Correlation of Bacterial Biofilm Grade with Clinical Features in Chronic Rhinosinusitis

Kronik Rinosinüzitte Bakteriyel Biyofilm Evresi ile Klinik Özelliklerin Korelasyonu

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ABSTRACT

Objective: The aim of this study was to determine the presence of bacterial biofilms in chronic rhinosinusitis (CRS) without polyps using scanning electron microscope (SEM) and to investigate whether there was correlation between grade of biofilm formation and clinical features of patients.
Methods: This was a prospective observational study with two groups: the first group was composed of 20 CRS patients undergoing endoscopic sinus surgery (ESS) and the control group included 15 patients without CRS, undergoing septoplasty or septorhinoplasty surgery. Clinal data were recorded preoperatively; mucosal samples and culture materials were obtained intraoperatively. Specimens were investigated for detection of biofilms with SEM. A biofilm grading system from grade 0 to 4, according to biofilm prevalence on the surface, was proposed. Symptom score, allergy presence, previous ESS history, Lund-Mackay computed tomography (CT) score and culture results of patients were compared according to biofilm grade.
Results: Biofilm formation was found in 16/20 (80%) CRS patients, yet none in 15 controls. Among CRS group, number of patients without biofilms (n=4) was too low to compare CRS patients with and without biofilms with each other, statistically. However, higher biofilm grades (grade 3 and 4) seemed to correlate with previous ESS and culture positivity but not with preoperative symptom score, Lund-Mackay CT score or allergy.
Conclusion: A grading system for biofilms is essential and it should be established in order to perceive CRS pathophysiology and find new treatment targets.

Key Words: Biofilms, sinusitis

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ÖZET

Amaç: Bu çalışmanın amacı, polip olmayan kronik rinosinüzit (KRS) hastalarında bakteriyel biyofilm varlığını taramalı elektron mikroskopu (TEM) ile göstermek ve biyofilm evresi ile hastaların klinik özellikleri arasında korelasyon olup olmadığını araştırmaktı.
Yöntemler: Bu prospektif klinik çalışmada iki grup yer aldı: ilk grup endoskopik sinüs cerrahisi uygulanacak olan 20 KRS hastasından; kontrol grup ise KRS'si olmayan ve septoplasti ya da septorinoplasti cerrahisi uygulanacak olan 15 hastadan oluştu. Ameliyat öncesinde klinik bilgiler kaydedildi; cerrahi sırasında ise mukoza örnekleri ve kültür materyalleri elde edildi. Spesmenler biyofilm varlığı açısından TEM ile incelendi. Yüzeydeki biyofilm prevalansına göre, evre 0 ile 4 arasında biyofilm evrelemesi önerildi ve kullanıldı. Hastaların semptom skoru, alerji varlığı, önceki ESC öyküsü, bilgisayarlı tomografideki (BT) Lund-Mackay skoru ve kültür sonuçları ile biyofilm evresine göre karşılaştırıldı.
Bulgular: Biyofilm varlığı KRS hastalarının 16/20 (%80)'sında gözlenirken, kontrol gruptaki hiçbir hastada tespit edilmedi. KRS grubunda biyofilm varlığı mevcut olmayan hasta sayısının düşük olması (n=4) nedeniyle, bu grupta biyofilm olan ve olmayan hastaların birbiri ile istatistiksel karşılaştırması yapılmadı. Ancak yüksek biyofilm evresi (evre 3 ve 4) ile önceki ESC öyküsü ve kültür pozitifliği arasında bağlantı olabileceği, ameliyat öncesi semptom skoru, Lund-Mackay BT skoru ve alerji öyküsü ile ise korelasyon olmadığı izlenimine ulaşıldı.
Sonuç: KRS patofizyolojisini daha iyi anlamak ve yeni tedavi hedefleri belirlemek için bir biyofilm evreleme sisteminin oluşturulması gerekmektedir.

Anahtar Sözcükler: Biyofilm, sinüzit

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INTRODUCTION

Chronic rhinosinusitis (CRS) is a common health problem affecting 10-15% of European and US population. Despite high prevalence and socioeconomical burden, etiopathogenesis still remains unclear (1). Main pathogenetic hypotheses include fungal infections, Staphylococcal superantigens, inflammatory cytokine system abnormalities and bacterial and/or fungal biofilms (2).

A bacterial biofilm is a complex organisation of bacteria which are encased in a self produced extracellular polymeric material formed by "quorum sensing" (3). Irreversible attachment to inert or living surfaces, decreased requirement for oxygen and nutrients and the genes that are transcribed provide additional resistance to antibiotics and host immunity (4). Therefore, serving a protective mode, of all bacteria, 99% live in biofilm form. It is also estimated that 65% of all human infections involve biofilms (5).

Bacterial biofilms have been demonstrated in many chronic otolaryngologic infections (6). In the light of recent studies, bacterial biofilms are regarded as one of the essential etiological factors in CRS (7-8). Whereas, impact and contribution of biofilms to CRS pathophysiology still necessisates further investigations. Besides, biofilm formation and its relation with clinical features of CRS is not fully understood.

The aim of this study was to determine the presence of bacterial biofilms in CRS without polyps with scanning electron microscope (SEM) and to investigate whether there was correlation between grade of biofilm formation and clinical features of patients.

METHODS

Study design and patient selection

The patients who have admitted to Hacettepe University Otolaryngology Department between February 2006 and September 2007 and diagnosed to have CRS were enrolled in this prospective study. Endoscopic sinus surgery (ESS) was performed on the study group which consisted of 20 patients. Control group included 15 patients who had undergone septoplasty or septorhinoplasty surgery. CRS diagnosis was based on the criteria of "2003 Chronic Sinusitis Task Force" (9). Patients with cystic fibrosis, immunosuppressive conditions (diabetes mellitus, HIV positivity, transplantation, systemic steroid use) and unwilling to participate were excluded from the study. Moreover, history of topical steroid or antibiotic usage in the last 6 weeks was another exclusion criteria. The study was approved by the local ethics committee and informed consent was obtained from both patient groups.

Clinical data acquirement

Preoperative medical overview of patients included age, gender, existance of allergic rhinitis, previous sinus surgery, smoking and history of antibiotic or topical/systemic steroid use in the last 6 weeks.

A symptom score system assesing the most common complaints of CRS patients was used: need to blow nose, nasal discharge, postnasal dripping, facial pain/headache and disorders of smell. Patients in both groups were asked to state a score between 0 to 5 for each symptom and overall symptom score values were recorded.

Lund-Mackay CT scores were also detected preoperatively. Endoscopic examination was performed in order to support diagnosis of CRS and rule out presence of polyps in study group. Absence of CRS was documented in control group with history, endoscopic findings and paranasl CT, if already exists.

Sample collection

Mucosal samples were obtained from ostium of maxillary sinus, middle meatus, anterior and posterior ethmoid sinus of each patient with CRS during ESS. Sample size ranged between 5x5 mm to 10x10 mm. For the control group, tissue biopsy site was middle meatus or inferior turbinate. Mucosa specimens were immediately fixed in 2.5% gluteraldehyde and transported to laboratory for SEM investigation. Besides, materials were obtained by swabs for aerobic culture from the mentioned anatomic sites.

Tissue preperation and SEM

Mucosal samples were fixed in 2.5% gluteraldehyde for 24 hours. Then, treated in phosphate buffer (pH 7.4) and macroscopic traces were removed (e.g. mucus and clot). For dehydration, increasing concentrations of alcohol (25% to 100%) which are sensitive for solid biological materials was used. Afterwards, specimens were left to air-dry for 12 hours and mounted on metal stubs with double-sided adhesive type. Last step was coverage of the samples with 180-200 Å layer of gold in a BIO-RAD (Hercules, CA) sputter apparatus. The images were acquired by JOEL SEM ASID-7200 EX (Tokyo, Japan) and ZEISS EVO LS SEM (Oberkochen, Germany). During SEM examination, 5-80 kV voltage range and 50x to 6000x magnification range was used. The entire mucosal surface was scanned for detection of biofilms. Two blinded investigators conducted SEM study independently.

Biofilm grading

Biofilms were identified according to previous descriptions, as bacteria assebled in clusters and towers, embedded in polysaccharide matrix, 0.5-2 µm in diameter and attached to surfaces (10-11).

Biofilms were graded in order to evaluate whether there is correlation between biofilm abundance and severity of CRS. Grading has been made on basis of the maximum field seen with 75-150x magnification which equals to 12.25 mm² area. For instance, grade 1 was defined as 25% (3 mm²) surface area covered by biofilms. When different grades were observed at seperate samples of the same patient, the mean of all specimens was accepted as the grade of that certain case. Photographic images were obtained from distinguished areas with 250-6000x magnification.

Quantitative biofilm grading according to distribution throughout the investigated surface area is as follows: grade 0, no biofilm formation (Fig.1); grade 1, <25% of the surface area covered by biofilms (Fig. 2); grade 2, 26-50% of the field baring biofilms (Fig. 3); grade 3, 51-75% of the area (Fig. 4,5,6); and grade 4, 76-100% of the surface occupied by biofilm formation (Fig. 7,8) (Table 1).

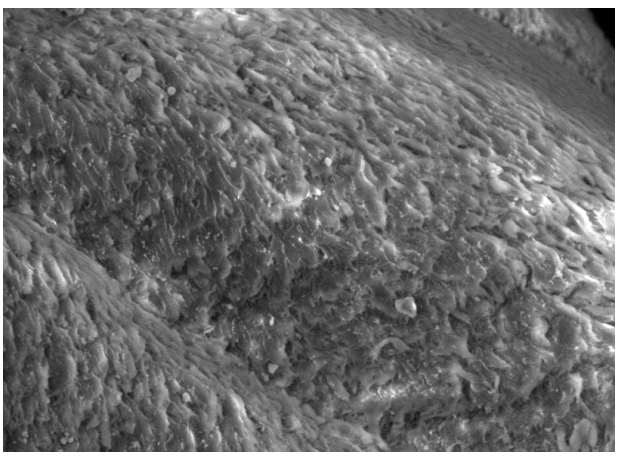

Figure 1. A sample of grade 0 from control group shows the healthy sinus mucosa (1000x).

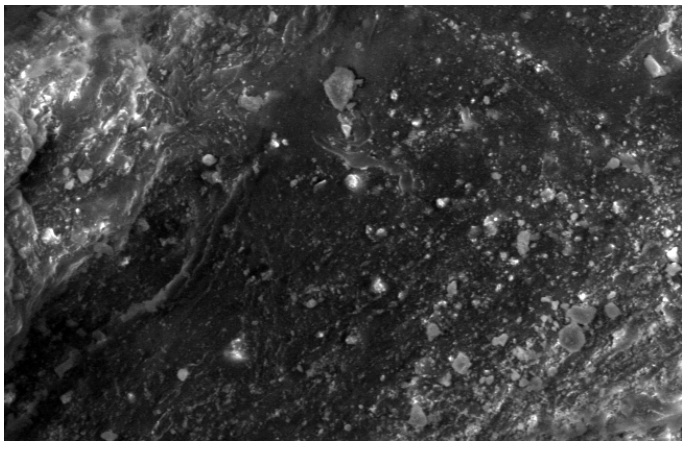

Figure 2. A sample of grade 1 shows sparse biofilm layers together with epithelial remnants (1000x).

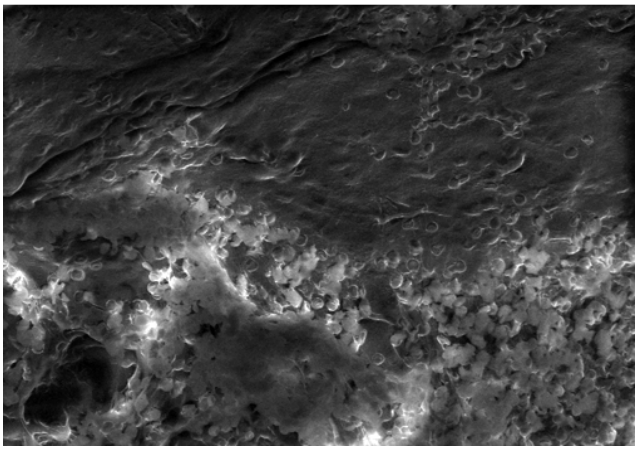

Figure 3. A sample of grade 2 demonstrates that biofilms are abundant compared to image of grade 1 specimen (1000x).

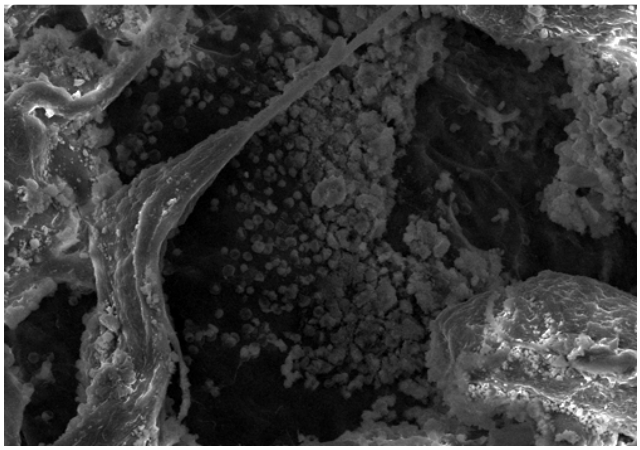

Figure 4. A sample of grade 3 in magnification 1000x.

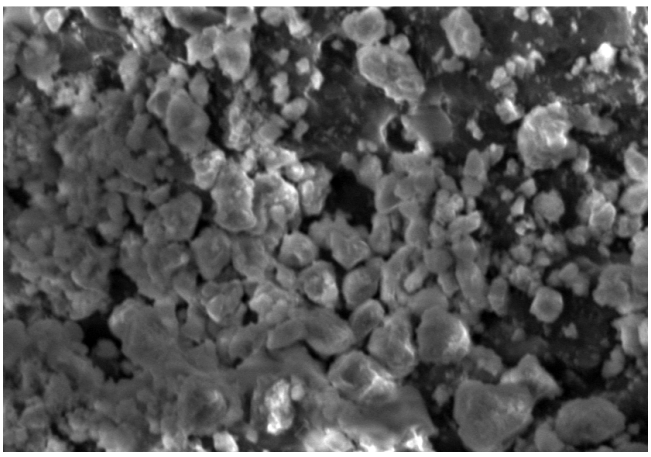

Figure 5. A **s**ample of grade 3 in magnification 2500x. Typical tower shaped morphology of biofilm formation with SEM is noticed.

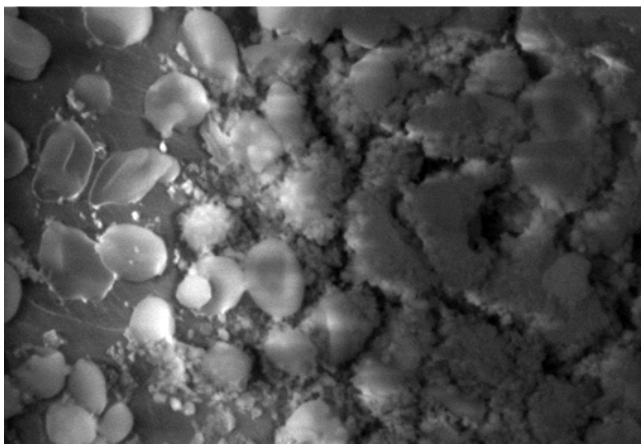

Figure 6. A sample of grade 3 in magnification 6000x. Note the 3-D biofilm structures and red blood cells in between.

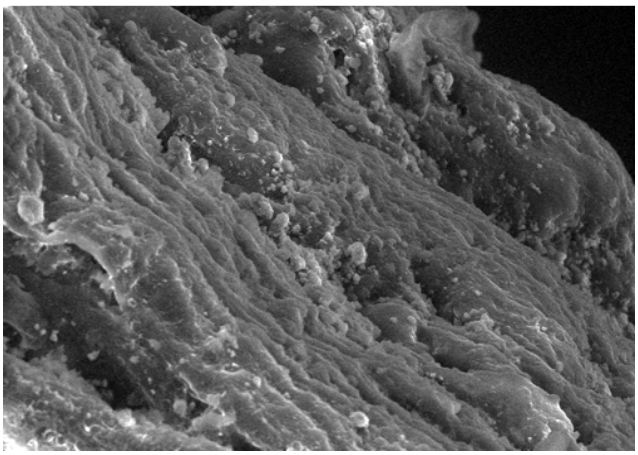

Figure 7. A sample of grade 4 with 1000x magnification. Whole mucosal surfaces are covered by biofilm layers.

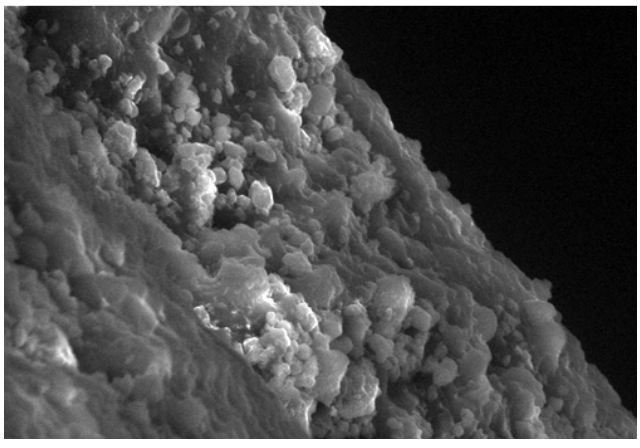

Figure 8. A sample of grade 4 with 3000x magnification. Whole mucosal surfaces are covered by biofilm layers.

Statistical tests were conducted using SPSS version 10.0 (SPSS, Inc, IBM Company, Chicago, Illinois). Continous data were analyzed using Student's *t*-test. In all analyses, p values <0.05 indicated statistical significance. In the study group, CRS patients lacking biofilms on their specimens were very few in number therefore statistical analyses were not reliable and descriptive statistical values were stated.

Table 1. Biofilm grading

Max. Surface area (75-150x) with SEM	Biofilm prevalence
Grade 0	None
Grade 1	<25%
Grade 2	26-50%
Grade 3	51-75%
Grade 4	>76%

RESULTS

Basic characteristics

The study group consisted of 20 patients with CRS, without polyps and the control group was 15 patients who underwent septoplasty or septorhinoplasty surgery. Presence of CRS was ruled out in the second group with aid of history, endoscopic examination and CT, if existed.

CRS group had 12 male, 8 female patients with a mean age of 38.4 (range, 18-67 years) and control group had 6 male and 9 female patients; mean age of 32.1 (range, 18-42 years). Mean symptom score of 20 CRS patients was 10.6 (range, 7-13); whereas mean symptom score of control group was found to be 5.0 (range, 3-8). In the study group, 7 patients (35%) had allergic rhinitis, 6 (30%) had previous sinus surgery history and 6 (30%) were smokers. On the hand, among 15 control patients, 5 (33.3%) had allergic rhinitis, none had previous sinus surgery and 5 (33.3%) stated habit of smoking. No significant difference was found in means of age, allergy, previos sinus surgery and smoking between two groups with and without CRS. However, symptom score was significantly higher in CRS patients compared to controls (Table 2).

Table 2. Basic characteristics of patients with and without CRS.

Characteristics	Patients with CRS (n=20)	Patients without CRS (n=15)	*p* value
Age (years)	38.40±11.50	32.07±7.17	0.70
Symptom score	10.6	5.0	<0.01
Allergic rhinitis	7/20	5/15	0.921
Previous ESS	6/20	0/15	0.190
Smoking	6/20	5/15	0.839

ESS: endoscopic sinus surgery, Lund-Mackay scores of CRS patients ranged between 9-17 with a mean of 12.65.

SEM findings and biofilm grades

Out of 20 CRS patients, biofilm formation was detected in 16 (80%). Two (12.5%) had grade 1, three (18.75%) had grade 2, six (37.5%) had grade 3 and five (31.25%) had grade 4 biofilms. Remaining four (20%) patients had grade 0 which means no biofilm formation. All 15 patients in control group were grade 0 in terms of biofilm occurance.

In CRS group, mean symptom score of 16 patients with biofilms was 10.69 (range, 7-15) and it was 10.25 (range, 9-11) for the rest 4 patients without biofilms (Table 3). Among 16 CRS patients with biofilms, 6 (37.5%) had allergic rhinitis and 4(25%) had previous sinus surgery. Within 4 CRS patients without biofilms, 1 (25%) had allergic rhinitis and 2 (50%) had previous sinus surgery (Table 3). Lund-Mackay scores of 16 CRS patients with biofilms ranged between 9-18 with a mean of 13.12 whereas it was found to be between 8-13 with a mean of 10.75 for the 4 CRS patients without biofilms (Table 3).

Materials obtained for culture yielded positivity in 14/20 (70%) patients. Among these 14 patients, 12 (85.8%) had biofilm formation whereas 2 (14.2%) were negative for biofilms (Table 3). Culture results revealed 4 different microorganism species: Staphylococcus aureus, Haemophylis influenza, Pseudomonas aeruginosa and Streptococcus pneumoniae.

When focused on CRS patients with biofilms, it was noticed that 9/16 (56.25%) had S. aureus, 2/16 (12.5%) had S. pneumoniae and 1/16 (6.25%) had H. influenza on culture. Culture results of CRS patients without biofilms revealed S. aureus in 1/4 (25%) patient and P. aeruginosa in another one (25%).

Table 3. CRS patient characteristics

Patient	Age	Gender	SS	Allergy	Previous ESS	LM	Culture	Biofilm grade
1	26	M	10	-	-	9	S. pneumoniae	4
2	42	M	7	-	+	11	S. aureus	4
3	67	F	11	-	-	10	S. aureus	-
4	18	F	12	-	-	13	S. aureus	2
5	34	F	11	+	-	15	-	2
6	40	M	12	+	-	17	S. aureus	4
7	34	M	13	-	-	10	-	3
8	49	M	15	+	-	14	S. aureus	4
9	42	M	8	-	+	14	-	4
10	41	M	10	+	+	12	P. aeruginosa	-
11	31	M	13	+	+	15	S. aureus	1
12	50	M	10	-	-	12	-	2
13	38	F	10	-	-	11	H. influenza	1
14	27	F	10	-	-	9	S. pneumoniae	3
15	36	F	13	-	+	18	S. aureus	3
16	47	M	8	+	-	13	S. aureus	3
17	41	M	9	-	-	12	S. aureus	3
18	32	F	10	+	-	17	S. aureus	3
19	52	M	11	-	+	13	-	-
20	21	F	9	-	-	8	-	-

SS – Symptom score, LM – Lund – Mackay score, ESS – Endoscopic sinus surgery.

Characteristics of CRS patients according to biofilm grades are summarized at Table 4.

Table 4. Characteristics of CRS patients according to biofilm grades

Biofilm grade (n=20)	Mean SS	Allergic rhinitis	Previous ESS	Mean LM	Culture positivity
Grade 0(n=4)	10.25	n=1	n=2	10.75	n=2
Grade 1(n=2)	11.50	n=1	n=1	13.0	n=2
Grade 2(n=3)	11.0	n=1	n=0	13.3	n=1
Grade 3(n=6)	10.50	n=2	n=1	13.2	n=5
Grade 4(n=5)	10.40	n=2	n=6	13.0	n=4

SS: Symptom score, LM:Lund – Mackay score, ESS:Endoscopic sinus surgery.

DISCUSSION

After the initial study of Cryer et al, which demonstrated the presence of bacterial biofilms on mucosa of CRS patients, many other investigations supported this finding (12-17). However, there is great incompatibility regarding the prevalence of biofilms in these researches. Biofilm detection rate ranges between 25-100%, mainly affected by the method of visualization (14,17-18). Other possible factors responsible from this wide range are inclusion criteria of CRS patients (e.g. recent use of antibiotics or steroids), number of samples and experience of the investigators on that particular imaging modality.

In this study, biofilm existance was found in 16 of 20 (80%) CRS patients with SEM. On the other hand, there was no biofilm formation in any of 15 controls. These findings are consistent with recent reports (8,19-20). Despite a few number of reports indicating presence of biofilms on healthy sinus mucosa, this evidence stil requires confirmation (21-22).

Currently, confocal scanning laser microscopy (CSLM) comes out in detection of biofilms on mucosa samples. Although, with CSLM, tissue preperation is easy and integrity of biofilms might be kept more successfully, SEM offers some advantages, too (23-24).

When tissue preperation techniques are properly applied, artifacts can be minimized and SEM reveals images of three dimensional (3-D) biofilm structures reliably (14,19,21,25-26). In this study, SEM was chosen as the image modality considering that it is a simple and rapid technique and particularly the authors are experienced with it. Nowadays, new screening methods such as BacLight/CSLM, FISH/CSLM or optical coherence tomography (OCT) are emerging for biofilm detection (7,27-29). Despite the advantages these techniques offer, their clinical utility is somehow limited due to high cost and tissue preperation complexity.

Correlation between biofilm formation and CRS severity and prognosis is the subject of recent researches. You et al. stated that, prognosis of CRS patients with biofilms is worse than those who do not have biofilm expression after ESS, in means of visual analogue scale (VAS) and Lund-Kennedy score (19). Furthermore, there are certain studies indicating more severe CRS in whom biofilms are present. Poorer radiological and endoscopic scores are reported in these publications for patients with biofilms (30-32). On the other hand, certain authors did not find any correlation between severity of inflammation in CRS and biofilms. Chen et al. showed that, biofilm formation occured in 54.2% of their study group; patients with and without biofilms had similar preoperative Lund-Mackay CT and Johansson endoscopic scores (26). Likewise, Hochstim et al. investigated biofilm prevalence with hematoxylin-eosin staining and FISH/CSLM in CRS patients and reported that, biofilm presence was strongly associated with persistent mucosal inflammation after ESS; however, was not related to prior ESS history or allergy (27). Also, Hai et al. proved that prevalence of bacterial biofilms can be reduced by ESS but this did not alter outcome measures of CRS patients (33). In vitro biofilm formation was investigated by Zhang et al. and they suggested that biofilm formation was not associated with polyps, allergy, Samter's triad, sleep apnea, smoking status, age or gender. The factors related to biofilm formation were prior sinus surgery, nasal steroid use and positive culture results in that cross-sectional study (34).

In our study, we also tried to figure out relationship of certain clinical features with biofilm formation in CRS patients. The additional aim of our investigation was to determine whether there was correlation between biofilm grades and clinical characteristics. It is obvious that a grading system is required in order to understand the effect of biofilm amount on outcomes of CRS patients. The number of publications attempting such grading scales is very limited. Hochstim et al. proposed a rough classification as "extensive" (>50% of mucosal surface in a sample) and "present" (<50% of surface) (27). Li et al. scored biofilm amount in 5 grades from 0 to 4 in increasing order and investigated correlation between grades and clinical features. It was stated that, biofilm scores were better correlated than biofilm existance with symptom score, endoscopy score and symptom duration.[28] In the current study, we also graded biofilm prevalence in 5 grades: grade 0 (no biofilm) to grade 4 (>76% of surface covered with biofilms). Due to limited number of patients without biofilms (4/20), ststistical analyses were not reliable thus descriptive statics were indicated. Although, statistically not proven, higher biofilm grades (grade 3 and 4) seemed to correlate with previous sinus surgery and culture positivity. This hypothesis should be supported with larger sample sized studies. However, there was an impression that, biofilm grades were not associated with symptom score, allergy and Lund-Mackay score. Similar findings of the mentioned studies warrant further investigation (26-27,33-34).

Tatar et al. used a similar biofilm grading scale to ours. In that study, they evaluated response of CRS patients to medical treatment and concluded that macrolides achieved regression of biofilms; however nasal steroids did not add to outcomes. They claimed that reduction of biofilm grades might be an indicator of response to treatment (25).

In our study, S. aureus was the most prevalent microorganism cultured which is a consistent finding with literature (34-36). If association of different microorganisms with severity of CRS is a concern, bacteria in biofilm structures should be identified thus CSLM may be more appropriate for that purpose. The major limitation of our study is small sample size which does not allow statistical analyses of CRS patients with and without biofilms. It should be noticed that the main reason is the inclusion criteria of CRS patients. One of the aims was to form the most possible homogenous group of CRS patients therefore patients who used antibiotics or nasal steroids in the last 6 weeks were excluded. This criteria was the most difficult to met in this particular disease. This current study still contributes literature by emphasizing the importance of creating a biofilm grading system.

If correlation of biofilm scores and CRS severity could be understood fully, it might lead to development of new treatment targets and strategies.

CONCLUSION

Biofilms were detected on sinus mucosa of 80% CRS patients with SEM. A biofilm grading system was proposed in order to determine correlation of biofilm prevalence and severity of CRS. Higher biofilm grades (grade 3 and 4) seemed to be related with previous sinus surgery and culture positivity; however not with preoperative symptom score, Lund-Mackay CT score or allergy. Further studies should be conducted on grading scales of biofilms to assess possible outcomes and new treatment options of CRS patients.

Conflict of Interest

No conflict of interest was declared by the authors.

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