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QUANTIFICATION OF BIOFILM FORMATION ON SILICONE INTRANASAL SPLINTS: AN *IN VITRO* STUDY

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Objectives: Biofilms are associated with persistent infections and resistant to conventional therapeutic strategies. The aim of this study was to investigate the quantity of biofilm produced on silicone intranasal splints. *Methods:* Quantity of biofilm formation on silicone splints (SS) was tested on 15 strains of *Staphylococcus aureus* and *Moraxella catarrhalis*, respectively. Antimicrobial susceptibility testing was performed in accordance with European Committee on Antimicrobial Susceptibility Testing recommendations. *Results:* All tested strains formed different amounts of biofilm on SS: 66.7% *S. aureus* and 93.3% *M. catarrhalis* were weak biofilm producers and 33.3% *S. aureus* and 6.7% *M. catarrhalis* were moderate biofilm producers. *S. aureus* formed significantly higher quantity of biofilm compared with *M. catarrhalis* ($p < 0.05$). Multidrug resistant *S. aureus* produced significantly higher amount of biofilm compared with non-multidrug resistant strains ($p < 0.05$). *Conclusion:* Quantity of biofilm on SS is highly dependent on bacterial species and their resistance patterns. Future studies are needed to ascertain another therapeutic option for prophylaxis prior to SS placement.

Keywords: silicone intranasal splints, biofilm formation, quantification, *Staphylococcus aureus*, *Moraxella catarrhalis*

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Introduction

Nasal septal surgery, one of the commonest operations performed in otorhinolaryngological practice, is often followed by nasal packing. There are no generally accepted standards regarding the materials that should be used for nasal packing after septoplasty, how long the packing should be left in place, or the indications for nasal packing [1]. Furthermore, various complications, such as postoperative infections, were reported to be associated with the use of nasal packings [2, 3]. Intranasal splints have been used recently as alternatives to classical nasal packing. The nasal airway splint is designed to provide septal support and allow nasal breathing postoperatively through the integral airway [4]. Silicone intranasal splints are commercially very popular and have a widespread use around the world [5]. Splints are typically removed within 4–7 days after surgery, during which period bacterial nasal residents can colonize splints and form biofilm [4, 6, 7].

Biofilm is a highly organized multicellular community encased in an extracellular polymeric matrix that is affixed to a surface. Bacterial populations within a biofilm, as opposed to their planktonic counterparts, have a reduced growth rate and a distinct transcriptome. Moreover, they exchange genetic material at an increased frequency, thereby augmenting their ability to acquire traits favorable to their persistence [8]. Bacteria in a biofilm have substantially increased resistance not only to the effectors of innate and acquired immunity, but also to the action of antibiotics as well [8, 9]. Biofilm is involved in the pathogenesis of various upper respiratory tract infections that are very difficult to treat with antibiotics and are often chronic and recurrent in nature [10]. Previously, it was demonstrated that biofilm formation on silicone splints (SS) increases significantly after 48 h following the placement [6]. However, to the best of our knowledge, there has been no study investigating the quantity of biofilm produced on silicone intranasal splints.

Nasal mucosa is a reservoir of many pathogens that inside bacterial biofilm inhabit mucous membrane. It is generally known that the prevalence of nasal carriage varies according to the age, geographic area, concomitant respiratory tract illness, antibiotic consumption, and other factors [11–13]. *Staphylococcus aureus* colonizes the nasal mucosa of approximately 30% of the population [14]. Due to the emergence of resistance to methicillin and other beta-lactam antibiotics in the early 1960s, and acquisition of other resistance genes during the past several decades, multiresistant strains of *S. aureus* have become a serious therapeutic problem [15]. Besides staphylococci, *Moraxella catarrhalis* can also reside as a nasal commensal both in children and adult population [16, 17]. Over the last three

decades, there has been a dramatic increase in the acquisition of beta-lactam resistance in *M. catarrhalis* strains, which implicates high empiric treatment failures with beta-lactam antibiotics for upper respiratory tract infections [16, 18].

The aim of this study was to determine the quantity of biofilm produced on silicone intranasal splints by two common nasal residents, *S. aureus* and *M. catarrhalis*.

Materials and Methods

Bacterial strains

In the period between May 2014 and December 2014, 15 strains of *S. aureus* and *M. catarrhalis*, respectively, were isolated from nasal swabs of the patients prior to rhinosurgery at the Clinic of Otorhinolaryngology and Maxillofacial Surgery, Clinical Centre of Serbia, Belgrade, Serbia. The Clinic represents a referral centre for otorhinolaryngology diseases in Serbia and a part of the largest University medical centre. This study was approved by the Institutional Ethical Committee, and all patients signed the informed consent form prior to their inclusion in the study.

Identification of the strains was performed in accordance with standard microbiology procedures and confirmed by automated Vitek2 System (bioMérieux, France). Antimicrobial susceptibility testing (AST) was performed by disk diffusion method for *S. aureus* [cefoxitin, ciprofloxacin, clindamycin, fusidic acid, erythromycin, gentamicin, mupirocin, penicillin, tetracycline, and trimethoprim/sulfamethoxazole (Bio-Rad, USA)] and *M. catarrhalis* strains [amoxicillin–clavulanic acid, erythromycin, nalidixic acid, tetracycline, and trimethoprim/sulfamethoxazole (Bio-Rad, USA)] in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [19]. AST of *M. catarrhalis* strains to cefuroxime and ceftriaxone was performed by Etest (bioMérieux, France) and detection of beta-lactamase production by Cefinase test (bioMérieux, France) [19]. Multidrug resistance was defined as a resistance to three or more distinct antimicrobial classes.

Biofilm assay

To detect and quantify the production of biofilm, the bacterial suspensions of grown *S. aureus* and *M. catarrhalis* cultures were prepared in sterile saline and adjusted to the density of 0.5 McFarland standard.

Preliminary screening of strains' ability to form biofilm was tested by a commonly used microtiter plate method in 96-well polystyrene plates (Sarstedt, USA) [20].

Capacity of biofilm production of 15 strains of *S. aureus* and *M. catarrhalis*, respectively, was tested on silicone double airway splint I (Websinger, Wolkersdorf, Austria). SS were cut under aseptic conditions into 1-cm pieces and placed in separate wells of 24-well microtiter plates (Sarstedt, USA) with 1,800 μL of tryptic soy broth (bioMérieux, France) supplemented with additional 1% glucose. 200 μL of previously prepared bacterial suspension was added to each well. Negative control for each plate represented only the medium with and without SS. After 24 h of incubation at 35 °C in aerobic conditions, plates with SS were decanted and gently rinsed three times with 2,000 μL of sterile phosphate-buffered saline (pH 7.2). After air-drying, plates with SS were fixed with 2,000 μL methanol per well for 20 min, dried, and stained with 2,000 μL of 2% crystal violet per well (bioMérieux, France) for 15 min. Unbounded dye was rinsed with water. After air-drying, SS were transferred into new 24-well microtiter plates and dye bound to biofilm formed on SS was released with 2,000 μL of 96% ethanol per well for 20 min at room temperature with gentle tapping. Extracted dye was transferred to 96-well microtiter plates (150 μL per well) and optical density (OD) was measured at 570 nm using a microtiter plate reader (ICN Flow Titertek Multiskan Plus, Germany). The results were calculated according to Stepanović et al. [20]. Each assay was repeated three times on three consecutive days. OD value of negative control (SS cultivated in medium without bacteria) was subtracted from the measured OD values of all tested strains, and mean OD values from three experiments were calculated. To calculate the category of biofilm production, the cutoff optical density (ODc) was determined as three standard deviations above the mean OD of the negative control. According to the obtained results, all tested strains were divided into four groups: $OD \leq ODc$ – category 0 (no biofilm producer, 0); $ODc < OD \leq 2 \times ODc$ – category 1 (weak biofilm producer, +); $2 \times ODc < OD \leq 4 \times ODc$ – category 2 (moderate biofilm producer, ++); and $4 \times ODc < OD$ – category 3 (strong biofilm producer, +++).

Statistical analysis

The data obtained in this study were analyzed in SPSS statistical program (PASW statistics for Windows, Version 18.0, Chicago: SPSS Inc., USA) using methods of descriptive statistics, χ^2 test, and Mann–Whitney *U* test.

Results

Susceptibility of analyzed *S. aureus* and *M. catarrhalis* strains to antibacterial agents is presented in Table I. Seven (46.7%) strains of *S. aureus* were multidrug resistant, while 46.7% strains were resistant only to penicillin and aminopenicillins. Among the tested *M. catarrhalis* strains, all (100%) produced beta-lactamase and were, according to the EUCAST guidelines, resistant to penicillin and aminopenicillins, 100% of strains showed intermediate susceptibility to cefuroxime and 20% to trimethoprim–sulfamethoxazole. None of the *M. catarrhalis* strains were multidrug resistant.

Preliminary screening revealed that all tested *S. aureus* and *M. catarrhalis* strains possessed the ability to form biofilm (Table I): 20% of *M. catarrhalis* strains were weak biofilm producer (category 1, +), 60% of *S. aureus* strains and 73.3% of *M. catarrhalis* strains were moderate biofilm producers (category 2, ++), and 40% of *S. aureus* strains and 6.7% of *M. catarrhalis* strains were strong biofilm producers (category 3, +++).

S. aureus and *M. catarrhalis* strains formed significantly lower amount of biofilm on SS compared with their initial biofilm formation capacity ($p < 0.05$) (Table I): 66.7% of *S. aureus* strains and 93.3% of *M. catarrhalis* strains belonged to the category of weak biofilm producers, and 33.3% of *S. aureus* strains and 6.7% of *M. catarrhalis* strains belonged to the category of moderate biofilm producers. None of the tested strains of both bacterial species belonged to the category of strong biofilm producers. *S. aureus* strains formed significantly higher quantity of biofilm on SS compared with *M. catarrhalis* ($p < 0.05$) (Figure 1a).

Eight strains (53.3%) of *S. aureus* and eleven strains (73.3%) of *M. catarrhalis* that were previously categorized as moderate biofilm producers reduced the capacity of biofilm production on SS to weak biofilm production. Six (40%) strains of *S. aureus* and one (6.7%) strain of *M. catarrhalis* reduced the quantity of biofilm formation on SS from category 3 to category 2.

Multidrug resistant *S. aureus* strains produced significantly higher amount of biofilm on SS (category 2, ++) compared with non-multidrug resistant strains (category 1, +) ($p < 0.05$; Table I, Figure 1b). One (6.7%) *S. aureus* strain resistant to penicillin, ampicillin, and tetracycline, and three (20.0%) *M. catarrhalis* strains belonging to the same resistotype produced the same amount of biofilm both on control plate and SS (Table I).

Discussion

Nasal septoplasty procedure may be concluded with nasal packing using SS. Intranasal splints are widely used after septal surgery for the prevention of

Table I. Susceptibility pattern and biofilm formation on microtiter plate and silicone intranasal splints of *Staphylococcus aureus* and *Moraxella catarrhalis* strains

Strain	S	R	MP	NS
S. aureus 1	A+K, C, G, Cip, E, Cl, T, Fa, M	P, A	++	+
S. aureus 2	Cip, T, Fa, M	P, A, A+K, C, G, E, Cl	+++	++
S. aureus 3	A+K, C, G, Cip, E, C, T, Fa, M	P, A	++	+
S. aureus 4	A+K, C, G, Cip, Fa, M	P, A, E, Cl, T	++	+
S. aureus 5	A+K, C, G, Cip, E, C, T, Fa, M	P, A	++	+
S. aureus 6	A+K, C, G, Cip, E, C, T, Fa, M	P, A	++	+
S. aureus 7	Cip, T, Fa, M	P, A, A+K, C, G, E, Cl	+++	++
S. aureus 8	A+K, C, G, Cip, Fa, M	P, A, E, Cl, T	+++	+
S. aureus 9	A+K, C, G, Cip, E, C, T, Fa, M	P, A	++	+
S. aureus 10	A+K, C, G, Cip, Fa, M	P, A, E, Cl, T	+++	+
S. aureus 11	A+K, C, Cip, Fa, M	P, A, G, E, Cl, T	+++	++
S. aureus 12	A+K, C, G, Cip, E, Cl, Fa, M	P, A, T	++	++
S. aureus 13	A+K, C, G, Cip, E, C, T, Fa, M	P, A	++	+
S. aureus 14	A+K, C, G, Cip, E, C, T, Fa, M	P, A	++	+
S. aureus 15	Cip, T, Fa, M	P, A, C, G, E, Cl	+++	++
M. catarrhalis 1	A+K, Na, E, T+S, CTX	P, A, CXM[a]	++	+
M. catarrhalis 2	A+K, Na, E, T+S, CTX	P, A, CXM[a]	++	+
M. catarrhalis 3	A+K, Na, E, CTX	P, A, CXM[a], T+S	++	+
M. catarrhalis 4	A+K, Na, E, T+S, CTX	P, A, CXM[a]	++	+
M. catarrhalis 5	A+K, Na, E, T+S	P, A, CXM[a]	++	+
M. catarrhalis 6	A+K, Na, E, T+S	P, A, CXM[a]	++	+
M. catarrhalis 7	A+K, Na, E	P, A, CXM[a], CTX[a], T+S	+++	++
M. catarrhalis 8	A+K, Na, E, T+S, CTX	P, A, CXM[a]	++	+
M. catarrhalis 9	A+K, Na, E, T+S, CTX	P, A, CXM[a]	++	+
M. catarrhalis 10	A+K, Na, E, T+S, CTX	P, A, CXM[a]	++	+
M. catarrhalis 11	A+K, Na, E, T+S, CTX	P, A, CXM[a]	+	+
M. catarrhalis 12	A+K, Na, E, T+S, CTX	P, A, CXM[a]	+	+
M. catarrhalis 13	A+K, Na, E, T+S, CTX	P, A, CXM[a]	+	+
M. catarrhalis 14	A+K, Na, E, T+S, CTX	P, A, CXM[a]	++	+
M. catarrhalis 15	A+K, Na, E, CTX	P, A, CXM[a], T+S	++	+

Note: S = susceptible, R = resistant, MP = microtiter plate, NS = nasal splint, P = penicillin, A = ampicillin, A+K = amoxicillin–clavulanic acid, C = cephalosporin, G = gentamicin, Cip = ciprofloxacin, E = erythromycin, Cl = clindamycin, T = tetracycline, Fa = fusidic acid, M = mupirocin, Na = nalidixic acid, T+S = trimethoprim–sulfamethoxazole, CXM = cefuroxime, CTX = ceftriaxone.
[a]Intermediate susceptible.

intranasal adhesions between the nasal septum and lateral nasal wall and to support the septal position [7]. Although these endonasal materials play an important role during the postoperative period, they are synthetic and foreign objects in the human body. As with all medical devices, silicone intranasal splints can be colonized with bacteria and biofilms could be formed after a short period. Acar et al. conducted the first comprehensive study on biofilm formation on SS and presented that 12%, 56%, and 100% of splints exhibited microbial biofilm formation on their surface at 48, 72, and 96 h after nasal placement, respectively [6].

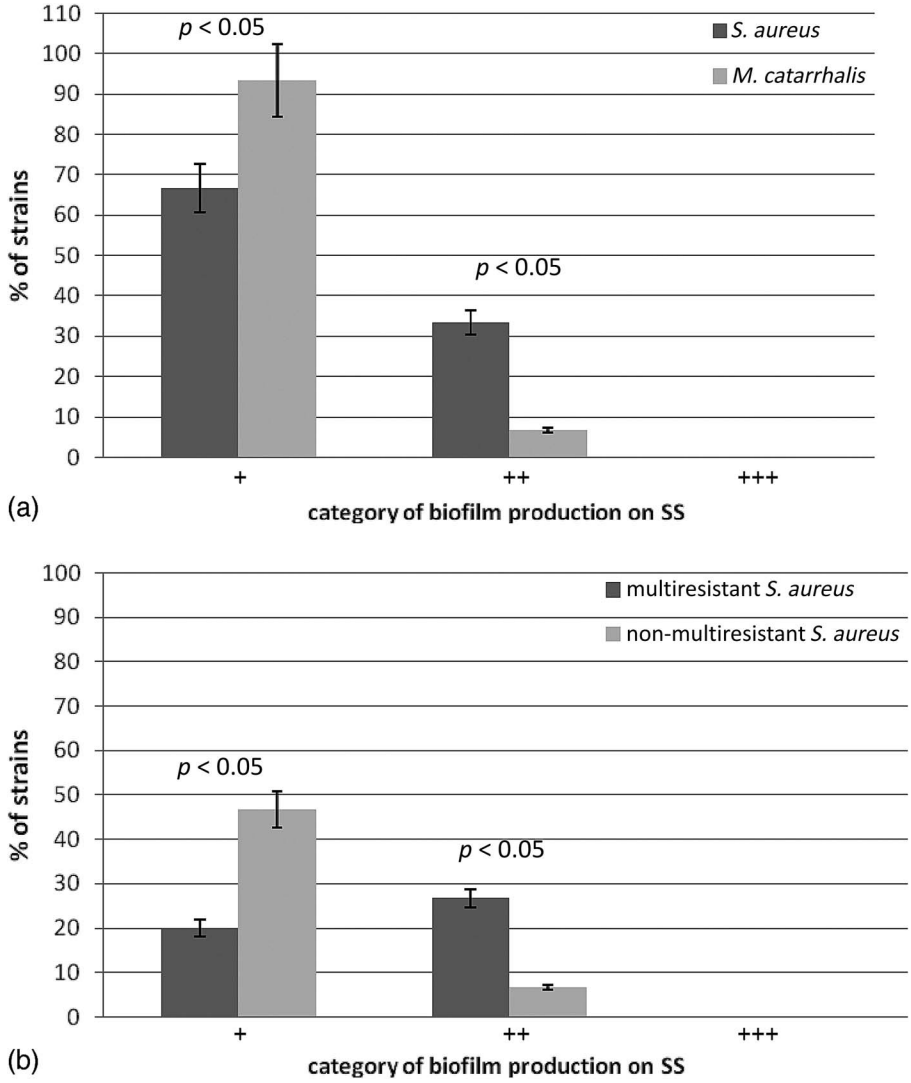

Figure 1. Category of biofilm production on silicone intranasal splints. (a) Biofilm production on silicone intranasal splints by *Staphylococcus aureus* and *Moraxella catarrhalis*. (b) Biofilm production on silicone intranasal splints by multiresistant and non-multiresistant strains of *S. aureus*. Results are presented as percent of strains ± standard deviation; 0, no biofilm producer; +, weak biofilm producer; ++, moderate biofilm producer; and +++, strong biofilm producer

In their study, visualization of biofilm formation on intranasal splints was achieved by scanning electron microscopy, using semi-quantitative method for *in vivo* determination of biofilm production. Thus, we performed modified *in vitro* microtiter plate method on SS and confirmed not only the presence of biofilm on splints but also its quantities. In this study, all investigated strains of two common nasal colonizers, *S. aureus* and *M. catarrhalis*, had the ability to produce biofilm on intranasal splints with different capacities (Table I).

Postoperative nasal packing may be performed by various types of packing materials including Vaseline gauze, bismuth iodoform paraffin paste, Merocel nasal packs, SS, and others [7, 21]. Dag et al. [5] investigated biofilm formation capacity of two different nasal materials [Merocel packs (made of polyvinyl acetal) and SS] and showed that biofilm formation on Merocel is significantly higher than on SS, mainly due to the different texture and surface properties of these materials. Similar findings were obtained in the present investigation showing that both *S. aureus* and *M. catarrhalis* strains expressed lower capacity of biofilm productions on SS compared with polystyrene plastic. Hence, it is of great importance to choose the most appropriate packing material of known adhesive capacity for the conclusion of nasal septoplasty, or, in the case of novel material, to investigate the surface sensitivity for biofilm formation by *in vivo* and/or *in vitro* studies before its introduction in operating theatre. Moreover, these results indicate the importance of performing quantitative *in vitro* biofilm investigation on biomaterials, rather than widely used classic quantitative biofilm method in order to obtain reliable and accurate results [20].

Biofilm is a complex microbial community with multiple species of bacteria coexisting in a matrix of extracellular polysaccharide substance. Besides the characteristics of the surface bacteria attach to and the environmental conditions, the process of biofilm formation is highly dependent on the characteristics of bacteria that produce biofilm [8]. *S. aureus* and *M. catarrhalis* belong to normal bacterial nasal flora, both in adults and children, with significant difference between age groups: *S. aureus* together with coagulase-negative staphylococci represents predominant flora in adults, while *M. catarrhalis* with *S. pneumonia* and *Haemophilus influenzae* in children population. As common nasal residents, these pathogens can easily contaminate the surface of silicone intranasal splints and form biofilm. Accordingly, the capacity of biofilm formation on splints was investigated with *S. aureus* and *M. catarrhalis* nasal colonizer strains in this study. The obtained results showed significantly greater capacity of *S. aureus* strains in biofilm production compared with *M. catarrhalis* strains. Drago et al. [22] reported similar findings with *S. aureus* and *M. catarrhalis* strains isolated from upper respiratory tract; however, to the best of our knowledge, this investigation is the first one examining different capacities of biofilm formation of respiratory pathogens on

silicone intranasal splints. Our research confirmed that strains of the same species have different potentials to form biofilm, which implies that biofilm production is strongly associated with phenotypic and genotypic characteristics of the strains, as it has recently been reported investigating other medical implants [23].

Common characteristics of *S. aureus* and *M. catarrhalis* are the emergence of resistant and multiresistant strains in the past several decades. Both bacteria show the capability to produce beta-lactamase or other acquired mechanisms of resistance [18, 24]. In this study, multiresistant *S. aureus* strains and *M. catarrhalis* strain resistant to two classes of antibiotics demonstrated a propensity to form a larger amount of biofilm on intranasal splints compared with other isolates. This would suggest that the ability to form a superior biofilm structure may be an important virulence factor in resistant bacterial strains, which is a result of great importance for future research studies.

The use of antibiotics for surgical procedures is a common practice among otorhinolaryngologists. A survey carried out among the Members of the US Rhinology Society showed that 66% of ENT doctors used antibiotics as a routine practice in postoperative period of septoplasties, and the most common reason for prophylactic use is the prevention of postoperative infections [25]. Amoxicillin–clavulanic acid is the most frequently administered antibiotic prior to septoplasty with nasal packing [5, 6, 21]. Although all strains tested in this study were sensitive to this drug, microbiology methods typically determine the susceptibility of planktonic and not sessile form of bacteria (i.e., bacteria in a biofilm). Bacterial biofilms exhibit a reduced susceptibility and/or resistance to antibiotics [8, 9]. The penetration of antibiotics into biofilm is significantly reduced, and the bacteria in a biofilm are exposed to the drug in gradually increasing doses. This gradual exposure can result in stress-induced metabolic or transcriptional changes in bacteria and increased resistance to antibiotic therapy [26]. Moreover, in all previous investigations, prophylactic administration of amoxicillin–clavulanic acid did not prevent biofilm formation on SS. Another possible treatment for biofilm is low-dose macrolides. Low-dose clarithromycin therapy has been shown to alter the structure of biofilms [27].

Further studies are needed to develop methods that block the information pathway between bacteria and gene transcription, which controls the attachment and production of biofilms.

Conclusion

Quantity of biofilm formation on silicone intranasal splints is highly dependent on bacterial species that produce biofilm and also their resistance

patterns. Further studies are needed to ascertain other therapeutic option for prophylaxis prior to silicone intranasal splints placement that would inhibit biofilm formation on biomaterials, and also to identify the factors that affect the formation and treatment of biofilms.

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Conflict of Interest

The authors declare no conflict of interest.

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