

Comparison of scanning electron microscopy findings regarding biofilm colonization with microbiological results in nasolacrimal stents for external, endoscopic and transcanalicular dacryocystorhinostomy

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Abstract

• **AIM:** To compare bacterial biofilm colonization in lacrimal stents following external dacryocystorhinostomy (EX-DCR), endoscopic dacryocystorhinostomy (EN-DCR), and transcanalicular dacryocystorhinostomy (TC-DCR) with multidiode laser.

• **METHODS:** This prospective study included 30 consecutive patients with nasolacrimal duct obstruction who underwent EXT-, EN-, or TC-DCR. Thirty removed lacrimal stent fragments and conjunctival samples were cultured. The lacrimal stent biofilms were examined by scanning electron microscopy (SEM).

• **RESULTS:** Eleven (36.7%) of the 30 lacrimal stent cultures were positive for aerobic bacteria (most commonly *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*). However anaerobic bacteria and fungi were not identified in the lacrimal stent cultures. Twenty-seven (90%) patients had biofilm-positive lacrimal stents. The conjunctival culture positivity after the DCR, biofilm positivity on stents, the grade of biofilm colonization, and the presence of mucus and coccoid and rod-shaped organisms did not significantly differ between any of the groups ($P>0.05$).

However, a significant difference was found when the SEM results were compared to the results of the lacrimal stent and conjunctival cultures ($P<0.001$).

• **CONCLUSION:** Type of dacryocystorhinostomy (DCR) surgery did not affect the biofilm colonization of the lacrimal stents. SEM also appears to be more precise than microbiological culture for evaluating the presence of biofilms on lacrimal stents.

• **KEYWORDS:** biofilms; nasolacrimal duct obstruction; epiphora; dacryocystitis; scanning electron microscopy

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INTRODUCTION

Nasolacrimal duct obstruction (NLDO) is a common cause of epiphora ^[1]. The standard treatment for NLDO is dacryocystorhinostomy (DCR), in which a permanent fistula is formed from the lacrimal sac to the nasal cavity to drain tears ^[2]. External DCR (EXT-DCR) is the traditional therapeutic procedure. Endoscopic DCR (EN-DCR) and transcanalicular DCR (TC-DCR) are also approved treatments ^[3,4]. Silicone stents are frequently used in DCR to improve the patency of the newly created fistula and to repair the tear drainage function.

Biofilms are organized communities of individual planktonic bacterial cells within an extensive exopolymer matrix ^[5,6]. Biofilm formation leads to numerous changes, including the development of a reservoir of bacteria that may cause a chronic infection by releasing microorganisms into the body, increased bacterial resistance (a greater resistance to antibiotics and host defenses), and a chronic inflammatory response at the site of the biofilm ^[6-8]. Additionally, the existence of a bacterial biofilm on nasolacrimal stents could lead to prosthetic failure by occluding the stent^[9].

Some studies have reported bacterial colonization of the outer and inner surfaces of lacrimal stents based on scanning electron microscopy (SEM) observations^[9,10]. The purpose of the present study was to compare, by SEM bacterial biofilm colonization in lacrimal stents implanted during EXT-, EN-, and TC-DCR with multidiode laser and to compare the biofilm presence to the results of lacrimal stent and conjunctival cultures to detect uncultivable biofilm organisms. To the best of our knowledge, this is the first article to analyze bacterial biofilms on lacrimal stents according to type of DCR surgery.

SUBJECTS AND METHODS

Study Design This prospective, non-randomized, comparative clinical study was conducted in the Departments of Ophthalmology and Otorhinolaryngology, Bagcilar Education and Research Hospital, Istanbul, Turkey, from January 2011 to June 2011. The study protocol, which adhered to the tenets of the Declaration of Helsinki, was approved by the Institutional Review Board, and informed consent was obtained from each participant. Thirty patients with NLDO underwent DCR surgery. These patients were non-randomly assigned into one of three groups according to the type of dacryocystorhinostomy surgery: the EXT- ($n=9$), EN- ($n=10$), and TC-DCR groups ($n=11$). The surgical choice among the three types of DCR was based on the patient's choice and their intranasal surface anatomy. The surgical techniques performed as standard EX- and EN-DCR surgery or TC-DCR with multidiode laser as reported in the literature^[11,12].

Methods

Lacrimal stent –related procedures Same perforate lacrimal silicone tubes (rve lovis armond, Paris cedex15, France) were inserted in all types of DCR surgeries. Oral antibiotherapy was used for 1wk postoperatively. A combination of antibiotic-steroid eye drops was applied four times per day, and a nasal corticosteroid spray was used twice daily after self-administered nasal irrigation with 0.9% normal saline for 3wk postoperatively. At the 8th postoperative week, the loop of lacrimal silicone tubes between the superior and inferior lacrimal puncta was cut with sterilized microscissors without local anesthesia and the area of the stent (approximately 10 mm in length) that sit on the conjunctiva was cut into 1-2 mm pieces. Then, the remaining part was withdrawn from the nose to remove the silicone tubes. These fragments were immediately placed onto aerobic, anaerobic and fungal media, and one was processed for SEM. Care was taken during the entire process to avoid contact between the lacrimal stent and the ocular surface, which could have led to bacterial contamination. At the same time, conjunctival cultures were obtained from the same eye. During this procedure, patients were not on steroids and antibiotics. Postoperative anatomic patency was also observed in all patients.

Conjunctival and silicone stent culture Conjunctival samples were inoculated onto 5% sheep blood agar, chocolate agar, MacConkey agar and Sabouraud dextrose agar media (Premed, Turkey). Silicone stent fragments were also inoculated on the same media used for conjunctival samples as well as anaerobic agar and in thioglycollate broth media. Blood agar and chocolate agar were incubated at 37°C in 5%-10% carbon dioxide for 24-72h; MacConkey agar was incubated at 37°C in ambient air for 24-72h; and Sabouraud Dextrose agar was incubated at both 25°C and 37°C for 21d. Anaerobic agar and thioglycollate broth media were incubated at 37°C under anaerobic conditions using the GasPak Anaerobe Pouch System (BD, USA) for 48h. Thioglycollate broth was subcultured on to anaerobic agar and 5% sheep blood agar after 48h incubation. Organisms isolated in the media were Gram stained. For Gram positive cocci, catalase, oxidase, bile solubility and optochin susceptibility tests were done. For the identification of Gram negative bacteria, oxidase and string test were carried out. For Haemophilus spp. X and V growth factor requirement was investigated. The organisms isolated from the samples were identified to genus and species level using the Vitec 2 compact system (bioMerieux, Marcy-l'Etoile, France).

Material preparation for scanning electron microscopy observation For the scanning electron microscopic investigation, the samples were fixed in 4% phosphate-buffered glutaraldehyde (0.13 mol/L and pH 7.4) for 4h and post-fixed with 1% OsO₄ for 1h more. The samples were then dehydrated in a graded alcohol series, put into amyl acetate, dried with liquid CO₂ under pressure with a critical point dryer (Bio-Rad E 3000) and covered with gold particles (Bio-Rad SC502). The sections were observed under a scanning electron microscope (SEM; Jeol 1200 JSM, Tokyo, Japan) by an experienced histologist who was unaware of the experimental groups.

Evaluation of biofilm growth on silicone stents and the presence of mucus If coccoid and/or rod-shaped structures were detected by SEM, the intensity of the biofilm colonization of the stents was graded on a scale from 0-3 (absent, mild, moderate, or severe). Additionally, the existence of mucus was assessed (0, absent; 1, present).

Statistical Analysis The statistical analysis was performed using IBM SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as the means \pm standard deviation and were controlled for normal distribution by the Shapiro-Wilk test. Categorical data were represented as the count and percentage. An analysis of variance (one-way ANOVA) was used to determine the difference in age among the groups. Categorical variables were compared among groups using the Freeman-Halton extension of Fisher's Exact Test (R-Project Program). The grade of biofilm colonization was compared among groups

Biofilm comparison in lacrimal stents

Table 1 Age, gender, laterality, surgery type, electron microscopy findings regarding lacrimal stent biofilm colonization and the results of culture of lacrimal stents and conjunctiva for each patient

Case no.	Age (a)	Gender	Laterality	Surgery type	Electron microscopy findings regarding lacrimal stent biofilm colonization			Lacrimal stent cultures	Conjunctival cultures
					Coccioid-shaped organisms	Rod-shaped organisms	Grade of biofilm colonization		
1	67	F	L	EX	+	-	3	—	<i>Staphylococcus epidermidis</i>
2	86	M	R	EX	+	+	1	—	—
3	70	F	L	EN	+	+	2	—	—
4	73	F	L	EX	+	+	3	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
5	51	F	R	TC	+	+	3	—	—
6	46	F	R	EN	+	-	2	—	—
7	4	M	L	TC	+	-	3	—	—
8	51	F	L	TC	+	+	4	<i>Corynebacterium</i> species	—
9	64	M	L	TC	-	-	0	—	—
10	41	F	L	TC	+	+	4	<i>Haemophilus influenzae</i>	—
11	30	F	R	EN	-	-	0	—	—
12	16	F	L	EN	+	-	2	—	—
13	31	M	L	EN	+	-	4	—	—
14	55	F	R	EN	+	-	1	—	—
15	33	F	L	TC	-	+	2	—	—
16	39	F	R	EX	+	+	4	<i>Corynebacterium jeikeium</i>	—
17	49	F	L	EX	+	+	4	<i>Staphylococcus epidermidis</i>	—
18	70	F	L	EX	+	-	4	—	—
19	83	M	R	TC	+	+	4	<i>Pseudomonas aeruginosa</i>	—
20	48	F	R	TC	+	+	3	<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>
21	31	F	L	EN	+	-	4	—	<i>Haemophilus influenzae</i>
22	14	F	L	EN	+	-	3	<i>Moraxella catarrhalis</i>	—
23	45	F	L	TC	+	+	3	—	—
24	45	F	L	TC	-	-	0	—	—
25	79	F	L	EN	+	+	4	<i>Streptococcus pneumoniae</i>	—
26	56	F	R	EX	+	+	2	—	—
27	33	M	R	TC	+	-	4	<i>Staphylococcus epidermidis</i>	—
28	45	F	R	EN	-	+	1	—	—
29	5	F	R	EX	+	-	2	—	—
30	55	F	L	EX	+	+	4	<i>Streptococcus</i> species	<i>Streptococcus</i> species

EX: External dacryocystorhinostomy; EN: Endoscopic dacryocystorhinostomy; TC: Transcanalicular dacryocystorhinostomy with multidiode laser; +: present, -: absent.

using the Kruskal-Wallis test. The results of the biofilm colonization and the results of the conjunctival and stent cultures were compared using the McNemar test. $P \leq 0.05$ was considered statistically significant.

RESULTS

The study involved 24 (80%) female and 6 (20%) male patients with a mean age of 46.9 ± 21.9 (range: 4-86y). Age, gender, laterality, surgery type, electron microscopy findings regarding biofilm colonization, the results of culture of lacrimal stents and conjunctiva for each patient are demonstrated in Table 1.

There were no statistically significant differences among the groups with respect to age or gender ($P=0.402$, one-way ANOVA; $P=0.233$, Chi-square test). In the EX-, EN- and TC-DCR groups, the descriptive statistics of the patients, including age, gender, the biofilm stent colonization rate, the microorganism growth rate on stents and conjunctiva, and the presence of mucus and coccoid and rod-shaped organisms, are shown in Table 2.

A microbiological analysis performed on a sample of the lacrimal stent material was positive for aerobic bacteria (36.7%) (Table 3), but no anaerobic bacteria or fungi were found. The differences in the rates of lacrimal stent culture positivity among the DCR surgery groups were not statistically significant ($P=0.454$, Freeman-Halton extension

of Fisher's Exact Test). Furthermore, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* were the bacteria most frequently isolated from the lacrimal stents (6.7% and 6.7%, respectively).

The differences in the rates of culture positivity of the conjunctiva after DCR surgery were not statistically significant ($P=0.392$, Freeman-Halton extension of Fisher's Exact Test). Moreover, *Staphylococcus epidermidis* was the most frequently detected aerobic species in the conjunctiva (6.7%) (Table 4).

The biofilm colonization of stents, the grade of biofilm colonization, and the presence of mucus and coccoid and rod-shaped organisms did not significantly differ among the groups ($P>0.05$). Using SEM, coccoid organisms were detected on all of the lacrimal stents implanted during EX-DCR. By contrast, coccoid organisms were detected on approximately 70% -80% of the lacrimal stents implanted during EN- and TC-DCR. In addition, SEM displayed rod-shaped organisms on approximately 65% of the lacrimal stents implanted in EX- and TC-DCR and on 30% of the lacrimal stents implanted in EN-DCR.

The SEM findings significantly differed from the microbiological cultures of the lacrimal stents ($P<0.001$, McNemar test). When we analyzed the lacrimal stents of 30 patients, both methods were negative in 3 patients (10%) and

Table 2 Descriptive statistics of patients including age, gender, the biofilm colonization rate on lacrimal stents, the microorganism growth rate in lacrimal stents and conjunctiva, and the presence of mucus and coccoid- and rod-shaped organisms

Demographic data and clinical findings	EX-DCR (n=9)	EN-DCR (n=10)	TC-DCR (n=11)	Total (n=30)	P
Age ¹	55.6±25.20	45.3±19.67	41.7±21.58	46.9±21.9	³ 0.402
F/M ratio	8/1	9/1	7/4	24/6	⁴ 0.309
Biofilm colonization rate on stents, [n (%)]	9 (100)	9 (81.8)	9 (90)	27 (90)	⁴ 0.756
Grade of biofilm colonization ²	3 (1-4)	2 (0-4)	3 (0-4)	3 (0-4)	⁵ 0.536
Microorganism growth rate in stents, [n (%)]	4 (44.4)	2 (20)	5 (45.5)	11 (36.7)	⁴ 0.454
Microorganism growth rate in the conjunctiva, [n (%)]	3 (33.3)	1 (10)	1 (9.1)	5 (16.7)	⁴ 0.392
Presence of mucus on stents detected by SEM, [n (%)]	0 (0)	1 (10)	2 (18.2)	3 (10)	⁴ 0.756
Presence of coccoid-shaped organisms on stents detected by SEM, [n (%)]	9 (100)	8 (80)	8 (72.7)	25 (83.3)	⁴ 0.327
Presence of rod-shaped organisms on stents detected by SEM, [n (%)]	6 (66.7)	3 (30)	7 (63.6)	16 (53.3)	⁴ 0.203

¹Values are presented as the mean±standard deviation; ²Values are presented as the median (minimum-maximum range); ³One-Way ANOVA; ⁴Freeman-Halton extension of Fisher's Exact Test; ⁵Kruskal Wallis Test. EX-DCR: External dacryocystorhinostomy; EN-DCR: Endoscopic dacryocystorhinostomy; TC-DCR: Transcanalicular dacryocystorhinostomy with multidiode laser; SEM: Scanning electron microscopy.

Table 3 Culture results of lacrimal stents

Pathogens	EX-DCR (n=9)	EN-DCR (n=10)	TC-DCR (n=11)	Total (n=30)	n(%)
<i>Staphylococcus epidermidis</i>	1 (11.1)	0	1 (9.1)	2 (6.7)	
<i>Staphylococcus aureus</i>	0	0	1 (9.1)	1 (3.3)	
<i>Streptococcus pneumonia</i>	0	1 (10)	0	1 (3.3)	
<i>Streptococcus species</i>	1 (11.1)	0	0	1 (3.3)	
<i>Haemophilus influenzae</i>	0	0	1 (9.1)	1 (3.3)	
<i>Pseudomonas aeruginosa</i>	1 (11.1)	0	1 (9.1)	2 (6.7)	
<i>Corynebacterium jeikeium</i>	1 (11.1)	0	0	1 (3.3)	
<i>Corynebacterium species</i>	0	0	1 (9.1)	1 (3.3)	
<i>Moraxella catarrhalis</i>	0	1 (10)	0	1 (3.3)	
No bacteria isolated	5 (55.6)	8 (80)	6 (54.5)	19 (63.3)	

Values are given as a number (percentage). EX-DCR: External dacryocystorhinostomy; EN-DCR: Endoscopic dacryocystorhinostomy; TC-DCR: Transcanalicular dacryocystorhinostomy with multidiode laser.

Table 4 Culture results of conjunctiva

Pathogens	EX-DCR (n=9)	EN-DCR (n=10)	TC-DCR (n=11)	Total (n=30)	n(%)
<i>Staphylococcus epidermidis</i>	1 (11.1)	0	1 (9.1)	2 (6.7)	
<i>Streptococcus species</i>	1 (11.1)	0	0	1 (3.3)	
<i>Haemophilus influenzae</i>	0	1 (10)	0	1 (3.3)	
<i>Pseudomonas aeruginosa</i>	1 (11.1)	0	0	1 (3.3)	
No bacteria isolated	6 (66.7)	9 (90)	10 (90.9)	25 (83.3)	

Values are given as a number (percentage). EX-DCR: External dacryocystorhinostomy; EN-DCR: Endoscopic dacryocystorhinostomy; TC-DCR: Transcanalicular dacryocystorhinostomy with multidiode laser.

positive in 11 patients (36.7%). In 16 patients (53.3%), the microbiological culture was negative, but the SEM results were positive; the reverse was not observed.

The difference between the rates of positive lacrimal stent SEM and positive conjunctival cultures was statistically significant ($P < 0.001$, McNemar test). When we analyzed 30 patients by both methods according to the results of the lacrimal stent SEM and conjunctival cultures, both methods were negative in 3 patients (10%) and positive in 5 patients (16.7%). In 22 patients (73.3%), the microbiological results were negative, but the SEM results were positive, the reverse

was not observed.

DISCUSSION

The frequency of infections caused by biofilms has been reported from 65% -80% [13]. Many prosthetic device-associated ocular infections due to biofilm formation on abiotic or biotic materials implanted in the eye including conjunctival infection and erosion associated with scleral buckles, endophthalmitis related to biofilms on intraocular lenses, keratoprotheses and glaucoma drain implants and infectious crystalline keratopathy have been stated [6,7,14-18]. Additionally, diffuse lamellar keratitis after the use of

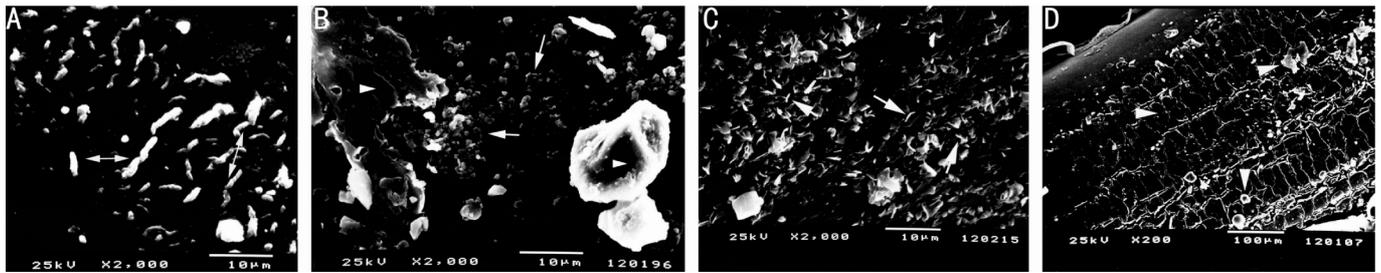


Figure 1 EN group A: the prominent presence of rod-shaped organisms on the stent (double-headed arrow); B: coccoid organisms (arrows) within the mucus (arrowheads) on the stent; C: the aggregation of rod-shaped organisms (arrows); D: the mucus coat appears similar to pavement (arrowheads) on the stent; note the mucoid drops.

contaminated surgical instruments in LASIK and bacterial keratitis associated with contact lens usage are ocular infections that result from biofilm formation on abiotic materials that come into contact with the eye [19,20]. Similarly, in our study, biofilm colonization was demonstrated on lacrimal stents, which are abiotic surfaces that come into contact with the eye.

Bacteria in biofilms cannot be viewed microbiologically. However, they may be detected by electron microscopy if special fixation techniques are used at the time of handling the fresh specimen [5]. Similarly, in the results of the bacterial cultures of conjunctival swabs and of the lacrimal stent fragments between the lacrimal puncta were largely negative compared with the SEM results.

Biofilm organization on lacrimal stents was described for the first time using SEM and confocal laser scanning microscopy by Parsa *et al* [10]. Springer and Roth [21] reported that electron microscopy could not identify biofilms prepared with conventional fixation, while fixation with ruthenium red permitted the visualization of the bacteria within an exopolysaccharide matrix. However, this claim was refuted by Tanner *et al* [22]. Although ruthenium red may preserve the glycocalyx during processing, there is no technical reason for the failure to detect bacterial biofilms using the routine processing techniques adopted by Pan *et al* [23] and others [22,24]. We identified biofilm formation without using ruthenium red. In our study, stent fragments were fixed and prepared for SEM by the procedure described in the materials and methods. Pan *et al* [23] and Sugita *et al* [24] reported that a multi-technique approach including SEM and TEM was necessary and that bacterial culture may be included in the study. We also advocate a multi-technique approach including SEM and microbiological techniques to identify and treat bacteria when chronic infection and stent occlusion are likely.

Biofilm colonization and mucus formation are potential risk factors for stent occlusion. Kim *et al* [25] reported that pus discharge during extubation was significantly related to final surgical failure. Ibáñez *et al* [9] identified biofilms in all specimens of failing polyurethane nasolacrimal stents, and

five of these seven stents were occluded with mucus or granulation tissue. They stated that the role of biofilm colonization of nasolacrimal stents could be major in prosthetic failure leading to stent occlusion. Authors reported also that mucus, granulation tissue and irregularities of the biomaterial could facilitate biofilm colonization [9]. In our SEM investigation, bacterial biofilms were detected on 90% of the removed lacrimal stents. When comparing the biofilm colonization of the lacrimal silicon stents implanted in the different types of dacryocystorhinostomy surgery, including EN- (Figure 1), EX- (Figure 2), and TC-DCR (Figure 3), there was no clear distinction among the appearances of the three types of lacrimal surgeries. In addition, coccoid organisms were detected more frequently than rod-shaped organisms, and mucus was detected on 10% of stents by SEM.

Staphylococcus epidermidis is currently recognized as the major etiological agent in the conjunctival flora [26]. *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* have also been reported as major colonizing bacteria of the biomaterials [24,27,28]. Sousa *et al* [29] explained this by demonstrating the greater likelihood of *Staphylococcus epidermidis* to adhere to the surface of silicone due to its higher surface hydrophobicity and the roughness of the silicone. Further, the adhesion of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* was determined to be affected by the free energy and roughness of pyrolytic carbon surfaces [30]. Kim *et al* [25] detected that the most frequently growing organisms on the surfaces of lacrimal silicone tubes removed from DCR were *Saphylococcus aureus* (71.8%), *Pseudomonas aeruginosa* (12.8%), and Diphtheroids (12.8%). They also showed significant relation between *Pseudomonas aeruginosa* infection and membranous obstruction of nasal mucosa that led to final surgical failure. As a reply to this study, Kamal *et al* [31] reported that the patients with pseudomonas infection should have given appropriate antibiotics after tube removal to prevent further damage and antibiotherapy might have changed this study result. Similarly, in our study, *Staphylococcus epidermidis* (6.7%) and *Pseudomonas aeruginosa* (6.7%) were detected on the

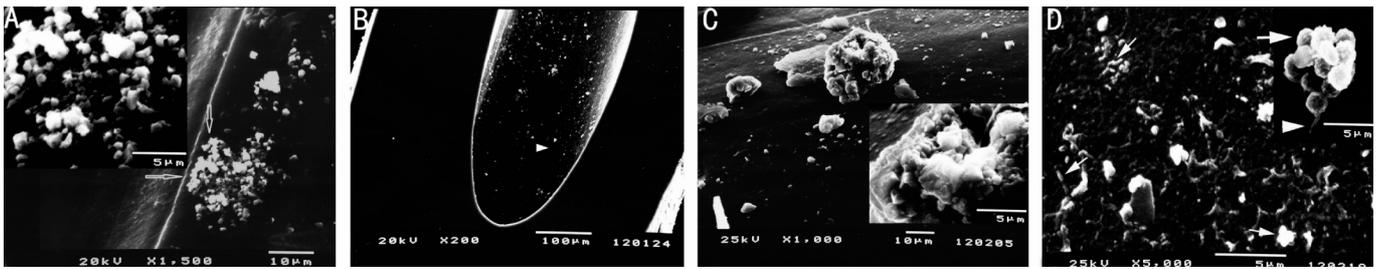


Figure 2 EXT group A: the accumulation of both coccoid and rod-shaped organisms (arrow); B: the interior of the stent tube (arrowhead) appears to be clean; C: aggregations of the coccoid organisms dominate the stent; D: in some areas, the coccoid organisms appear to have adhered to each other (arrows) and the rod-shaped organisms (arrowhead).

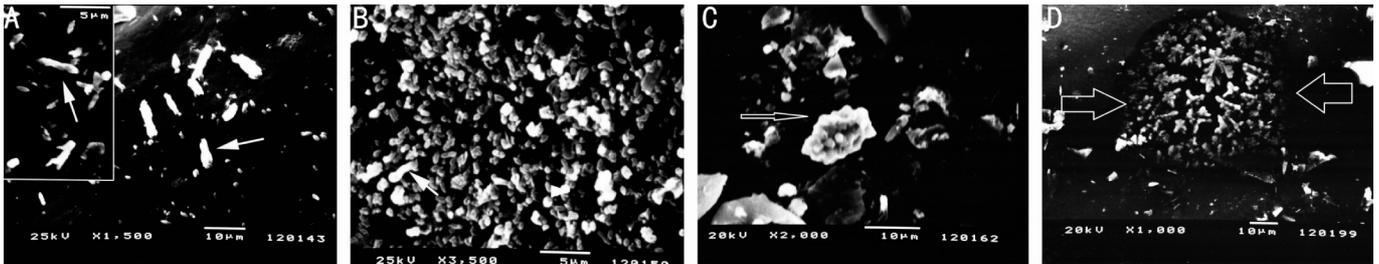


Figure 3 TC group A: rod-shaped organisms are apparent (arrows) but do not dominate; B: the prominent accumulation of coccoid organisms (arrow) next to a mild mucous coat (arrowhead); C: rosette-like shapes formed by the coccoid organisms (arrow); D: the combination of both coccoid and rod-shaped organisms on the stent (arrows).

lacrimal silicone stents. This difference might be related with the technique used for detection of microorganisms.

Biofilms on silicone stents in patients with NLDO may function as reservoirs for pathogenic bacteria that cause bacterial infections of the eye, including chronic or acute conjunctivitis [7]. In light of these discoveries, biofilm colonization on lacrimal stents may be prevented by the use of topical antiadhesive agents. Antibiotic models that inhibit biofilms are also mentioned in the literature^[32-34].

This study has some limitations that should be noted. First, our sample size is small due to too high cost of the study materials. However this is a preliminary study shedding light to further studies about this subject. In addition, creating a balance among the DCR groups in terms of patient assignment is very difficult. Because patient assignment was based on the patient's choice on surgery type and their intranasal surface anatomy. It should be better if ocular and nasal cavity inflammatory signs before DCR surgery and tube removal compared with the results of biofilm colonization. That is another limitation of our study.

In conclusion, this study is the first to evaluate biofilm colonization on lacrimal stents following different types of DCR surgery using SEM. The type of DCR surgery did not affect biofilm colonization of lacrimal stents. Additionally, SEM appears to determine more precisely the presence of biofilms on lacrimal stents than microbiological cultures. Biofilms can impact ocular health. Although our data represent a small-scale pilot study that may act as a seed for better understanding of the biofilm formation in case of lacrimal stents. Further studies on this subject by comparing

the various methods of biofilm detection and the relation between lacrimal stents in different DCR surgeries and biofilm in a large study population are needed.

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Biofilm comparison in lacrimal stents

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