Evaluation of the in vivo efficacy of topical tobramycin against Pseudomonas sinonasal biofilms

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Background: There is increasing evidence that bacterial biofilms are present within the sinonasal mucosa of patients with medically recalcitrant chronic rhinosinusitis. The antibiotic concentrations needed to eradicate biofilms are much higher than those commonly used and pose an increased risk for systemic side effects. Topical medications are an alternative approach to deliver high concentrations to the mucosa while limiting systemic side effects. This is the first study to evaluate the effects of a topical antibiotic on Pseudomonas biofilms in an animal model of sinusitis.

Methods: Pseudomonas sinusitis was established in nine rabbits. An irrigation catheter was placed within the lumen of the maxillary sinus, and the rabbits were irrigated with 7 days of normal saline or varying concentrations of tobramycin diluted in normal saline. Bacterial cfu were determined for nasal lavage fluid as well as physically disrupted mucosa, and scanning electron microscopy (SEM) of the mucosal surface was performed at the end of the study.

Results: Increasing concentrations of topical tobramycin resulted in the eradication of viable bacteria within the lumen of the sinus but did not eradicate Pseudomonas attached to the mucosa. SEM detected biofilms within the mucosa even in those rabbits treated with high concentrations of topical tobramycin.

Conclusions: Bacterial biofilms attached to the sinonasal mucosa are resistant to topical saline and tobramycin irrigation. SEM was useful in the identification of biofilms, but did not provide a quantitative evaluation for efficacy of treatment.

Keywords: rhinosinusitis, animal model, bacteria

Introduction

Pseudomonas exacerbation of chronic rhinosinusitis following functional endoscopic sinus surgery (FESS) is a common condition seen in over 30% of endoscopically obtained sinonasal cultures. These infections are frequently relapsing and often refractory to oral antibiotics. Colonization with Pseudomonas aeruginosa and subsequent biofilm formation have been demonstrated to occur in the lower airways and within the sinonasal mucosa. Persistence of this infection often leads to chronic mucosal inflammation, altered sinonasal ciliary function and nasal polyp formation. Pseudomonas colonization of the sinus mucosa has also been implicated in diminished lower airway function and has been identified as one of the main reasons for pulmonary complications following lung transplant in cystic fibrosis (CF) patients.

The treatment of biofilm- and non-biofilm-forming Pseudomonas rhinosinusitis remains largely ineffective and vastly understudied. There are few clinical trials or animal studies looking at this subject, and as a result, many patients are forced to undergo multiple sinus surgeries and prolonged courses of systemic medications that have been ineffective in preventing the colonization of Pseudomonas, formation of biofilms or disruption of existing biofilms.

Bacterial biofilms, which are microcolonies encased in the extracellular polysaccharide material, lack the susceptibility to antibiotics that planktonic bacteria possess. The minimal biofilm eradication concentration (MBEC) for Pseudomonas biofilms has been demonstrated to be 60-fold greater than the MIC of gentamicin and >1000-fold for ceftazidime and piperacillin. Systemic treatment with such high concentrations may result in significant systemic side effects and preclude their use.
Topical tobramycin against sinonasal biofilms

Topical medications are an alternative treatment method aimed at delivering antibiotics directly to the sinus mucosa. This approach has been utilized in a nebulized form for pulmonary treatments in CF patients. The obvious advantage of topical preparations of intravenous (iv) antibiotics is mucosal exposure to high therapeutic concentrations with limited systemic side effects, thereby effectively treating pseudomonal biofilms. Topical aminoglycosides have been used in the sinuses in CF patients undergoing pulmonary transplantation, resulting in decreased incidence of pulmonary complications following surgery. Additionally, they have been used anecdotally in humans with sinusitis after FESS, but their effects have not been objectively measured.

The goal of this study was to objectively measure the in vivo effects of a topical aminoglycoside on sinonasal mucosal Pseudomonas biofilms. We have previously described the use of scanning electron microscopy (SEM) in the description and identification of bacterial biofilms in an animal model of Pseudomonas sinusitis. We have adapted this model to incorporate an indwelling single lumen catheter with the ability to deliver aqueous medications directly to infected sinus mucosa. Using this model, we will set out to determine the in vivo efficacy of topical tobramycin against sinonasal mucosal biofilms.

Materials and methods

Animal model

A widely accepted animal model was used to induce Pseudomonas biofilm sinusitis. Briefly, Pasteurella-free, female, New Zealand white rabbits (2–4 kg) were used and acclimatized to the animal facility 1 week before study initiation. Each rabbit was separately caged and had free access to standard pelleted food and water throughout the experiment. The protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee and the investigation was conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, DDH, Publ. No. 85-23.

Surgical technique for sinusitis

The rabbits were anaesthetized with intramuscular ketamine and vital signs were recorded. No rabbits were intubated or given any inhalational anaesthetic agents. The skin overlying the dorsum of the nose was incised with a no. 15 blade in a vertical line down the midline. A laterally based flap of periosteum was elevated from the nasal dorsum and brought out through a stab incision at the vertex. The hub of the tubing was capped and secured to the skin with a vicryl sutures were used to drape the periosteum over the catheter. The sinus was then inoculated through the surgical antrostomy with 0.2 mL of wild-type P. aeruginosa (PAO-1 with gentamicin resistance) grown up that day and collected during log-phase growth. The choice of PAO-1 with gentamicin resistance had been made on the basis of its firm establishment as an inducer of biofilm sinusitis. The concentration was adjusted to an OD_{600} = 0.6 in sterile saline (4.0 × 10^8 cfu). One rabbit served as a control in which a surface attachment defective (sad) mutant strain of Pseudomonas, sad-36 (provided by Dr George O’Toole, Dartmouth University), incapable of forming biofilms, was introduced into the sinus. Next, the perioveal flap was replaced over the surgical antrostomy and fixed in place by ‘spot welding’ with a small application of Vet Bond to create an airtight closure. Finally, the skin incision was closed with a running vicryl stitch.

Surgical technique for irrigating catheter placement

After 1 week, the rabbits were taken back to the operating room. The midline incision and periosteal flaps were re-opened, and the cotton pledges were removed from the infundibulum by the attached string, relieving the osteal obstruction. A culture was obtained from the sinus to confirm Pseudomonas infection, and 100 μL of pus removed and placed on ice for quantification of bacteria. Single lumen tubing (0.32 cm diameter and 30 cm long) was used as the irrigating catheter. Tubing length was sized to fit within the previously made antrostomy, tunnelled under the skin of the nasal dorsum and brought out through a stab incision at the vertex of the cranium between the ears of the rabbit. Along its course, vicryl sutures were used to drape the periosteum over the catheter.

Study design: irrigations

Following placement of the irrigating catheter, nine rabbits, eight of which were infected with biofilm-forming PAO-1 Pseudomonas and one of which was infected with the sad-36 mutant, were exposed to either normal saline or various concentrations of iv tobramycin diluted in normal saline up to 400 times the established MIC for PAO-1 of tobramycin (1 mg/L). The choice of tobramycin was based on its popularity in clinical use for Pseudomonas sinus and pulmonary infections in the CF population.

Prior to irrigating each rabbit with the study medication, the irrigation line and sinus were flushed with 5 mL of saline. Then, 5 mL of the study medication was irrigated through the catheter once a day for 7 days. The irrigation catheter was connected to a peristaltic pump that delivered the irrigation at a rate of 20 mL/min. The rabbits were held in a rabbit holder and no anaesthetics were used. Three rabbits, including the rabbit infected with a sad-36 mutant, received 7 days of 5 mL of normal saline through the irrigation catheter. Three groups of two rabbits each were irrigated with 7 days of 5 mL of the following concentrations of tobramycin mixed in normal saline: 80 mg/L (80×MIC), 160 mg/L (160×MIC) and 400 mg/L (400×MIC).

Objective measures

On treatment days 1, 3, 5 and 7, 5 mL of saline was irrigated through the catheter and the fluid that emerged through the snout was collected. This fluid was then subjected to centrifugation. The bacterial pellet was then resuspended in saline and serial dilutions were plated on LB agar gentamicin plates (100 mg/L) and colony...
counts were performed the following day resulting in 50–200 colonies/plate.

At the end of 7 days of irrigation (day 14 from inoculation), all the rabbits were euthanized. In each rabbit, the mucosa was extracted from the surrounding bone of the maxillary sinus. The sad-36 mucosa, as well as the mucosa from one rabbit of each study arm (normal saline and 80, 160 and 400× MIC of tobramycin), was prepared for study under SEM for biofilm identification. Formalin-fixed mucosal specimens were dehydrated in a series of increasing ethanol concentrations, up to 100%. The mucosa was then critical-point dried in CO₂, mounted on SEM stubs and sputter coated with gold palladium to a depth of 12 nm. Next, the mucosa was examined with an AMR-1400 SEM at an accelerating voltage of 20 kV. Representative photomicrographs of the stubs (captured as TIFF files) were taken at various angles to most effectively display the specimen so that any error in assessment is minimized because of the tilt of the specimen or other artefacts. These images were taken at a variety of magnifications, from 200× to 5000×. Photomicrographs were then evaluated for morphological evidence of appropriate-sized (0.5–2 μm diameter) and rod-shaped bacteria existing in towers with intertwining water channels. Representative pictures, blinded to the study group, were then analysed by the three senior authors of the study (A. G. C., J. N. P. and N. A. C.) for the presence of biofilms.

The mucosa of the other four rabbits, each being treated with normal saline or 80, 160 or 400× MIC of tobramycin, was evaluated for viable bacteria adherent to the mucosal surface. The mucosa was washed gently three times with normal saline and vortexed in 1 mL of saline. The saline group and each tobramycin concentration group, including those receiving 400 mg/L, grew bacteria, demonstrating that, despite clearance of viable bacteria in the sinus lumen, there was persistence of viable bacteria attached to the sinonasal mucosa. We may be able to infer that this is due to persistence of biofilms within the mucosa.

SEM was used to analyse the mucosa of five rabbits receiving normal saline or tobramycin irrigation. Bacterial biofilms, characterized by the morphological evidence of appropriately-sized (0.5–2 μm diameter) and rod-shaped bacteria existing in towers with intertwining water channels, were identified in the rabbits irrigated with normal saline and 80, 160 and 400 mg/L of tobramycin (Figure 2). Bacterial biofilms were not identified in the control rabbit infected with the non-biofilm-forming sad-36 mutant (Figure 3).

Discussion

This is the first reported study that attempts to examine the in vivo efficacy of antimicrobials in the treatment of sinonasal mucosal bacterial biofilms. Biofilms in biomedical contexts are insidious because of their resistance to antibiotic therapy and physical attachment to biotic surfaces. The persistence of biofilms is largely due to their encasement method of growth, whereby P. aeruginosa grows in microcolonies surrounded by an extracellular matrix of the exopolysaccharide alginate. Recent studies in our lab,11 and subsequent confirmation by others,12–14 have identified biofilms in the sinus mucosa of patients with chronic rhinosinusitis. The logical progression after identification of biofilms is the development of treatment options to prevent their formation and eradicate pre-formed biofilms.
Topical tobramycin against sinonasal biofilms

Bacterial biofilms are a complex organization of bacteria anchored to a surface. They begin as a random collection of independent free-floating, planktonic bacteria, which then attach to a surface and begin to form microcolonies. When bacterial density reaches a critical point, interbacterial crosstalk triggers a phenomenon known as ‘quorum sensing’. Quorum sensing in turn initiates a cascade of protein expression that ultimately leads to the biofilm phenotype. This phenotype is marked by the formation of towers composed of layers of embedded live bacteria with intertwining water channels nourishing the individual bacteria and demonstrates a functional heterogeneous community. The ‘mortar’ for these structures comprised a bacterially extruded exopolysaccharide matrix, which makes up as much as 90% of the biofilm.15

Existing in a biofilm phenotype, bacteria can evade host defences and demonstrate decreased susceptibility to systemic and local antibiotic therapy. Finally, biofilms can deliberately release bacteria in a planktonic form, causing new acute infections in remote sites.

The MBEC for Pseudomonas has been demonstrated to be >60× the MIC of gentamicin. Systemic concentrations at this strength will result in considerable morbidity. Topical medications are an alternative method to delivering high concentrations of iv antibiotics directly to the diseased mucosa while limiting systemic absorption.

To better study the effects of topical antibiotics on Pseudomonas biofilms, we first modified the animal model for Pseudomonas biofilm sinusitis and developed a catheter delivery system that allows for the instillation and evaluation of efficacy of topical medications. We have demonstrated that after 7 days of irrigation with normal saline, there is a histological persistence of purulence, mucosal inflammation and underlying inflammatory bony changes indistinguishable from that of rabbits infected with biofilm-forming Pseudomonas but not receiving any irrigation.7

A second study was performed to determine the effects of 7 days of irrigation with increasing concentrations of topical tobramycin.16 Histopathological analysis of sinonasal complexes from rabbits treated with increasing concentrations of topical tobramycin demonstrated a dose-dependent response in terms of decreasing inflammation and infection in the sinus lumen, sinonasal mucosa, as well as the underlying bone. At doses 80× the MIC (80 mg/L) and currently the concentration recommended for clinical use in humans, topical tobramycin did not have an effect on the histological degree of infection within the mucosa and sinus lumen. The only effective concentration that demonstrated a significant histological improvement was 400× MIC or 5 times the normal clinical concentration used in humans. Despite the improvement and clearance of infection within the maxillary sinus lumen, there was still a persistence of mild mucosal inflammation and underlying bony inflammation.

Using sinonasal lavage to account for viable bacteria, we have shown in this study a clearance of viable bacteria from the maxillary sinus lumen with increasing concentrations of topical tobramycin, indicative of an eradication of planktonic bacteria. However, after isolating and sonicating the sinonasal mucosa, there was a positive bacterial growth indicative of viable bacteria adherent to the mucosa but not present within the lumen of the maxillary sinus. This was seen even in the animals irrigated with 400× MIC of tobramycin. These findings are consistent with the model of bacterial biofilms, in which the bacteria are adherent to the mucosa in biofilm structure, only to release planktonic bacteria and cause recurrent infection at the same or within a remote site. This may also help to explain the clinical scenario in which a patient feels better while on an antibiotic, only to quickly relapse with symptoms when the course of antibiotics has finished.

In this study, SEM was used as the main imaging tool to assess the presence of bacterial biofilms. SEM has previously been shown to be helpful in aiding in the identification of biofilms adherent to sinus mucosa. In this study, SEM was able to identify the persistence of bacterial biofilms after treatment with saline and tobramycin nasal irrigations. Although we were able to use it as a tool for identification, SEM was unable to provide a quantitative response to therapy.

SEM provides high-resolution images of the mucosal surface and its associated structures, but there are several shortcomings that make this technique a less than ideal modality for evaluation of therapeutic response. One potential source of bias is in the subjective nature of reading SEM. The slides were read independently by three separate and blinded authors. Only those slides in which the definite presence or lack of biofilms was agreed upon by all three authors were included in this study. Both mucus lining the mucosal surface and biofilm matrix encompassing the bacteria are composed of long-chain polysaccharides.17 These can look very similar under SEM and may have been the cause of multiple slides being dismissed owing to a lack of concordance between the three reviewing authors.

SEM also has other drawbacks in its lengthy preparation time and orientation of the tissue specimens. Only small areas of 10–20 μm of tissue are analysed at any one time. Therefore, the quantitative evaluation of biofilm structures within the mucosa is nearly impossible to achieve in a 1–2 cm sample of tissue.

Transmission electron microscopy provides greater depth of examination, but has similar flaws as SEM in that small sections of tissue are analysed at any one time. Confocal scanning laser microscopy using green-fluorescent-tagged bacteria, live–dead and alginate staining has been used for in vitro studies of bacterial biofilms and may be a more reliable identifier of bacterial...
biofilms. This technique would also help better illustrate the presence of *Pseudomonas* within the mortar of the biofilm. This modality too only examines microns of tissue at a time, making the quantitative evaluation of large mucosal areas of biofilms problematic. At this point in time, studies examining the therapeutic efficacy of medications against biofilms are mostly relegated to identifying the presence or lack of bacterial biofilms, as opposed to the percentage of biofilm forms eradicated. Additionally, the percentage of residual biofilms needs to be correlated with clinical relevance and thus additional efforts are needed in order to better quantify treatment success.

**Conclusions**

Sinonasal *Pseudomonas* biofilms are difficult to eradicate with currently used topical preparations. Despite the clearance of bacteria within the lumen of the maxillary sinus after treatment with high concentrations of topical tobramycin, there was a persistence of viable bacteria attached to the sinus mucosa. We have inferred this to represent the presence of bacterial biofilms, which were later identified on SEM. This is the first report to examine the effects of topical medications on sinonasal bacterial biofilms. SEM has proven to be a valuable tool to identify biofilms, but further research techniques are needed to provide *in vivo* quantification of biofilm response to therapy.

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**References**


