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THE INVESTIGATION OF THE BIOFILM FORMATION IN CHILDREN WITH AND WITHOUT OTITIS MEDIA WITH EFFUSION UNDERGOING ADENOIDECTOMY

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ABSTRACT

Present study was carried out to investigate the biofilm formation on the adenoid surface of children undergoing adenoidectomy and to analyse whether this formation is associated with serous otitis media by electronmicroscopic findings. Fifty children with adenoid vegetation, of which half of them have 'otitis media with effusion (OME)'were studied, at Eskisehir Osmangazi University Medical Faculty and Eskisehir Yunus Emre State Hospital, between Sep 2011 and Feb 2012**.** Adenoid tissues were investigated by by using light, scanning (SEM) and transmission electron microscope (TEM). The biofilm formation was detected in all samples at varying degrees by SEM. Although the biofilm stucture was not observed directly by TEM, but inflammatory or degenerative changes were determined. Statistically, there was no significant difference between the two patient groups (Group 1: adenoid hyperthrophy+serous otitis media; Group 2: only adenoid hyperthrophy) for the presence of biofilm ($p>0,05$). By the TEM examinations, biofilm stucture was not observed directly. However, inflammatory or degenerative changes were determined.

Further investigations should be performed in order to determine that whether biofilm formation may be an important factor in the pathogenesis of OME. Since surgery was decided for the cases resistant to medical therapy, biofilm may detect in all samples. In fact, sampling the adenoids of the cases of OME that respond to the therapy may changed the

results of the study. By the TEM examinations, biofilm stucture was not observed directly. However, inflammatory or degenerative changes were determined.

Keywords: Adenoid, Otitis Media with Effusion, Biofilm and Electron Microscope

INTRODUCTION

Adenoidal hypertrophy and otitis media with effusion (OME) are the most frequent indications for surgery in children **[1, 2]**. Especially in children, they may cause severe symptoms and complications. In cases of adenoidal hypertrophy and its related symptoms, adenoidectomy has been the treatment of choice **[3]**. OME is also characterized by a nonpurulent effusion of the middle ear that may be either mucoid or serous. Hearing loss associated with OME can potentially have a detrimental effect on speech and language development **[4]**. The chronic nature of these infections makes also the situation more difficult with regard to diagnosis and management.

As known, biofilms are organized communities of adherent microorganisms that are encased in a complex extracellular polymeric substance matrix **[5]**. The most notable clinical features of cells within a biofilm are their high resistance to antimicrobial drugs and host defense **[6]**. They are formed on biotic surfaces and some may develop on living tissues **[7]**. Since the presence of biofilm may play a major role in microbial resistance, it may have a significant contribution to the otolaryngologic infections **[8]**. Despite this, there are insufficient data regarding the presence of biofilms on the surfaces of adenoid tissues, especially in children **[9, 10]**. Various imaging techniques have been employed in the investigation of biofilms **[11]**. Each method has advantages and disadvantages. Confocal laser scanning microscopy (CLSM) is the reference technique, because it provides both morphological and functional information with excellent spatial resolution, however it is very costly and is available at very few research center **[11]**. Scanning electron microscope (SEM) is more affordable technique and its main advantage is the ability to acquire images in high magnification, with structural microscopic details **[12]**. Transmission electron microscope (TEM) can also elucidate ultra structural details of biofilms, like the composition and the interaction of extracellular matrix with the surface and cells in the vicinity **[13]**.

The aim of our study is to investigate microbial biofilm formation on the adenoid surface of patients with and without OME undergoing adenoidectomy and to reveal whether biofilm formation is associated

with OME, by electron microscopic examinations.

MATERIALS AND METHODS

This study was approved by the ethics committee of the Faculty of Medicine, Eskisehir Osmangazi University. Tissue samples were obtained from 50 children who underwent adenoidectomy in the Eskisehir Osmangazi University Medical Faculty and Eskişehir Yunus Emre State Hospital during the period between September 2011 and February 2012. 25 cases in the first group had adenoidal hypertrophy and OME and 25 cases in the second group had only adenoidal hypertrophy. So, we planned to investigation of biofilm formation in adenoidal tissue between the first and second groups. In our study, adenoidectomies were performed with adenotoid curette, and the specimens were obtained from different parts of the nasopharyngeal surface of adenoid tissues with a knife. The biofilm studies of adenoid tissues was examined by using light, SEM and TEM, by the same researcher, at the Electron Microscope Laboratory, Eskisehir Osmangazi University.

Tissue Collection

Adenoid specimens were obtained during routine adenoidectomy from children (n=50). The specimens were cut in several parts to be respectively prepared for SEM and TEM.

Transmission Electron Microscopy (TEM)

Adenoid tissues were prepared by fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 24 hours at 4˚C and then rinsed with phosphate buffer. Specimens postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 2 hours at room temperature. All specimens were then dehydrated in graded solutions of ethyl alcohol (30%, 50%, 70%, 90%, 96% and 100%) and embedded in epon-araldite resin. Several semithin sections (700 nm) were collected at a variety of depths of the sample and stained with toluidine blue. The sections were examined by light microscope (Olympus BX50) to select appropriate areas for ultrastructural analysis. Later, ultrathin sections (60 nm) were taken from regions containing and suspected biofilms on an ultramicrotome (Leica Ultracut R) and counterstained with uranyl acetate-lead citrate. They were examined and photographed using a TEM (JEOL JEM 1220) with digital imaging capabilities **[14]**.

Scanning Electron Microscopy

The adenoid samples was immediately placed in 2.5% glutaraldehyde (prepared in 0.1M phosphate buffer, pH 7.4) for 24 hours at 4 ºC as a prefixation step. It was then rinsed twice with 0.1M phosphate buffer (pH 7.4), postfixed using 1% osmium

tetroxide for 1 hour at room temperature and finally rinsed with distilled water. Following that, the specimen was dehydrated using graduated concentrations of ethyl alcohol (30%, 50%, 70%, 90%, and 96%) for 15 minutes each followed by absolute alcohol for 30 minutes. After that, the specimen was dried using the critical point dryer (Polaron CPD Critical Point Dryer). For mounting, carbon conductive paint was used; for specimens, gold coating with Polaron SC7620 Sputter Coater. Finally, each specimen was examined using a JEOL scanning electron microscope (JEOL JSM-5600LV) **[9, 15]**.

Statistical Analysis of Data

The data from experiments were analysed using the linear-by-linear association method.

RESULTS

There were 27 male and 23 female patients in the present study; their ages ranged from 3 to 13 years. The mean age of the groups were 5.600±2,309 in the first group (adenoidal hypertophy and serous otitis media) while 7,320±2,868 in the second group (only adenoidal hypertrophy). In the first group, 18 male (72,0%) and 7 female (28,0%) patients were taken to study; in the second group, 9 male (% 36,0) and 16 female (% 64,0) patients were taken.

In our study, we determined the biofilms as the areas where multilayered remnants of tissue and microorganisms exist by SEM. With respect to the average biofilm extension, grade 1, grade 2, grade 3, and grade 4 biofilm formations were determined when less than 25%, 25% to 49%, 50% through 75% and more than 75% of sample surfaces were involved, respectively **[16]**. Generally, the biofilm formation was detected in all samples in the range of grades 1 to 4. **Figure 1** shows an adenoid sample in the range of biofilm grade 4 at different magnifications. Sometimes, small clusters of bacteria colonies were also seen in adenoid tissue.

However, as seen in **Table 1**, there was no statistically significant difference between the two groups we studied **(Table 1)** (p>0, 05).

According to our SEM micrographs, the distribution of bacterial microcolonies was not homogenous throughout of the tissue surface. In some areas, extracellular material was seen connecting the bacteria and large of them were seen either in the vicinity of the crypts or on the outer surface of adenoids. Therefore cilia disorganization on the surface of adenoid tissue was observed **(Figure 2 a-b and Figure 3)**.

By the TEM examinations, biofilm structure was not observed directly. However, inflammatory or degenerative

changes were determined **(Figure 4 a-e)**. While intracytoplasmic odeme has been

seen in some of the cells, necrotic changes have also been encountered. Generally, it was observed that cell membranes have disappeared and in some regions of nucleus, there have been nucleus membrane degenerations. In addition, apparent organel degeneration, lenfocyte accumulation and dense vacuolisation were seen. However, it was not possible to determine the organels between the vacuoles . It couldn't be determined clearly that whether biofilm structure has an additional effect on these degenerations which are observed because of inflammation.

Table 1: The Distribution of the Patients According to Biofilm Grades of Adenoid Tissues

		Group 1 [a]		Group 2 [b]		*p*
		n	%	n	%	
Biyofilm formation, samples, No (%)	Grade 1 (<25)	3	% 12,0	5	% 20,0	0.182
	Grade 2 (25-49)	2	% 8,0	6	% 24,0	
	Grade 3 (50-75)	15	% 60,0	10	% 40,0	
	Grade 4 (>75)	5	% 20,0	4	% 16,0	

[a]Group 1 had Adenoid Hypertophy and Serous Otitis Media, [b]Group2 had Only Adenoid Hypertophy

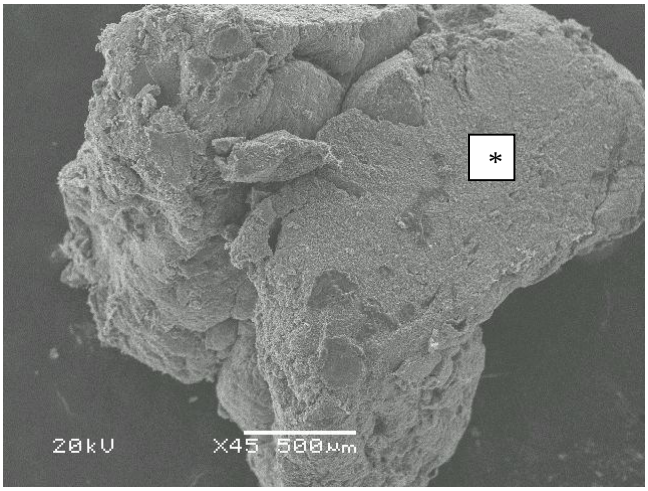

Figure 1(a)

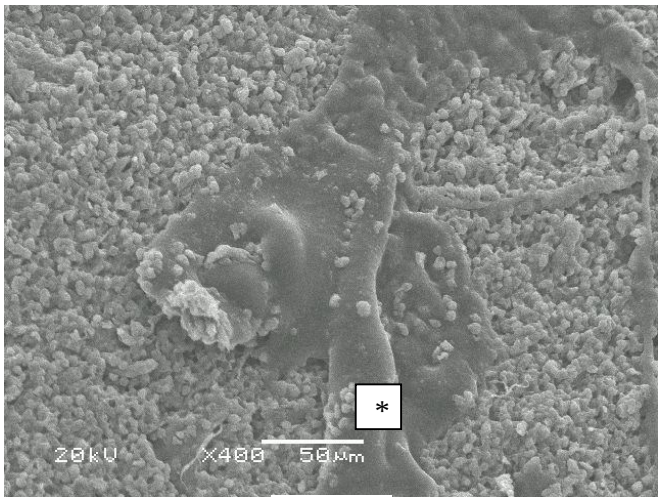

Figure 1(b)

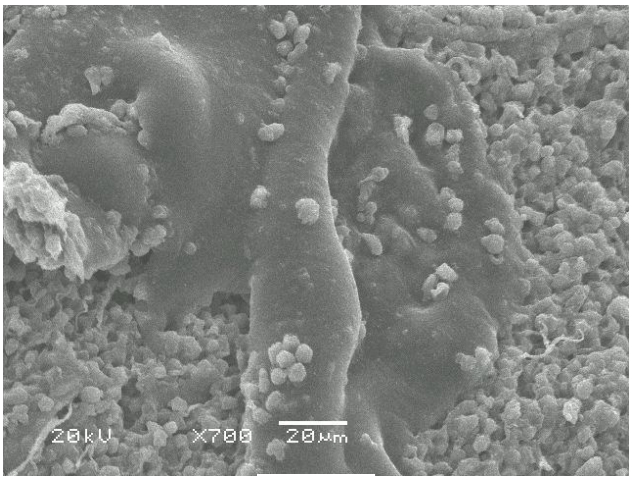

Figure 1(c)

Figure 1 (a, b, c): Biofilm Formation Graded as 4 can be Seen on These Images. Asteriks indicates biofilm Area on the Adenoid Surface

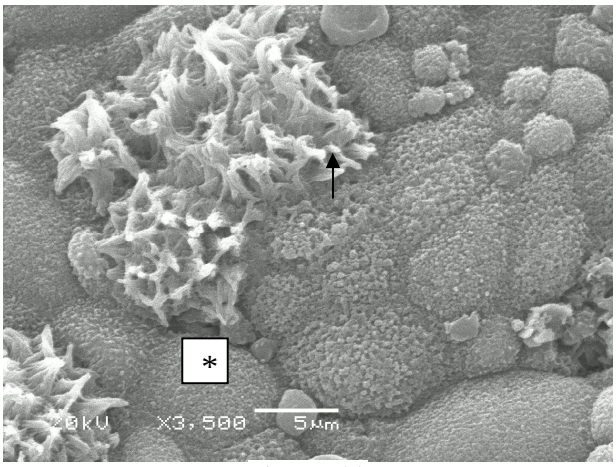

Figure 2(a)

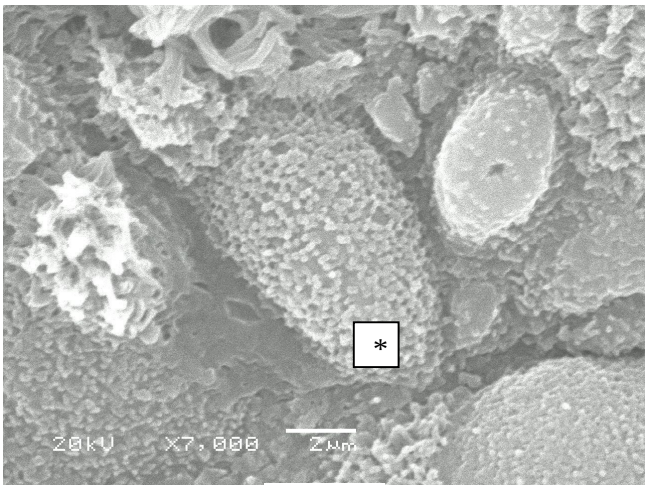

Figure 2(b)

Figure 2 (a, b): Scanning Electron Microscopy Images of an Infected Adenoid Covered With Biofilm (asteriks) and Disorganized Cilia (Arrow)

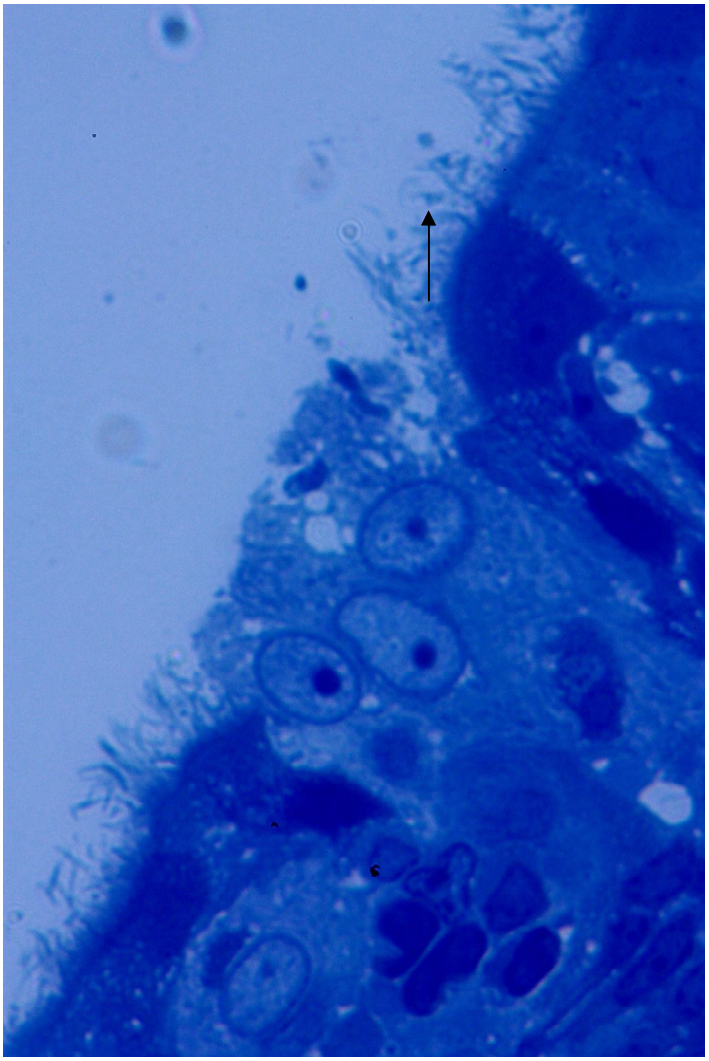

Figure 3: Semihtin Section Stained with Toluidine Blue of Adenoid Tissue Sample (Biofilm grade 4). Disorganization and Marked loss of Cilia on Surface

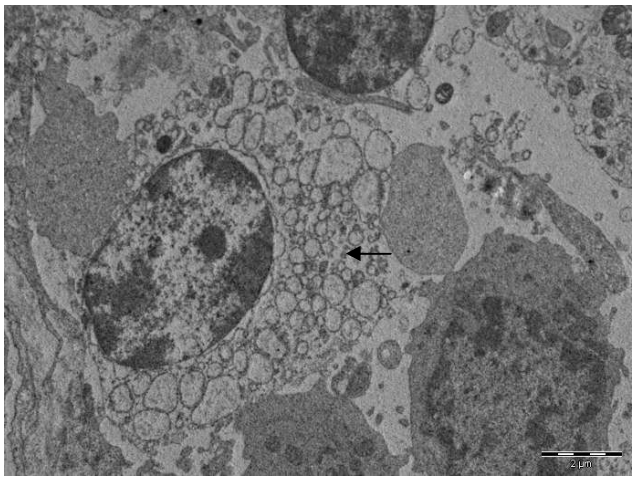

Figure 4 (a)

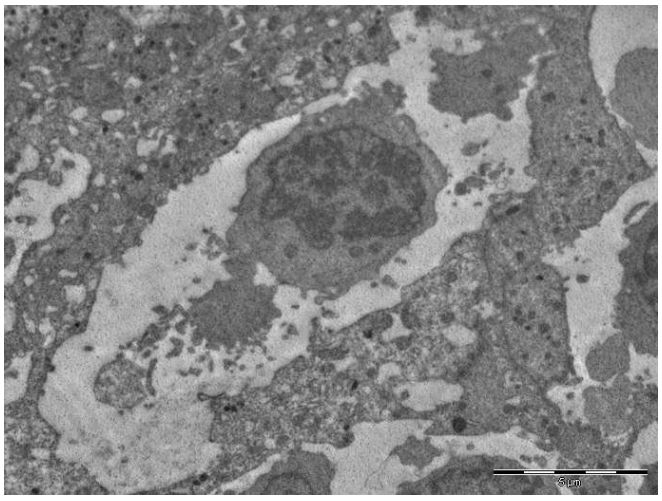

Figure 4 (b)

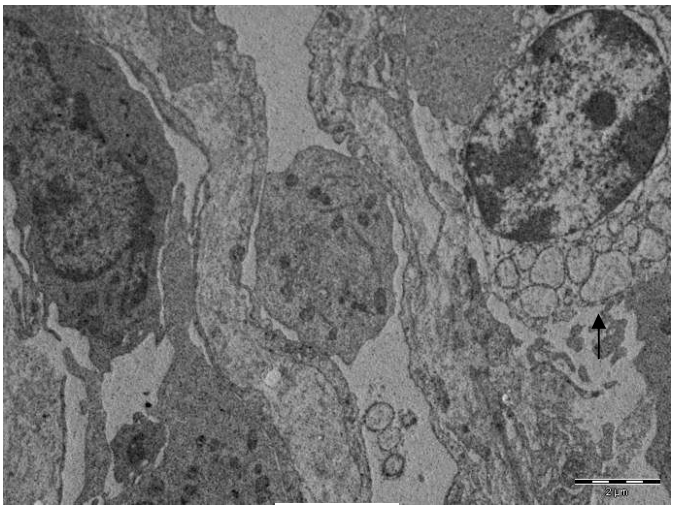

Figure 4 (c)

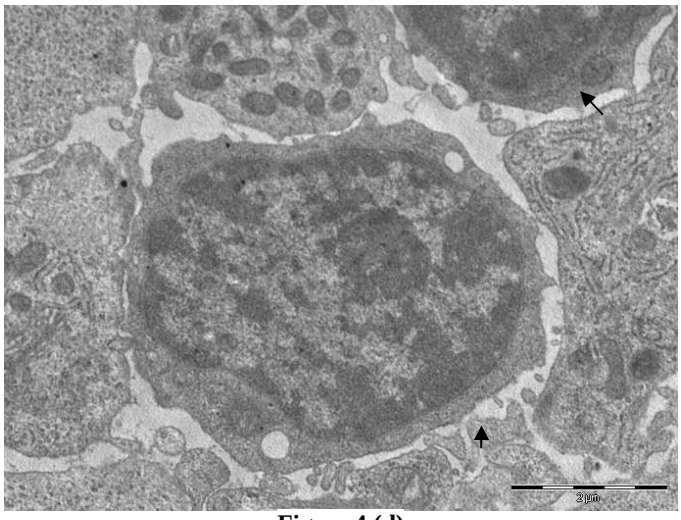

Figure 4 (d)

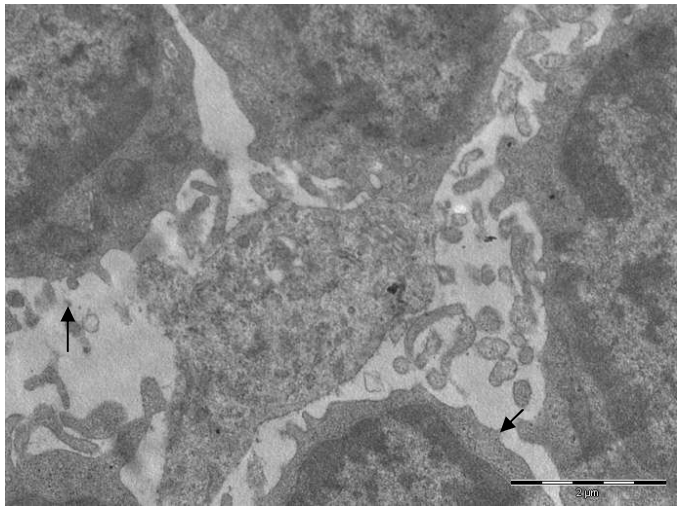

Figure 4 (e)

Figure 4: Transmission Electron Microscopic Findings: (a) Dense Vacuolization (b) Cell Membrane is Missing (c) Nucleus Cell Membrane Degeneration (d), (e) Lenfocyte Accumulation

DISCUSSION

The adenoid is a bacterial reservoir that contributes to chronic upper and lower airways infections **[17]**. Otitis media with effusion is one of the most common infection during childhood, and it usually also coexists with the adenoid hypertrophy **[18, 19]**. Most of the time, origin of initial infection in the middle ear and recurrence has been allocated to adenoid tissues. Recent studies have been focused on the presence of biofilms in adenoid tissue. The eradication of biofilms are difficult owing to their resistance to immunologic defense mechanisms and antibiotics, resulting in persistent infections **[20]**.

Microbial biofilms are involved in the chronicity of infections and resistance to antibiotic treatments, so they pose a serious problem for public health **[21]**. Especially, it

has been shown that play a role in upper respiratory tract diseases, including acute and chronic middle ear diseases **[8]**. As known, biofilm infection is suggested if three criteria are met: direct visualization of pathogenic bacteria in clusters contained within a surface matrix, anatomically localized infection, and recalcitrance to antibiotics **[22]**. Currently, as evidence supporting the role of biofilms in these infections and continues to grow, the techniques employed to study biofilms are also expanding **[23]**. This study aimed to investigate the biofilm formation on the adenoid surface of children undergoing adenoidectomy and to analyse whether this formation is associated with OME by transmission and scanning electronmicroscopic findings.

As known, SEM allows visualization of surface structures with a three-dimensional appearance at very different resolutions **[24]**. It also provides a clear visualisation of bacteria within a biofilm. Our data also indicates that the SEM is a reliable and practical for determining the biofilm formation of adenoid tissues.

TEM is used for the evaluation of ultrathin sections. An important advantage of TEM is high resolution, however the contrast is rather low. In our studies, biofilm stucture was not observed directly by TEM, however, cell degeneration were seen in most samples. On the other hand, it is not clear whether this situation is associated with biofilm formation. But, this probability should also be taken into consideration. From these findings we concluded that when investigating biofilm prevalence detailed, TEM and SEM should be used combined.

So far, the presence of biofilms on adenoids or tonsils has been reported. Al-Mazrou and Al-Khattaf have studied the biofilm formation on the epithelial surfaces of tonsils and adenoids in children undergoing adenotonsillectomy **[9]**. Similarly, Galli et al. used SEM to show structures resembling biofilms in 21/25 patients with adenotonsillitis **[25]**. Investigators indicated that the presence of biofilms in a significantly higher proportion of patients with chronically inflamed tonsils and adenoids, so an association between the presence of biofilms and chronic inflammation **[9]**. In a study of 19 patients, Chole and Faddis found biofilms in 15 patients with chronically diseased tonsils (74%) **[26]**. Another study reported the presence of biofilms on adenoids in 6 of 16 patients with sinusitis (44%) **[27]**. Direct evidence for bacterial biofilms due to *H. influenzae* in the middle ear comes from animal models, where *H. influenzae* has been shown to grow in biofilms on middle ear mucosa. Using a chinchilla model, SEM

demonstrated biofilms in all specimens from day 1 to 21 days post infection **[28]**. In also the study of **[16]** the role of adenoid surface biofilm formation was investigated in children with chronic otitis media with effusion (COME). They were also detected the biofilm formation on all samples. According to the their findings, adenoids removed from patients with COME had higher grade biofilm formation than the other group. So, investigators indicated that the adenoid surface biofilm formation may be associated with COME etiopathogenesis **[16]**. Although our study was conducted with a larger group of patients (n=50), there was no statistical difference between the two patient groups for the presence of biofilm (p>0,05). Nevertheless, the adenoid samples from children with OME had higher-grade biofilm formation (grade 3 (60%) and grade 4 (20%), totally 80%) than those without effusion (grade 3 (%40) and grade 4 (4%), totally 56%). In the present study, all samples were taken during the surgery, so, patients were resistant to theraphy. For this purpose, differences between groups may result meaningful.

CONCLUSION

As a result, the recognition of the role of biofilms in otolaryngologic diseases such as serous otitis media is critical to our understanding of the disease and to developing more effective treatment strategies. In the present study, the biofilm formation was detected in all samples. Since these patients are resistant to medical therapy with regard to adenoid vegetation, biofilm may have been detected in all samples. Further investigations should be performed in order to determine that whether biofilm formation may be an important factor in the pathogenesis of OME. In fact, sampling the adenoids of the cases of OME that respond to the therapy may changed the results of the study. However, this is impossible to perform as these children are not indicated for an operation. If any nonsurgical and easy method introduce to the clinical practice, diagnose of biofilm would be easier and unnecessary surgical intervention is avoided.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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