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Characterization of fungi in chronic rhinosinusitis using polymerase chain reaction and sequencing

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Abstract The role of fungi in chronic rhinosinusitis (CRS) remains unknown. Fungi were also determined as one of the responsible agents in the etio-pathogenesis, while several studies found fungi in 6–93% of the cases. The aim of this study is to test the presence of fungi in samples taken from the middle meatus of patients with CRS, using traditional culture methods and polymerase chain reaction (PCR), and to compare the efficacy of these methods. Thirty patients diagnosed with CRS, with or without nasal polyposis, undergoing an operation in the Otorhinolaryngology Clinic, were prospectively included in the study. Nasal mucosa samples from ten patients, who were operated for pathologic evaluation, and without CRS, were used as controls. Nasal samples were taken from each patient by swabbing with a cytology brush. Middle meatus culture samples were taken by using nasal cotton swab, and the polyp and/or sinus mucosa samples were taken during endoscopic sinus surgery. Fungal specific PCR, using 18S rRNA primers and standard cultures, were performed on every sample. All amplicons were sequenced. There was no fungal growth in the Sabouraud dextrose agar (SDA) medium from middle meatus samples and tissue parts. Of 30 tissue and brush samples, 3 and 2 were positive for fungal DNA, respectively. Sequence analysis showed that four amplicons were homologus to *Cladosporium herbarum* and one to *Aspergillus amstelodami*. We concluded that fungal etiology is overestimated and fungi rarely play a role in patients with CRS. Large-scale studies should be done using molecular methods.

Keywords Polymerase chain reaction · Sequence analysis · Chronic rhinosinusitis · Fungi

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Introduction

Chronic rhinosinusitis (CRS) is a common, long-term illness, characterized by chronic inflammation of the nasal and paranasal sinus mucosa and is associated with mucosal alterations ranging from inflammatory thickening to gross nasal polyp formation. The role of fungi in CRS remains unknown. The recognition and diagnosis of fungal rhinosinusitis has increased dramatically over the last 2 decades [14]. Fungi were also determined as one of the responsible agents in the etio-pathogenesis, while several studies found fungi in 6 and 93% of the cases [1, 14]. Ponikau et al. [14] have determined positive results in 96% of fungal cultures of nasal secretions in patients with or without polyp. They have also determined positive results in 100% of fungal cultures of healthy individuals. Braun et al. [2] have detected growth formation in 91.3% of fungal cultures in sinus secretions of healthy individuals and patients diagnosed as CRS. Based on their study, they suggest that nearly everyone has fungi in their noses. It has not been clarified yet whether fungi are found as a pathogen in nasal mucosa or they are just a part of the normal flora.

The different incidences of fungal pathogens may contribute to the difficulties in the isolation of fungi and the

limited number of methods used. In the traditional fungal culture methods, a period of 4 or 6 weeks time is required for evaluation and it is thought that sensitivity of cotton swabs is low [3]. The gold standard for diagnosis of fungal sinusitis is histopathologic evaluation and culture of nasal biopsy [12]. Another problem is the site and method of sampling for cultures. While some authors sampled nasopharyngeal mucosa using irrigation fluid through nasal passage, others have obtained samples from inferior turbinate and nasal septum using a brush or a swab [2, 3, 14]. Gillespie et al. [5] recommended middle turbinate biopsy as a safe and effective method for timely diagnosis of fungal sinusitis. Endoscopically guided middle meatal bacterial cultures correlate with cultures from maxillary sinus aspiration in 85–93% of samples [6]. Rao et al. [15] recommend middle meatus mucosal samples for diagnosis of fungal sinusitis, because they believe that the mucosa of the middle meatus represents paranasal sinuses more than the nasal cavity.

Polymerase chain reaction (PCR) is the most sensitive evaluation method of our age, being capable of detecting DNA from ten or lesser fungi. PCR technique is shown to be useful for the culture-independent diagnosis of various microbial infections, including mycoses [9, 15]. PCR has begun to be widely used in research of the pathogens that cause rhinosinusitis and/or nasal polyp, and in those illnesses whose etiology has still been controversial [3, 17].

The aim of this study is to test the presence of fungi in samples taken from middle meatus of patients with CRS, using traditional culture methods and PCR, and to compare the efficacy of these methods.

Patients and methods

Informed consent of the patients was taken for all interventions. Thirty patients, diagnosed as CRS with or without nasal polyposis, undergoing an operation in the Otorhinolaryngology Clinic, were prospectively included in the study. Patients treated with systemic antifungal agents, cystic fibrosis patients, and lastly patients suspected for malignancy, were excluded from the study. Before the operation, samples were taken from each patient by swabbing with a cytology brush. To get brush biopsy samples, brushes sterilized with ethylene oxide were introduced into middle meatus by an endoscope and were turned in the middle meatus at least ten times. With the same method, middle meatus culture sample was taken by using nasal cotton swab, and cultured in the Sabouraud dextrose agar (SDA). The clinicians have paid attention to avoid any anesthetic exposure to the nasal mucosa before the brush biopsy and culture samples were taken. Sinus mucosa samples, taken during endoscopic sinus surgery, were immediately sent to a mycology laboratory in a sterilized container. Nasal mucosa samples from ten patients who were operated for pathologic evaluation and without CRS were used as controls.

Fungal cultures

Middle meatus culture samples and tissue parts taken from the patients were cultured in two tubes containing SDA. One of the tubes was incubated at 37°C for a week, while the other was incubated at 26°C for 3 weeks.

DNA isolation

DNA extraction was done with NucleoSpin Tissue (Macherey-Nagel, Germany) extraction kit from tissue biopsies. First of all, 25 mg of tissue samples were cut with a sterile lancet and placed into eppendorf tubes. An extraction was made according to the kit procedure. Brush biopsy tips were cut with sterile scalpels and placed into a sterile eppendorf tube for DNA isolation. After 180 µl of TE (10 mM Tris-Cl, 1 mM EDTA pH 8.0) buffer samples were added, they were vortexed for 5 min. Then, DNA was extracted from samples using NucleoSpin Tissue kit as recommended by the manufacturer; DNA extracted from *Candida albicans* ATCC 90028 and from a clinical *Aspergillus flavus* strain were used as positive control.

PCR

First of all, in order to show that adequate DNA was extracted from all samples, PCR was applied by using GJB2 and GJB2b primers (Table 1). With these primers, gab junction beta 2 gene, found in human cells, was amplified. The master mix was prepared for PCR, the final volume being 45 µl. 10× Taq buffer with KCL (Fermentas, Canada) was 5 µl $MgCl_2$ 4 µl (25 mM), dNTP (10 mM) mix 1 µl, 1 µl from GJB2 and GJB2b primers (10 pM), and Taq polymerase 1.5 U (Fermentas, Canada). Lastly, 5 µl of extracted DNA was added. Eppendorf tubes are placed into the thermal cycler (Eppendorf Mastercycler Gradient, Germany). After the first denaturization at 94°C for 10 min, 35 cycles were kept at 94°C for 30 s, at 50°C for 30 s, and at 72°C for 30 s. Finally, it was kept waiting at 72°C for 7 min and passed into +4°C. The amplicons were colored in ethidium bromide, after being separated in 2% agar gel (Sigma, Germany), by electrophoresis for an hour at 80 V. The bands were visualized using gel projection system (Vilber-Lourmant, France).

Presence of fungal DNA was tested using FF2 and FR1 primers (Table 1) specific for amplification of 18S rRNA gene. A mixture was prepared, its final volume being 19 µl for PCR. Taq buffer 2 µl (10×), $MgCI_2$ 1 µl (25 mM), dNTP

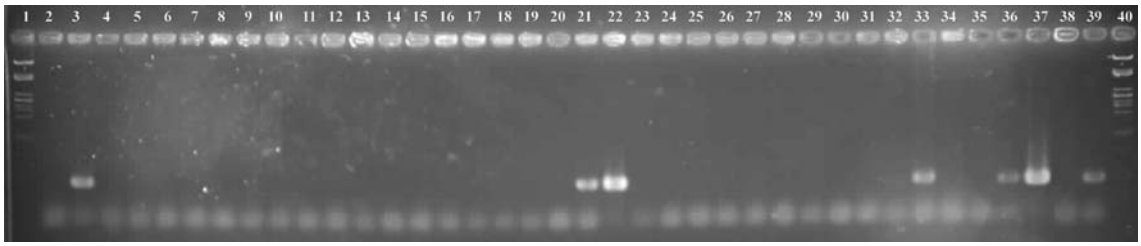

Fig. 1 Amplification of fungal DNA using FF2 and FR1 primers. *Lines 1* and *40* Lambda PstI marker, *line 2* negative control, *line 3 Aspergillus flavus* positive control (425 bp), *line 39 Candida albicans* ATCC 90028 positive control, *line 21*, *22*, *33*, *36*, *37* samples from fungal-positive patient. *Other lines* sample from fungal-negative patient

Table 1 Primers used in this study

Primer	5′ → 3′	References
GJB2 (CX26a24)	TCT TTT CCA GAG CAA ACC GCC	16
GJB2 (CX26b589)	GCC TTC GAT GCG GAC CTT C	16
FF2	GGT TCT ATT TTG TTG GTT TCT A	15
FR1	CTC TCA ATC TGT CAA TCC TTA TT	15

(10 mM) mix 0.5 µl, 1 µl each from FF2 and FR1 primers (10 pmol), and Taq polymerase 2.5 U. Finally, 1 µl of extracted DNA was added. The eppendorf tubes were placed into the thermal cycler. After the first denaturization at 94°C for 2 min, 50 cycles were applied at 94°C for 15 s, at 55°C for 20 s, and at 72°C for 30 s. The products, which have been amplified, are colored with ethidium bromide, after being separated in a gel, containing 2% agar (Sigma, Germany), by electrophoresis for 1 h at 80 V.

Sequence analysis

Amplicons were purified by PCRCleanUp Kit (GeneMark, Taiwan) and were sequenced in both directions with FF2 and FR1 primers using CEQ 8000 genetic analysis system (Becman Coulter, CA, USA) as recommended by the manufacturer. Sequence homologies with Gene Bank were studied using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

The ages of the patients in the study ranged between 18 and 73 (The average was 43.28 ± 12.94.). The study group was made up of 25 (62.5%) male and 15 female (37.5%). Totally, 33.3% of patients had previously defined history of allergy. Four patients (13.3%) had asthma. Nine of 30 patients (30%) had endoscopic sinus surgery or polypectomy operations at least once. According to the preoperative endoscopic examination and CT, 26 (86.7%) patients had diffuse polyps and pansinusitis in the middle meatus.

There was no fungal growth in the SDA of the cultures of middle meatus samples and tissue parts in study and control patients. Success of the method used for DNA extraction from brush biopsy samples was tested by the amplification of a 565 base of fragment from GJB2 gene. GJB2 amplification was positive in all of 40 samples. Only two brush samples were positive for 18S rRNA amplification by PCR. Tissue sample from one of these patients confirmed the diagnosis and was positive by PCR but the other patient's tissue sample was negative (Fig. 1).

Ability of the method used for DNA extraction from mucosa was also tested using GJB2 primers and, as expected, PCR was positive for all samples. Fungal DNA was detected in three tissue samples (10%) by PCR using 18S rRNA primers (Table 1). The amplicons were sequenced using the same primers used for amplification. Sequence analysis showed that four amplicons were homolog to *Cladosporium herbarum* and one to *Aspergillus amstelodami*. One of the patients with *C. herbarum* also had allergic rhinitis and asthma. However, this was the patient's fifth operation due to nasal polyposis.

The sensitivity and specificity of brush biopsy were 33.3 and 96.3%, respectively, when tissue PCR was accepted as a gold standard method. None of the patients from control group was positive by tissue or brush PCR.

Discussion

The role of fungi in CRS remains unknown. Problems in diagnosing allergic fungal sinusitis (AFS) with precision stem from a poor understanding whether fungi are present as a pathogen or simply a part of normal flora at that time. Fungi are ubiquitous in nature. They are clearly found as normal flora in the oral cavity and presumably along the nasal mucosa. The nasal lavage fluid has been used to take samples in some of the studies to determine fungal etiology in nasal and paranasal sinus diseases [2, 14]. Studies performed by Ponikau et al. [14] and Braun et al. [2] showed a high level of positivity (>90%) for fungi in the nasal lavage

fluid. Catten et al. [3] had taken the samples for fungi isolation from the nasal septum and inferior turbinate by brush biopsy. They had detected 42% fungal DNA in the patients, and 40% in the control group, by PCR method. Lebowitz et al. [10] had isolated fungi in 56% of the patients with CRS by using standard isolation technique in specimens, such as polyp, sinus mucosa, and mucin, which were taken during the endoscopic sinus surgery. Gosepath et al. [7] demostrated fungal DNA in all 27 surgical specimens collected from patients with CRS using a universal primer and a second primer pair specific for *Alternaria*. Ten of 15 samples of healthy paranasal mucosa were also positive using their universal fungal primer. Rao et al. [15] studied the presence of fungi in sinus mucosa by conventional methods and PCR. Conventional culture methods yielded no growth with samples from patients and control group; however, fungal DNA was detected by PCR in 6.5% of the samples from patients and was negative among the control group [15]. In our study, we also studied presence of fungi by conventional methods as well as by PCR. No fungal growth was obtained among patients and control group. In addition to the previous studies, we have also tested the presence of fungi in samples obtained during operation and compared the results with samples obtained by brush biopsy. To show the accuracy of DNA extraction, samples were tested by GJB2 amplification. All samples were positive by PCR. In our study, brush and sinus tissue samples from two patients were positive (6.6%). None of the samples from control group was positive for both brush and tissue samples. The susceptibility of PCR with samples from brush biopsy was low when the tissue biopsy was considered (33.3%). Detection of fungal DNA by PCR was positive among 10% of patient samples when tissue samples were used. This is lower than the rates reported in the literature, but is congruent with the results obtained by Rao et al. This difference may be contributed by the humidity in the regions where these studies were performed. We believe that PCR analysis of tissue samples to study fungal etiology is better than samples obtained by brushing or other methods. More studies should be done with large number of patients to conclude the role of fungi in CRS.

Today, identification of species by molecular methods, particularly sequencing, is largely used. Determination of genetic sequence variations by sequencing is an alternative for detection and identification of fungi [13]. In our study, we identified the fungi by sequencing the amplified fragments from tissue and brush biopsies. The universal primer set used in this study was previously shown to amplify DNA sequences from six commonly found fungal species, including *Alternaria*, *Aspergillus*, *Candida*, and *Cladosporium* [4, 18]. Sequencing is more appropriate for identification than using species-specific gene amplification methods by PCR. In the present study, sequence analysis showed that the amplified fragments belonged to *Cladosporidium* and *Aspergillus*.

One of the most commonly found fungal organisms in AFS are *Aspergillus* spp., with detection rates varying from 13 to 27% [11]. *Cladosporium* spp. are causative agents of skin lesion, keratitis, onycomycosis, sinusitis, and pulmonary infection [8]. Park et al. [12] detected seven different fungal pathogens from endoscopic biopsy samples of 17 immunocompromised, pediatric invasive, fungal rhinosinusitis patients by a traditional culture method. While the most common fungus isolated was *Fusarium* spp., *Aspergillus* spp. was detected in two patients and *Cladosporium* spp. was detected in one patient [12].

Conclusion

We concluded that fungal etiology is overestimated and fungi rarely play a role in patients with CRS. Large-scale studies should be done using molecular methods. Universal PCR and sequencing may be an alternative to culturing for identification of fungi in diagnosis of allergic fungal sinusitis.

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