A rat model of Staphylococcus aureus biofilm in rhinosinusitis

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Abstract: Objective: Bacterial biofilms are thought to play a major role in recalcitrant chronic rhinosinusitis (CRS). The exact mechanisms by which bacterial biofilms negatively impact the outcome of CRS are unknown. Prevention and eradication of bacterial biofilms in individuals with CRS warrant further studies. Specifically, these studies require an accurate animal model to assess the interactions of biofilms and the host during CRS infections. Thus, the goal of this study was to develop a rat model of rhinosinusitis to study Staphylococcus aureus biofilms. Methods: We utilized 12 rats for this study. We performed surgery on these rats to identify the maxillary sinuses. We then treated the maxillary sinuses with a *S. aureus* suspension and harvested maxillary mucosa at days 7 (six mice, group A) and 21 (six mice, group B). A group of six untreated rats served as the control (group C). Bacterial biofilms were identified by scanning electron microscopy (SEM) and fluorescence in situ hybridization/confocal laser scanning microscopy (FISH/CLSM). Results: SEM and FISH/CLSM revealed *S. aureus* biofilm formation in all rats from groups A and B as early as day 7. No biofilm formation was observed in the control rats. Conclusion: This study provides a suitable rat model to study the role of *S. aureus* biofilms in rhinosinusitis. The animal model can be utilized in future studies to elucidate the role of biofilms in rhinosinusitis, thus leading to the discovery of new therapeutic targets for this infection.

Keywords: Staphylococcus aureus biofilms, rhinosinusitis, rat model

Introduction

Biofilms are a mechanism that allows bacteria to evade host defenses and protects them from systemic and local antibiotic therapy. For these reasons, biofilms are thought to play a major role in recalcitrant CRS [1-5]. However, the exact role bacterial biofilms play in rhinosinusitis aetiopathogenesis, the interactions between the host and biofilms, as well as the factors that drive biofilm formation are unknown. Future studies will be necessary to investigate these unknowns to develop therapeutics that can prevent and eradicate biofilms from the nasal mucosa. Therefore, the aim of this study was to create an appropriate animal model that closely mimics biofilms in rhinosinusitis to enhance our knowledge of biofilms and the aetiopathogenesis of this disease.

To date, several studies have focused on an appropriate animal model to investigate the role of biofilms in rhinosinusitis. In 2006, Perloff and Palmer utilized a rhinosinusitis rabbit model in which biofilms formed on rabbit maxillary mucosa infected with *Pseudomonas aeruginosa* at 20 days post-infection [6]. In 2007, Ha et al. utilized a rhinosinusitis sheep model and reported that sinus ostium occlusion substantially promoted biofilm formation in infected sinuses [7]. More recently, a rhinosinusitis sheep model of biofilm formation in the frontal sinuses has been utilized [8-11]. However, a rhinosinusitis sheep model is not practical due to the difficulty in working with this animal as well as the high costs. Alternatively, a rhinosinusitis rat model has been reported previously [12, 13]. To date, few studies have focused on the role of biofilms in CRS utilizing a rhinosinusitis rat model. Rat is a more practical animal model because they are readily available and easy to use. Therefore, in this study we aimed to develop a rhinosinusitis rat model to investi-
gate the role of S. aureus biofilms in rhinosinusitis.

Materials and methods

Animals and bacteria strain

Eighteen 10-12 weeks old healthy male SD rats weighing 180-200 g were obtained from Shanghai Super-B&K laboratory animal Corp. Ltd. The rats were housed in groups of three animals per cage and acclimated for 2 weeks. All of the animal experiments were conducted in compliance with the Ethical Guidelines for the Care and Use of Animal for Research Purposes. Surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

S. aureus, China General Microbiological Culture Collection Center (CGMCC) 1.2386 was used for the inoculation. The strain was provided by China Sciences Institute of Microbiology and stored at 80°C until use. After being cultured on blood agar at 37°C overnight, the bacterial suspension was harvested and adjusted to 0.5 McFarland in sterile physiological saline (equivalent to $1\times10^8$ CFU/ml), then transferred to test tubes and placed on ice ready for instillation.

Surgical procedure for maxillary sinusitis

Experimental groups: 12 rats underwent sinus surgery to identify their maxillary sinus. The maxillary sinuses were treated with S. aureus instilled. Maxillary mucosa were harvested at day 7 (6 mice, group A) and day 21 (6 mice, group B) respectively after bacteria instilled. Another 6 rats without any treatment were considered as control group (group C).

Surgical procedures: The animals were sedated with intraperitoneally administered pentobarbital (45 mg/kg). The nasal dorsum was shaved and sterilized with 75% alcohol. The skin overlying the dorsum of the nose was incised with a blade in a vertical line about 3 mm beside the midline. The bone of the maxilla was exposed and an antrostomy was made until the maxillary sinus can be visualized. A small absorbable gelatin sponge was introduced into the maxillary sinus through the surgical antrostomy, 0.1 ml prepared S. aureus suspension was injected into the sinus with a syringe. The periosteal flap was replaced over the surgical antrostomy, and finally, the skin incision was closed with a stitch. All the rats were sent for watch after recovery.

Tissue collection

Rats were painlessly euthanized with a lethal dose of i.m. phenobarbitone at day 7 or day 21 after bacteria inoculation. The skin and anterior table of maxillary sinus were removed, exposing the sinus mucosa. The mucosa was carefully dissected using sterile instruments under a microscope at low magnification. All mucosa specimens except some for H&E staining were divided into two parts for biofilm detection, each about 2 mm x 3 mm. One part was washed thoroughly in saline, and then fixed in 2.5% glutaraldehyde at 5°C overnight for scanning electron microscopy (SEM) use. The other was washed thoroughly in three separate beakers of sterile MilliQ water to remove any planktonic bacteria for FISH analysis.

H&E specimen preparation and staining

The mucosal specimens were fixed in 4% paraformaldehyde for 24 hours and then embedded into paraffin blocks according to routine procedures. The blocks were cut to 5 um sections and stained with H&E using standard procedures. Then, the stained sections were examined by light microscopy and images were acquired with a digital camera at 400 × magnification.

Scanning electronic microscopy detection

Specimens for SEM were prepared using the following techniques. After two rinses of 15 min each in 0.1 M PBS, the specimens were fixed with 1% osmium tetroxide for 1 h, followed by another two rinses of 15 min each in 0.1 M PBS. Then they were dehydrated through a graded ethanol series as follows: 50% for 15 min, 70% for 15 min, 80% for 15 min, and 100% twice for 15 min each time. The tissue was immersed in 100% acetone for 15 min and washed in 100% isoamyl acetate for 15 min, followed by critical point drying for 2 h. Finally, the specimens were mounted on SEM stubs, and sputter coated with gold preparation for imaging.

Observations were carried out in our SEM laboratory using a Stereoscan 260 microscope (Leica, Wetzlar, Germany) at an acceleration
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voltage of 15 kV. Structures characterized by water channels, 3D structure, and matrix embedded spherical or elliptical bodies were identified as evidence of biofilms. The entire area of each specimen was scanned for the presence of biofilms structures. Representative photomicrographs were taken at various angles to effectively display the specimens so that any error in assessment is minimized because of the tilt of the specimen or other artifact.

CSLM-FISH detection

Probe species-specific for S. aureus were commercially available (AdvanDx, 05113A-US). FISH protocol was followed as the manufacturer’s instructions. Briefly, single 2 mm×3 mm pieces of sinus mucosa were fixed to individual glass slides. One drop of the fixation solution (phosphate-buffered saline with detergent) and 20 minutes of heating at 55°C was used to fix each specimen, dehydrated in 90% alcohol for 10 minutes, and air-dried. The probe was applied to the tissue and hybridization at 55°C occurred for 60 minutes. Slides were then washed in the manufacturer’s wash solution for 30 minutes at 55°C and air-dried before CSLM examination.

The post hybridization slides were analyzed using a Confocal Scanning Laser Microscope (Zeiss LSM510, Germany), with scanning at an excitation of 488 nm. The FISH probe was collected with a narrow band filter (505-550BP). The entire tissue area and depth were scanned by a professional investigator. Images of

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Figure 1. The anatomy of rat nasal sinuses and a visual depiction of maxillary sinusitis. A: CT images of rat maxillary sinuses (previously published [14]). B: The anatomical relationship of the rat nasal cavity (small arrow) and maxillary sinus (large arrow). C: Location of the incision into the anterolateral wall of the left maxillary sinus (large arrow). D: Congestion and edema with purulent discharge observed in the maxillary sinus mucosa from rats in group A. E: Congestion and edema of the maxillary sinus mucosa of rats in group B. F: Normal maxillary sinus mucosa was observed in control rats from group C.
Biofilms were identified as clusters and towers of immobile, irreversibly attached, intensely fluorescing. Bright green coccal structures fluorescing, approximately 0.5 to 3 μm in diameter, were visualized as live bacteria. A less intense “blush” surrounding the areas of discrete brightly fluorescing areas was deemed to represent the exopolysaccharide matrix of the biofilm.

Results

Gross observation and histological findings

At 5 to 7 days post-infection, rats infected with S. aureus had an increased frequency of sneezing, nasal rubbing and nasal discharge compared to control rats. After S. aureus treatment, rats in groups A and B were euthanized at days 7 and 21, respectively. The control mice (group C) were euthanized on day 7. The maxillary
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Sinuses from control rats had healthy mucosa and no purulent nasal discharge, while rats in group A and B had mucosa with mild to intense congestion and edema. In group A, purulent nasal discharge overlying the mucosal surface could also be observed (Figure 1). H&E staining of the sinus mucosa from groups A and B indicated dramatic histological changes. The epithelial cells from the sinus tissue of rats in group A appeared damaged or disintegrated. Furthermore, the sinus mucosa was swollen with marked infiltration of inflammatory cells, including neutrophils, lymphocytes, and plasma cells, as well as the presence of necrosis in these areas (Figure 2A). In the mucosal tissue of rats from group B, we observed mucosal swelling, infiltration of inflammatory cells in the epithelial and subepithelial layers of tissue, as well as slight hypertrophy of epithelial cells (Figure 2B). In contrast to the experimental groups, tissue samples from the control group were observed to be normal respiratory mucosa with columnar ciliated epithelium and no significant infiltration of inflammatory cells infiltrated into the subepithelial layer of tissue (Figure 2C).

SEM observations

Under microscopy bacterial biofilms are characterized by the presence of three-dimensional structures, spherical bodies, and water channels. All of these characteristics were identified in the sinuses of 12/12 rats in the experimental groups. Specimens from group A and B appeared similar under SEM. In both groups, we observed disarrayed cilia and in the absence of cilia numerous large protrusions or pods appearing as towers of spherical bodies. In the control group, though disarray and absence of cilia were also observed in some areas of the tissue, the majority of the tissue had healthy cilia and no evidence of biofilm formation (Figure 3).

FISH/CLSM observations

We utilized FISH/CLSM to analyze the mucosal tissue of rats from groups A and B. In all 12 rats from both groups, we observed clusters of intense green fluorescence surrounded by an area of less-intense fluorescence; spots of bright green fluorescence were also observed, indicating the presence of live bacteria (Figure 4A, 4B). Collectively, these results mimic the characteristics of biofilms as described previously [7, 15, 16].

In group C, the control group, no green fluorescence was evident (Figure 4C).

Discussion

To develop the most appropriate rhinosinusitis model to study the involvement of biofilms in CRS, several factors need to be addressed. First, it is important to select a practical animal as well as the appropriate bacterium for this model. Second, it was necessary to determine the dose and duration of infection. Lastly, it is important to correctly identify qualitative and quantitative analyses to investigate the presence of biofilms.
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To identify an appropriate animal we considered availability, low cost, and ease of use. Sheep are problematic because of their high cost and difficulty in use. Alternatively, rats are favorable for use in medical research because of their small size, low cost, availability, fast reproduction rate, and ease of use. Rats share a high genetic homology with humans and can be genetically manipulated. Although mice have similar advantages to rats, their small sinus cavity presents a challenge for sinusitis studies. According to the literature, S. aureus biofilms have been closely associated with unfavorable outcomes in CRS patients [2, 17]. S. aureus biofilms are also known to negatively affect the outcomes of endoscopic sinus surgeries by causing mucosal inflammation and leading to postoperative infections. After careful analysis, we selected S. aureus for this model because of its ability to readily form biofilms and its role in affecting the outcome of sinus-related infections.

To investigate the success of our proposed model it was necessary to first locate the maxillary sinus in a rat. We observed that an antrostomy located about 3 mm beside the midline and 4 mm below the eye was the location of the maxillary sinus. In a previous study utilizing a rhinosinusitis sheep model, 1 ml of bacterial culture in a 0.5 McFarland suspension was injected into the frontal sinus and after 7 days biofilm formation on this sinus tissue was observed [7, 10, 11]. Another study that evaluated a rhinosinusitis rabbit model indicated that biofilms form within 5 days post-infection and can last for several weeks [6, 18]. According to previous studies utilizing a rat rhinosinusitis model [13], we injected 0.1 ml of a 0.5 McFarland S. aureus suspension into the maxillary sinus. We observed the presence of biofilms at days 7 and 21. Our results demonstrate the ability of S. aureus to form a mature biofilm in the maxillary sinus of rats.

Biofilms can be analyzed using a variety of techniques, including haematoxylin and eosin (H&E) staining [19, 20], scanning electron microscopy (SEM), transmission electron microscopy (TEM), and fluorescence in situ hybridization (FISH)/confocal laser scanning microscopy (CLSM) [21]. The combination of SEM and FISH/CLSM can greatly increase the sensitivity and specificity for detecting biofilms. SEM provides high-resolution images of the mucosal surface, highlighting three-dimensional structures. However, a limitation associated with this technique is the inability to quantitatively evaluate biofilm structures and identify bacterial species within the biofilm. The combination of FISH/CLSM has the greatest level of accuracy for detecting biofilms. Because the samples are processed immediately, there is no need to fix the tissue. This process increases the viability and integrity of the biofilm structure, thus enhancing our visualization of these structures. Live bacteria can be differentiated from other cells because of their smaller size and intense fluorescence. A species-specific FISH probe can be utilized to stain specifically for a particular bacterial strain. In our study, we used a probe for S. aureus that facilitated identification of this bacterium, as evidenced by the bright green-stained cocci. Furthermore, these images can be quantitatively assessed using the CLSM imaging software COMSTAT. In this study, we did not perform a quantitative analysis because of the small sample size. However, this technique has been reported to enable quantification of biofilms on the sinonasal mucosa [11]. Quantitative assessment may provide information regarding the degree of biofilm involvement in sinusitis and potentially facilitate development of a more-effective biofilm-targeted treatment.

Conclusions

In conclusion, we successfully developed an experimental rhinosinusitis rat model utilizing S. aureus biofilms to investigate the involvement of biofilms in rhinosinusitis. SEM and CLSM were enabled investigation of the presence of biofilms in tissue from maxillary sinuses. This model will prove useful for future studies of the role of biofilms in the aetiopathogenesis of rhinosinusitis, and facilitate development of a more specific therapeutic against biofilms for treating CRS.

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Disclosure of conflict of interest

None.

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