients with fungal rhinosinusitis, Aspergillus sp. was the most frequently isolated fungus in culture. In a similar study by Eyigor et al. (20), sequence analysis showed that the amplicons were homologous to Cladosporium herbarum and Aspergillus amstelodami. We also observed a only Aspergillus and Penicillium species in our study.

Study Limitations

The main limitation of the study is that it was conducted with a limited sample. Additionally, the long-term postoperative follow-up of patients may help evaluate the relationship between fungal colonization and nasal polyp recurrence.

Conclusion

The importance of fungal etiology in chronic sinusitis patients with NP is still a controversial issue. Microorganism rates isolated in fungal cultures are at similar levels in patients with nasal polyp rhinosinusitis and healthy individuals. This suggests that fungal dysbiosis may not be the only pathogenetic determinant of sinus inflammatory disease. In the findings we obtained in our study, we did not observe the superiority of the PCR-based sequencing technique to fungal culture. However, large case series are still needed.

Ethics Committee Approval: The study was approved at the University of Health Sciences Turkey, İstanbul Training and Research Hospital Institutional Review Board (approval number: 64, date: 11.02.2022).

Informed Consent: An informed consent form was obtained from all patients.

Peer-review: Externally peer-reviewed.

Authorship Contributions: Surgical and Medical Practices - O.Ö., A.K., T.K., M.A.K., E.Y., Ö.D.; Concept - O.Ö., A.K., T.K., M.A.K., E.Y., Ö.D., Ö.Y.; Design - O.Ö., A.K., T.K., M.A.K., E.Y., Ö.D., Ö.Y.; Data Collection or Processing - O.Ö., A.K., T.K., M.A.K., Ö.D.; Analysis or Interpretation - O.Ö., A.K., T.K., M.A.K., E.Y., Ö.D., Ö.Y.; Literature Search - O.Ö., A.K., T.K., M.A.K., E.Y., Ö.D.; Writing - O.Ö., A.K., T.K.

Conflict of Interest: No conflicts of interest was declared by the authors.

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