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Antibodies directed against Integration Host Factor Mediate Biofilm Clearance from Nasopore®

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Abstract

Objectives—Intranasal resorbable packing, such as Nasopore®, is commonly used during sinus surgery despite a paucity of evidence that demonstrates clinical benefit. We theorized that Nasopore supports bacterial growth and biofilm formation. The DNABII family of bacterial nucleic acid binding proteins stabilizes the extracellular polymeric substance of the biofilm, thus protecting bacteria from host defenses and traditional antibiotics. We tested the hypothesis that use of anti-IHF antibodies in conjunction with antibiotics would enhance biofilm eradication from Nasopore.

Study Design—*In vitro* experiments.

Methods—Non-typeable *Haemophilus influenzae* (NTHI) biofilms were grown on Nasopore. Following 24-hour incubation, biofilms were incubated for an additional 16 hours with either: medium alone, naïve rabbit serum, rabbit anti-IHF serum, amoxicillin/clavulanate or anti-IHF serum + amoxicillin/clavulanate. COMSTAT analysis was performed on images of biofilms obtained via confocal microscopy.

Results—NTHI readily formed a biofilm on Nasopore. Treatment with amoxicillin/clavulanate alone mediated an increase in biomass by 92% to $6.63 \mu^2/\mu^3$ compared to incubation in sterile medium alone ($3.46 \mu^2/\mu^3$). Treatment with anti-IHF alone reduced the biomass by 77% to $1.29 \mu^2/\mu^3$ compared to incubation with naïve rabbit serum ($5.53 \mu^2/\mu^3$). Anti-IHF + amoxicillin/clavulanate reduced biomass by 88% to $0.66 \mu^2/\mu^3$ ($p < 0.02$) compared to incubation with naïve rabbit serum.

Conclusion—Antibiotics alone were ineffective in eradicating NTHI biofilms that had formed on Nasopore *in vitro*. Anti-IHF antibodies plus amoxicillin/clavulanate therapy synergistically

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reduced biofilm biomass by 88%. These data support clinical studies for the use of anti-IHF combined with antibiotics to reduce biofilm formation on intranasal packing.

Keywords

anti-IHF; biofilms; non-typeable *Haemophilus influenzae*; Nasopore®; antibiotics

INTRODUCTION

Nasal packing has been used for decades in sinus surgery to assist in hemostasis, decrease synechial formation and prevent lateralization of the middle turbinate. Its use was first described in 1951, examining the efficacy of rubber pneumatic packs.¹ In the years since, a variety of packing materials have been used, including polyethylene glycol-containing polyurethane foams (Nasopore®), petroleum ointment-soaked ribbon gauze, fingerstall packs, polyvinyl acetate sponge (Merocel®), and balloon tamponade devices.^{2,3,4,5,6,7} However, these materials can serve as a nidus for inflammation and infection, cause pain and discomfort for patients post-operatively, and lead to mucosal trauma when removed from the nasal cavity. Over the years, numerous studies have been dedicated to determining whether there is a true clinical benefit to using intranasal packing, and if so, whether the clinical benefit is worth the cost, patient discomfort and risks imparted by their use. In a study conducted by Orlandi and Lanza in 2004, post-operative bleeding and complications were tracked in patients post-endoscopic sinus surgery. No significant benefits were seen in the 170 patients examined.⁸ In 2006, Eliashar et. al. examined a group of post-endoscopic sinus surgery patients, 92% of which did not receive nasal packing or hemostatic agents. There was no statistically significant difference in post-operative bleeding or complications between the two patient groups.⁹ Mo et al. conducted a study in 2008 in which 75% of patients did not receive intranasal packing. Their results showed that in unilateral endoscopic sinus surgery (ESS) cases, subjective estimated blood loss and mucosal inflammation were decreased in the no-packing group. However, for bilateral ESS, no statistically significant clinical differences were found.¹⁰ Despite the lack of clear, compelling evidence to support the use of nasal packing, Nasopore® (Stryker Corporation, Kalamazoo, MI) has become one of the most commonly used absorbable synthetic nasal packing materials in patients post-ESS. However, there is a paucity of evidence supporting Nasopore's clinical benefit, and conflicting evidence regarding its safety and risk profile. A recent study by Wang, *et al.*¹¹ concluded that there was a risk of excessive granulation tissue formation in patients treated with Nasopore over the more traditional nasal packing materials.

We theorized that bacterial biofilm formation on both the surface as well as within pores of nasal packing and its subsequent harm to nasal mucosa maybe responsible for the lack of strong evidence to support the clinical efficacy of such nasal packing. In recent years, biofilms have become the subject of heightened investigation as these microbiological structures are 1000-fold more resistant to standard antibiotic treatment.¹² The backbone of the biofilm communities is the extracellular polymeric substance, or EPS, which is involved in facilitating adhesion to surfaces and also acts as an encasement or barrier protecting the bacterial cells that reside within this matrix. The composition of the EPS is highly diverse,

however the EPS of many human pathogens contains a large amount of extracellular DNA of both bacterial and host origin, the latter of which originating via neutrophil ‘netting’. The extracellular DNA derived from bacteria is supported by a family of bacterial DNABII binding proteins; our laboratory previously identified one of these proteins, Integration Host Factor (IHF), and confirmed its essential role as a structural support for extracellular DNA in bacterial biofilms.¹³ Furthermore, we showed a synergistic effect between anti-IHF antibodies and otherwise ineffective traditional antibiotic treatment. The debulking of the EPS by anti-IHF antibodies proved sufficient to allow access of the antibiotics and immune modulators to the resident bacterial cells, thus enhancing biofilm eradication.¹³

The basis of this investigation lies on the fact that Nasopore is becoming an increasingly popular choice for middle meatal packing after ESS, yet the mesh-like structure of the dressing and its persistence in the nasal cavity until full absorption (average of 14 days) makes it a potential ideal scaffold on which bacterial biofilms can grow. We first determined if bacterial biofilms derived from NTHI, the most common pathogen in bacterial rhinosinusitis, would grow readily on Nasopore *in vitro*. In addition, given that IHF plays a crucial role in biofilm survival and that anti-IHF has strong therapeutic utility in biofilm clearance, we decided to investigate the efficacy of using anti-IHF in combination with amoxicillin/clavulanate (Augmentin™), a commonly used antibiotic in rhinosinusitis, to mediate clearance of biofilms from Nasopore samples.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A low passage clinical isolate of NTHI (strain 86-028NP) was grown on chocolate II agar (Fisher Scientific, Pittsburgh, PA) and incubated at 37° Celsius in a humidified environment under 5% CO₂ for 18 hours. Bacterial colonies were suspended in 2 mL of brain heart infusion (BHI) broth that had been supplemented with 2 µg each of heme (Sigma-Aldrich, St Louis, MO) and β-NAD (Fisher Scientific, Pittsburgh, PA) per ml (sBHI). A fluorescent NTHI reporter construct of strain 86-028NP was also utilized, This provided an additional method to visualize and quantify the biofilm bacteria while ensuring that the mass of the Nasopore was excluded from the COMSTAT analysis calculations (see “Visualization of the Biofilm” below for further discussion). The pRSM2211 plasmid contains the strong promoter for the outer membrane protein P2 which drives expression of green fluorescent protein (GFP). The resulting reporter isolate, strain 86-028NP/pRSM2211 was selected for use here due to its near constitutive expression of GFP which allows for direct, continuous, biofluorescent imaging of NTHI.¹⁴

Nasopore® preparation

A sterilely packaged Nasopore synthetic dressing (firm) was removed from its packaging and was sectioned into 2 mm cubes using a scalpel and sterile technique. These cubes were then placed into wells of a 96-well optical density plate (Fisher Scientific, Waltham, MA). The samples were saturated with 50 µl of pre-warmed sBHI and incubated at room temperature for 30 minutes prior to inoculation with NTHI.

***In vitro* Biofilm Growth**

Bacterial suspensions were adjusted to an optical density of 0.65 at 490 nm then diluted 1:6 in sterile supplemented medium. Cultures were then incubated statically for 3 hours at 37° Celsius in a humidified environment under 5% CO₂ to reach mid-log phase. Cultures were diluted 1:5000 with sterile, pre-warmed sBHI and 50 µl of this bacterial suspension was inoculated into pre-moistened sample of Nasopore in the 96 well plate (½ of the samples received the wild-type strain and ½ of the samples received the GFP-expressing strain). The bacteria were then incubated at 37° under 5% CO₂ for 1 hour to allow bacterial adherence to the Nasopore cubes. At this time, 100 µl of sBHI was added to each well and the plate was incubated an additional 16 hours under the same conditions. The media was then aspirated from the corner of each well and replaced with 200 µl fresh sBHI dispensed along the wall of the well as to not create liquid-mediated shear forces within the well that could disrupt the growing biofilms. The cultures were incubated at 37° under 5% CO₂ for 8 hours. At this time (24 hours post-inoculation), the medium was aspirated from the corner of each well and the samples were treated with 200 µl of one of the following solutions: sBHI alone, naïve rabbit serum diluted 1:50 in sBHI, rabbit anti-IHF diluted 1:50 in sBHI, amoxicillin/clavulanate diluted to 1µg/ml in sBHI or anti-IHF (1:50) + amoxicillin/clavulanate (1µg/ml) in sBHI. The plate was incubated for an additional 16 hours at 37° with 5% CO₂.

Visualization of Biofilm

After the final incubation, the medium was aspirated from the corner of all wells and the samples were washed twice with sterile saline. One half of the wells from each treatment group (containing wild-type NTHI biofilms) were stained with 200 µl of a viable stain [Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR)] for 15 minutes, the stain was aspirated and these samples were washed with saline twice more. These samples were then fixed with paraformaldehyde, glutaraldehyde, and acetic acid in phosphate buffer at pH 7.4. The remaining wells were stained with 50 uL of Filmtracer Biofilm stain (Life Technologies, Grand Island, NY). Filmtracer Biofilm stain was used in light of the fact that Live/Dead stain was taken up by Nasopore and thus resulted in an inability to subtract Nasopore mass when performing COMSTAT analysis between biofilms from confocal imaging. Filmtracer stain was not taken up by the Nasopore material, thus allowing us to properly perform COMSTAT analysis. The biofilms were imaged using a 63 × objective on a Zeiss 510 Meta-laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). All *in vitro* biofilm assays were repeated a minimum of three times, on separate days, and all individual biofilm assays included replicates of three chambers per assay condition on each assay day. Data are presented as mean values ± standard error of the mean.

Statistical Analysis

All data was reported as a mean value +/- standard error of the mean. We assessed efficacy of treatment based on differences in biofilm height, biomass and thickness determined by COMSTAT analysis (The MathWorks Inc., Natick, Massachusetts). We then calculated percent reduction in biofilm size when compared to control condition (either sBHI or naïve

rabbit serum). Significance was determined by unpaired Student's t-test, with a p-value of <0.05 considered significant.

RESULTS

NTHI biofilms readily grew on Nasopore at a greater height compared to standard chamber slides *in vitro*

COMSTAT analysis of confocal microscopy images of biofilm growth on Nasopore indicated a mean biofilm height of 96.6 μm $\pm$ (11.6 μm) at 36 hours post-inoculation, whereas biofilms grown on standard glass slides were approximately 35 μm $\pm$ (5 μm) in height 36 hours post-inoculation (Figure 1). Thus NTHI biofilms grown on Nasopore were significantly more robust, with an increase in height of nearly 3-fold.

Amoxicillin/clavulanate alone does not clear biofilms grown on Nasopore *in vitro*

COMSTAT analysis of confocal microscopy images taken from bacterial biofilms grown for 24 hours on Nasopore then treated with amoxicillin/clavulanate alone demonstrated an increase in biomass by 92% to 6.63 μ^2/μ^3 $\pm$ 1.11 μ^2/μ^3 compared to biofilms treated with control (sterile sBHI) of 3.46 μ^2/μ^3 $\pm$ 0.83 μ^2/μ^3 (Figure 2).

Amoxicillin/clavulanate plus anti-IHF significantly enhance clearance of 24 hour biofilms grown on Nasopore

COMSTAT analysis of confocal microscopy images taken from bacterial biofilms grown for 24 hours on Nasopore then treated with anti-IHF (at a dilution of 1:50) demonstrated a mean biomass of 1.29 μ^2/μ^3 . This was a 77% reduction of biomass compared to Nasopore treated with naïve rabbit serum (5.53 μ^2/μ^3) ($p < 0.06$).

Nasopore treated with anti-IHF plus amoxicillin/clavulanate (1 $\mu\text{g}/\mu\text{l}$) demonstrated a mean biomass of 0.66 μ^2/μ^3 . This was a significant decrease of 88% compared to Nasopore treated with naïve rabbit serum ($p < 0.02$) (Figures 2,3).

DISCUSSION

The use of absorbable biomaterials after otolaryngologic surgery gained notoriety in 1969 with the first reported use of oxidized regenerated cellulose.¹⁵ However, it was not until recently that otolaryngologists started favoring these resorbable materials over traditional packing methods post-ESS. The hope was that these materials would provide similar hemostatic qualities to traditional materials but cause less mucosal irritation and re-bleeding. In reviewing studies that compared resorbable packing, traditional packing and no packing at all, most studies found that resorbable materials have no statistically significant difference with regard to post-operative bleeding (Table 1). Studies examining the effects of resorbable materials on wound healing appear inconclusive. Fifty percent of the studies analyzed showed no significant difference in synechial formation, granulation tissue formation or stenosis when resorbable packing was compared to no packing; 30% of the studies showed increased inflammation and granulation tissue formation as well as delayed wound healing with use of thrombin/collagen (FloSeal®) or Nasopore rather than traditional packing; and

20% of the studies reviewed demonstrated decreased inflammation and improved wound healing with use of Merogel over traditional packing. Furthermore, there is a paucity of research investigating the potential for bacterial growth and/or biofilm formation on these materials used during nasal surgery.

The difficulty in demonstrating clinical efficacy for a resorbable material used perioperatively during ESS is likely due to the numerous requirements for this material: it must be hemostatic, promote wound healing, reduce adhesion formation, visibly and uniformly cover all healing surfaces, and minimize tissue trauma during future debridements. As a result, no single biomaterial has gained widespread acceptance, and significant effort has been devoted to developing novel biomaterials that may demonstrate these multiple requirements.^{16,17}

A major factor as to why resorbable materials may not be effective in reducing adhesion formation is that their meshwork structures provide an ideal attachment site for bacterial growth and biofilm formation. Bacterial biofilms are comprised of bacterial communities that adhere to natural and synthetic materials via a self-produced matrix of extracellular polymeric substance (EPS) generally composed of extracellular DNA, proteins, and polysaccharides. Bacteria undergo a phenotypic shift and differentially regulate many genes when switching to a biofilm mode of growth. The EPS and other components of this phenotype confer significant resistance to clearance of these biofilms, with 1000-fold resistance to antibiotic therapies demonstrated compared to their planktonic forms.¹² The EPS layer accounts for up to 90% of the mass of the biofilm and is semipermeable, in that it selectively allows diffusion of water, nutrients and other essential substances through the matrix and reduces access of antibiotics, host defense cells and other microorganisms to the underlying bacterial cells.¹⁸ Our laboratory has demonstrated in recent years – by means of the study of nontypeable *Haemophilus influenzae* (NTHI) biofilms produced *in vivo* – that the extracellular DNA in the EPS is in fact organized into an interwoven mesh-like pattern.¹⁹ Although this arrangement of eDNA may not be consistent from one microorganism to another, the complex architecture of the extracellular matrix (ECM) indicates that the eDNA is organized and that the structural integrity of this matrix is likely essential to the survival of the biofilm. We also recently demonstrated that members of the DNABII family of proteins are critical for the integrity of the EPS. Integration Host Factor (IHF), one of the members of this family of proteins, exists as a heterodimer and binds and compacts extracellular DNA within the EPS of bacterial biofilms. Exposure of these biofilms to antibodies directed against IHF mediated notable debulking of the EPS. As such, we suggest that antibodies directed against IHF might serve as an attractive novel therapeutic to debulk biofilms and render them susceptible to both traditional antibiotic therapy as well as a variety of innate and acquired immune effectors. Such treatment would likely mediate more effective clearance of bacterial biofilms from resorbable packing materials such as Nasopore without limiting its ability to reduce post-surgical mucosal trauma and/or promote healing.

In our first sets of experiments, we demonstrated that Nasopore acts as a suitable substrate for *in vitro* biofilm formation by a clinical isolate of NTHI, the most prevalent bacterial pathogen found in chronic rhinosinusitis. This study is thus the first to look at biofilm

growth on intranasal packing materials, and Nasopore in particular. We were surprised by the rapidity and size of the biofilm that formed on our samples, as these were significantly more robust than were those that formed on either glass surfaces or other substrates in our experience.

Interestingly, in this study the biofilms treated with amoxicillin/clavulanate alone showed an *increase* in biofilm biomass when compared to sterile medium alone. This finding supports previous research demonstrating that biofilm formation can be a specific, defensive reaction to the presence of antibiotics.²⁰ Importantly, only biomass on the Nasopore was examined, and total bacterial counts (which would include planktonic forms suspended in media) were not included. Although the reason behind this increased biomass in the presence of antibiotics is still being investigated, it was clear that this commonly used perioperative antibiotic alone was not able to mediate either a reduction or eradication of the NTHI-induced biofilm that had formed on cubes of Nasopore packing material. It is a bit concerning that amoxicillin/clavulanate had no treatment effect in our study, as this is one of the first-line agents used commonly in the perioperative period following ESS. However, given that it is well known that bacteria growing within a biofilm can be 1000-fold more resistant to the action of antibiotics¹², this observation is not unexpected and suggests that targeting the biofilm matrix for intervention is likely a wise strategy in terms of development of novel treatment regimens in situation wherein nasal packing materials are indicated for use.

Nonetheless, when amoxicillin/clavulanate was used in concert with anti-IHF antibodies, a strong synergistic effect was observed wherein almost complete dissolution of the biofilm on the incubated Nasopore was achieved. These results are consistent with previous studies demonstrating the synergistic effect of anti-IHF antibodies in enhancing clearance of biofilms.¹³ and which suggest that anti-IHF mediated collapse of the biofilm structural matrix mediates release of bacteria from the biofilm community. These planktonic bacterial cells are likely more susceptible to the action of antibiotics.

CONCLUSION

It is yet to be determined how these *in vitro* findings translate to elucidating the pathophysiology of adhesion formation in actual patients undergoing ESS. Important questions yet to be addressed include examination of the types, structures and integrity of biofilms that form on and/or within nasal packing material. It is unlikely that biofilm formation is unique to any single type of packing material, although different types of materials are likely to support bacterial growth and/or biofilm formation *in vitro* and *in vivo* that is microbe-dependent. Our results suggest great promise for the clinical utility of anti-IHF to be used in conjunction with the application of nasal implants such as Nasopore for use perioperatively with ESS. Further work to translate these promising findings *in vitro* to a product that is useful in the operating room or at the patient's bedside will be performed to improve clinical outcomes associated with intranasal packing use during nasal surgery.

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Figure 1.

Representative confocal microscopy image of Live/Dead staining of NTHI biofilms after 36 hours of growth on Nasopore packing. Biofilms grew to a mean height of $96.6 \mu\text{m} \pm (11.6 \mu\text{m})$ at 36 hours post-inoculation. Note in Figure 1 that the Nasopore takes up the Live/Dead stain and appears as the large green portion surrounding the biofilm. The majority of bacteria demonstrated uptake of live staining (green) with a very small percentage of bacteria demonstrating uptake of dead staining (red).

Figure 2.

Confocal microscopy images of biofilms stained with Filmtracer (which does not label Nasopore) demonstrating the effect of incubation with naïve rabbit serum (panel a), amoxicillin/clavulanate (panel b), rabbit anti-IHF (panel c) and rabbit anti-IHF plus amoxicillin/clavulanate (panel d). Biofilms treated with naïve serum demonstrated a mean biomass of $5.79 \mu^2/\mu^3$. Treatment with amoxicillin/clavulanate alone *increased* biofilm mass by $3.17 \mu^2/\mu^3$ to $6.63 \mu^2/\mu^3$ when compared to treatment with sterile sBHI alone. Treatment with anti-IHF reduced biofilm size by 77% to $1.29 \mu^2/\mu^3$ when compared to treatment with naïve rabbit serum. Treatment with anti-IHF and amoxicillin/clavulanate significantly reduced biofilm size by 88% to $0.66 \mu^2/\mu^3$ ($p < 0.02$) when compared to treatment with naïve rabbit serum.

A

B

Figure 3.

a) The mean biomass of biofilm growth on Nasopore after 24 hours of growth and 16 hours of treatment with naïve serum (control), amoxicillin/clavulanate, anti-IHF antibodies, or a combination of amoxicillin/clavulanate and anti-IHF. b) Percent reduction of biomass compared to control. Treatment with amoxicillin/clavulanate alone led to a 92% *increase* in biomass compared to sterile sBHI alone. Treatment with anti-IHF alone led to a 77% reduction in biomass compared to treatment with naïve rabbit serum. Treatment with anti-

IHF and amoxicillin/clavulanate led to a significant 88% reduction in biomass compared to treatment with naïve rabbit serum. * = $p < 0.02$

Table 1

Results of literature review examining post-operative bleeding and wound healing between use of resorbable packing compared to traditional packing material or no packing material

Packing Materials		Postoperative Bleeding			Wound Healing		
		Increased	Same	Decreased	Improved	Same	Decreased
Resorbable vs. Traditional	Nasopore® ²¹		×			×	
	Merogel® ²²		×		×		
	Nasopore® ²³	×					×
	Merocel® ²³		×			×	
	FloSeal® ³		×				×
	FloSeal® ⁷			×		×	
Resorbable vs. No Packing	Meropack® ²⁴		×			×	
	CMC ^{25,26}		×			×	
	MPH ⁶					×	
	Merocel® ⁹		×				
	Gelfoam ²⁷			×		×	
	Merogel® ²⁸					×	
Total		1/11	8/11	2/11	1/11	8/11	3/11

Abbreviations: CMC = carboxy-methylated cellulose, MPH = microporous polysaccharide hemospheres