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Original Research

Fungal biofilms in sinonasal polyposis: The role of fungal agents is notable?☆

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ABSTRACT

Chronic rhinosinusitis (CRS) is known by the inflammation of the sinonasal mucosa. Limited data are available on the fungal biofilms in CRS. This study aimed to investigate the role of fungal agents in sinonasal polyposis, and also to identify the dominant fungal pathogens by PCR and mycological studies. Sixty sinonasal biopsy specimens were examined; 31 sinonasal polyposis (SNP) as the study group and 29 concha bullosa without any history of chronic rhinosinusitis as the control. The specimens underwent histopathological examination for bacterial and fungal biofilms; routine mycological methods and then PCR using the internal transcribed spacer (ITS) region. Four (12.9%) specimens of the SNP group had a positive KOH preparation for fungal elements. The positive culture for *Aspergillus flavus* was obtained in 5 (16%) cases and one control. The identified isolates included one *Cryptococcus magnus* and two *Penicillium chrysogenum* in the study group and one *P. chrysogenum* in the control group. The fungal agents didn't have a significant role in the etiopathology of the SNP. Moreover, the results of mycological and histopathological methods had not any agreement with the molecular technique in CRS.

1. Introduction

Chronic rhinosinusitis (CRS) is known by the inflammation of the sinonasal mucosa. It is a common disease in the field of otolaryngology with a prevalence rate of 16% [1]. Today, it is one of the main causes of medical visits and one of the significant reasons for starting antibiotic therapy; it can even affect the life quality of such patients [2,3]. The underlying mechanism causing sinonasal polyposis (SNP) is still unknown. However, several theories have been raised, including chronic infections, aspirin intolerance, aerodynamic changes in the motion of gas and air by the entrapment of pollutants, clutter of epithelial tissues and food or inhalation allergies [4]. Allergy, bacterial and fungal infections and environmental pollutants have been proposed as the primary triggers which stimulate the inflammation of lateral nasal walls resulting in the development of SNP [4]. Biofilms are a thin layer of microorganisms adhered to a living or non-living surface which have been extracted from a matrix of extracellular polymeric substances (EPS) with the microbial origin [5]. Limited data are available regarding the properties of fungal biofilms in CRS. Fungal agents act as antigens in the mucosa of different individuals, resulting in the recruitment of inflammatory cells, namely eosinophils, and the release of major basic protein (MBP) which finally causes mucosal damage and infection by migration of other inflammatory cells into that site [6]. Moreover, biofilms cannot be entirely cultured and isolated by the conventional microbiological protocols [7]. On the other hand, identifying the causal genes in SNP is essential, because it can improve the path towards better prevention and treatment of this disease. Such results will hopefully lead to early intervention protocols and therefore targeted treatment for such patients, hopefully reducing the rate of surgical failure or recurrence.

The present study aimed to investigate the role of fungal agents in sinonasal polyposis, and also to identify of dominant fungal pathogens

☆ AsianAOMS: Asian Association of Oral and Maxillofacial Surgeons; ASOMP: Asian Society of Oral and Maxillofacial Pathology; JSOP: Japanese Society of Oral Pathology; JSOMS: Japanese Society of Oral and Maxillofacial Surgeons; JSOM: Japanese Society of Oral Medicine; JAMI: Japanese Academy of Maxillofacial Implants.

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by PCR and mycological studies.

2. Materials and methods

In this descriptive-analytical study, 60 sinonasal specimens were obtained from the patients visiting the Otolaryngology clinics of Ghaem and Imam Reza Hospitals, Mashhad, Iran. Among the specimens, 31 cases of sinonasal polyposis were accounted as the study group and 29 cases with the diagnosis of concha hypertrophia or concha bullosa with no history of chronic rhinosinusitis, were regarded as the control group. They were matched with the study group regarding age (≥3yrs). The 31 patients in the study group were initially enrolled with the diagnosis of bilateral nasal polyposis based on clinical and endoscopic findings. They had no history of recent antibiotic therapy or respiratory and oral corticosteroids consumption.

The study protocol was fully approved by the ethics committee of Mashhad University of Medical Sciences (MUMS), and informed consent was obtained from each participant before the study entrance (Ethics committee code: IR.MUMS.REC.1392.154).

2.1. Preparing the sinonasal tissue specimens

The biopsy specimens (the tissue around the concha bullosa in the control group, and the polypoid tissue in study group) were obtained during surgery from each patient. One specimen was sent to the pathology laboratory and examined by Hematoxylin-Eosin (H&E) staining. Another one was examined by direct microscopic examination with 20% potassium hydroxide (KOH) and cultured on a plate containing Sabouraud dextrose agar (SDA; Merck) containing chloramphenicol. The plates were incubated for 3–7 days at 35 °C.

DNA purification was done by a GeneAll kit (South Korea). The obtained genome was amplified in the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using a pan-fungal primer.

In order to perform the PCR, the pairs of primers ITS1 [5′-TCCGT AGGTGAACCTGCGG -3′] and ITS4 [5′- TCCTCCGCTTATTGATATGC -3′] [1] were used with the following conditions: D.W: 6 µl, 12 µl 2× premix (Amplicon, Denmark): 1 µl of each primer, 5 µl template DNA, and 6 µl distilled water (total volume 25 µl). The PCR program was as follows: initial denaturation, 95 °C for 5'; thermo-cycle file contained 25 cycles of denaturation, 95 °C for 30"; annealing, 95 °C for 30"; extension, 95 °C for 30"; final extension, 72 °C for 5'; 4 °C Φ.

A part of the PCR product was analyzed by agarose gel electrophoresis (the molecular weight of the expected DNA, around 600 bp) and the rest were sequenced for final identification. Eventually, the sequencing results were analyzed and arranged by the SEGMENT software and their genomic sequence was BLAST in the Genebank.

The data were analyzed by the SPSS ver. 16 and the significance level was set at $P < 0.05$.

3. Results

Of the sinonasal specimens examined, 31 specimens were obtained from the study group, 74.2% males ($n = 23$) and 25.8% females ($n = 8$); 29 specimens were obtained from the control group, 69% males ($n = 20$) and 31% females ($n = 9$).

Based on the statistical analyses performed by Chi-square testing, regarding sexual homogeneity, no meaningful difference was observed between the two groups ($P = 0.65$).

The mean age of the sinonasal polyposis group was 42.7 ± 13.3 yrs (range: 19–76 yrs) whereas the mean age of the control group was 28.2 ± 12.7 yrs (range: 16–61 yrs). The independent *t*-test showed a significant difference between the two groups based on age ($P < 0.001$).

The peak prevalence was in the 35 to 54-year age group in the sinonasal polyposis group; while the 15 to 25-year age group had the highest prevalence among the controls.

Table 1
The prevalence of nasal symptoms in the two groups of study and control among the patients with sinonasal polyposis.

Symptoms	Sinonasal polyposis group No (%)	Control group No (%)	P-value (Using chi-square test)
Nasal dripping	23 (74.2%)	2 (6.9)	< 0.001
Post-nasal discharge	15 (48.4)	2 (6.9)	< 0.001
Loss of smell	29 (93.5)	15 (51.7)	< 0.001

Table 2
The results of clinical laboratory examinations in the two studied groups among the patients with sinonasal polyposis.

Laboratory techniques	Sinonasal polyposis group No (%)	Control group No [%]	P-value (Using chi-square test)
Direct examination with KOH	4 (12.9)	0 (0)	0.04
Culture	5 (16.1)	1 (3.4)	0.27
PCR	3 (9.7)	1 (3.4)	0.028
Histopathological examination:			
fungal biofilm	1 (3.2)	0 (0)	0.999
bacterial biofilm	7 (22.6)	1 (3.4)	0.001

KOH: Potassium hydroxide; PCR: Polymerase chain reaction.

Nasal congestion was a common symptom in all patients of both the study and control groups. The prevalence of other symptoms in the two groups is summarized in Table 1; all indicated a meaningful difference between the two groups ($P < 0.001$).

The results of direct examination with KOH, culture, histopathological examination and PCR are summarized in Table 2. Meaningful difference was not detected between the study and control groups for all the performed clinical laboratory examinations in regard to the positive cases ($P = 0.742$).

A. flavus was isolated in 5 (16%) and one specimen in the study and control group by culture, respectively. Moreover, fungal agents were detected in 4 (6.7%) cases by the PCR method; 3 (9.7%) in the SNP group and 1 (3.4%) in the control group. The identified isolates included one *Cryptococcus magnus* and two *Penicillium chrysogenum* in the study group and one *P. chrysogenum* in the control group. Examination of H&E-stained sections also showed bacterial biofilms in 7 (22.6%) cases and 1 (3.4%) control. However, the structure of the fungal biofilm along with degenerated hype was diagnosed in one case of the SNP group by this technique. But, the control group did not show any fungal biofilm (Table 2).

4. Discussion

In the present study 60 sinonasal specimens were examined; 31 SNP cases and 29 controls with the diagnosis of concha hypertrophia or concha bullosa. We aimed to evaluate fungal biofilms and their causative agents by a simple and rapid molecular method; mycological and histopathological examinations.

CRS is a multifactorial disease which is characterized by the inflammation of the sinonasal mucosa; it is resistant to the available medical and surgical treatments [8]. Several studies have been performed to determine the underlying factors causing this condition. They have concluded that a variety of factors, including the defense mechanisms of the host and environmental triggers such as microorganisms cause the disease manifestations. Such colonization may even require previous mucosal injury or occlusion of the nasal sinuses [26]. Among the environmental triggers, fungi have been mostly discussed [8]. Although, the hyperresponsiveness of the sinonasal mucosa to fungal agents can be one of the underlying factors affecting the

development of sinonasal polyposis [4], yet fungi can act as a mechanical stimuli preserving chronic sinusitis as a foreign body [9]. Although specific treatments which can target the biofilms are not yet available, it is of great importance to prove the presence of a biofilm as it is strongly associated with treatment failure and sustainable symptoms. Therefore, it seems that biofilms have a vital role in the pathogenesis of CRS with SNP. Nevertheless, development of molecular-based diagnostic techniques for biofilm-related infections has the main role in the better discovery and identification of microorganisms and their role in the pathogenesis of this disease [5]. Nevertheless, development of molecular-based diagnostic techniques for biofilm-related infections has the main role in the better discovery and identification of microorganisms and their role in the pathogenesis of this disease [10]. Most researchers believe that PCR studies have priority over both culture and direct examination in the discovery of fungal agents [8]. In the present study by using PCR, 4 (6.7%) cases of fungal agents were isolated and identified. This method showed a meaningful difference between the study and control groups. They consisted of one *C. magnus* and two *P. chrysogenum* in the study group and one *P. chrysogenu* in the control group. *Penicillium* species are one of the types of fungi which are found in the decomposition of soil or plants, and only a few cases of its infection have been reported among humans. They were of the healthy and weakened immunity individuals in both of the groups [11].

In the study by Hashemian et al. in Hamedan city of Iran, fungal agents were detected during surgery in 16 of the 62 CRS cases; 9 cases of *A. fumigates,* 3 *A. niger,* one *Penicillium* spp. and one *Cladosporium* spp. [12]. In the study by Twaruzek et al., fungal agents were detected in 67% of the 107 cases; the most common fungi included *P. crustosum, P. citrinum*, *A. niger*, *C. claosporioides* and *Fusarium verticillioides* [13].

In another study by Biedunkiewic et al. on 100 veterinary students, nine fungal species in 4 genera were isolated that the most dominant fungus was *P. chrysogenum.* They concluded that even healthy individuals could be carriers [14]. In the present study, in total 5 cases in the study group were smokers compared to none in the control group, indicating no meaningful difference (P = 0. 053).

Penicillium rarely causes disease in humans. Given the isolation and identification of *Penicillium* species in this study and other similar studies, the fungus may play an important role in the pathogenesis of such infections. Therefore, it can even cause contamination in sterile specimens and the culture plates in the laboratory [15]. *C. magnus* is one of the *Cryptococcus* species which is present in the air, soil and plant decomposition. Despite this remarkable progress, it rarely causes disease in humans [16]. It has been reported in the upper respiratory tract and mainly the nasal cavity of cats and dogs in the studies conducted by Malik et al. and Danesi et al. [17,18].

In the study by Ziauddin Khan et al. on 63 children with cancer, PCR was performed on the specimens taken from the nasal cavity. In 3 cases with lymphoblastic leukemia, yeasts were isolated in their culture. Until now, two cases of *C. magnus* and one case of *C. chernovii* have also been reported in other studies [16]. Similar to our study, the mentioned colonies did not cultivate in the SDA medium and were only diagnosed by PCR. In the study by Razmpa et al. among the 50 cases with SNP, 21 (42%) and 24 (34%) became positive for fungi in direct examination and culture in SDA medium, respectively. Aspergillus species, especially *A. flavus* were isolated from the clinical specimens of SNP in culture [4]. Their results were in accordance with our findings. Similarly, in some studies, there is an agreement on higher prevalence of *A. flavus* isolated from clinical specimens of patients as pathogenic agents or colonization in Iran [19–23]. Also, this may be due to the high spread of *Aspergillus* conidia in indoor and outdoor airborne that can influence the health [24].

To date, *Aspergillus* species have been reported as the most common fungi infecting the sinuses in Europe and Northern America [2]. A few cases of fungal invasion or contamination with *Penicillium*, *Candida*, *Mucor*, *Alternaria* and *Neoscytalidium* have also been reported [2,25].

Biofilm culture may deliver false negative results, and such results should not be interpreted as the absence of biofilm-related infections because the microorganisms in biofilms are alive but nearly half are not cultivable [5]. It seems that the quantity of fungal load and special species in the mucosa is more important [8]. It may also be due to incorrect sampling strategies and handling, or even in correctly and enough obtained specimens.

However, one of limitations which have to be point in this study was the small sample size that may lead to bias. Hence, the further studies on larger sample sizes are highly recommended to better clarify the role of fungal agents in the development of the chronic inflammatory process of SNP. Our findings showed that fungal agents did not have a significant role in the etiopathology of SNP. Moreover, the results of the PCR method were not consistent with other conventional mycological and histopathological methods. However, SNP should be evaluated in terms of probable fungal infections for the successful management.

Conflict of interest

The authors declare that they have no conflict of interest.

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