# Investigating Bacterial Biofilms in Chronic Rhinosinusitis: An *In vitro* Study, *In vivo* Animal Study and a Examination of Biofilms in Human CRS

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The degree of Master of Surgery

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# ABSTRACT

**Introduction**

Bacterial biofilms have been implicated in the pathogenesis of Chronic Rhinosinusitis (CRS). This thesis consists of a number of separate studies. The results of each study were designed to help provide an evolution of knowledge that could be applied to our subsequent investigations on the topic of bacterial biofilms and chronic rhinosinusitis. In vitro studies were utilized to document the capacity of CRS bacteria to form biofilms as well as to investigate the efficacy of various antimicrobials at high concentrations. Additionally, an in vivo sheep model was developed to examine different biofilm detection techniques. Finally, a study of CRS patients was conducted to investigate the incidence of biofilm related sinus disease.

**Methods**

Our *in vitro* studies used 96 well crystal violet microtiter plate assays to determine the biofilm growth characteristics of S.aureus isolated from patients with CRS. Established biofilms were then subjected various antimicrobial agents, and the degree of biofilm reduction calculated to examine their potential for sinus biofilm treatment. A sheep sinusitis model involved performing endoscopic sinus surgery, occlusion of frontal sinus ostia and the introduction of bacteria. Mucosal specimens were subsequently examined for the presence of bacterial biofilms using transmission electron microscopy (TEM), scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM).

CSLM was also used in a prospective study to document the presence bacterial biofilms on the mucosa of patients with CRS compared to controls.

**Results**

The findings of in vitro experiments revealed that not all isolates were capable of forming biofilms. Of the antibiotics tested, only Mupirocin was capable of reducing biofilm mass by 90% in all isolates. The animal model showed considerable variation in biofilm detection rates. The CSLM biofilm detection rate was 100% in obstructed sinuses with bacteria introduced, whereas TEM detected only 66%. Both these objective measures failed to identify biofilms in control groups. SEM found biofilms in all experimental groups including controls. CSLM analysis of CRS patients found Bacterial biofilms in 44% and no biofilms in controls.

**Conclusion**

The demonstration of biofilms in the sheep model for sinusitis and biofilms on the mucosal specimens of patients with CRS, and the ability of bacteria in CRS to form biofilms in vitro, further supports the hypothesis that biofilms play a role in the pathogenesis of CRS. CSLM is the modality of choice in documenting the presence of bacterial biofilms on sinus mucosal surfaces due to the inherent flaws of sampling error and subjectivity of TEM and SEM. Finally, CRS is a multi-factorial disease, topical Mupirocin via nasal irrigation may be a therapeutic option in patients with likely S.aureus biofilms.

# DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Kien Rach Ha

1st December, 2008

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# PREFACE

A portion of the work described within this thesis has been published, as listed below.

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Ha, K.R.#, Psaltis, A.J#., Butcher, A.R., Tan, L & Wormald, P.J. A Sheep Model for the Study of Biofilms in Rhinosinusitis. *Am J Rhinol* **21**: 339-345 (2007).

Ha, K.R.#, Psaltis, A.J#., Beule, A.G., Tan, L.W. & Wormald, P.J. Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis. *Laryngoscope* **117**: 1302-1306 (2007).

# Co-First Authors

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# CHAPTER 1

# AIMS

The aims of this thesis were:

1. To develop an *in vitro* model for the study of biofilms and examine optimal *in vitro* biofilm growth conditions of *Staphylococcus aureus*.

2. To determine the *in vitro* biofilm growth potential of *Staphylococcus aureus* bacterial strains isolated from patients with chronic rhinosinusitis

3. To evaluate the *in vitro* effects of antibiotics Mupirocin, Ciprofloxacin and Vancomycin on established biofilms of *S.aureus* chronic rhinosinusitis clinical isolates.

4. To develop an *in vivo* animal model for the study of bacterial biofilms in chronic rhinosinusitis.

5. To evaluate the use of transmission electron microscopy, scanning laser microscopy and confocal scanning laser microscopy in the detection of bacterial biofilms on sinus mucosal surfaces.

6. To investigate the presence of biofilms in patients with chronic rhinosinusitis using confocal scanning laser microscopy.

# CHAPTER 2

# INTRODUCTION

## Chronic Rhinosinusitis

### Definition and Disease Burden

Chronic Rhinosinusitis (CRS) is a spectrum of disorders and infections characterized by mucosal inflammation of the nose and paranasal sinuses. In 2003, The Rhinosinusitis Task Force defined CRS as having symptoms and signs for greater than 12 weeks with confirmatory radiologic evidence and/or signs of inflammation on physical examination and naso-endoscopic examination evidence of inflammation.[1]

According to Benninger et al., the major signs and symptoms of CRS include: facial pain or pressure, nasal obstruction or blockage, nasal discharge or purulence or discolored postnasal discharge, hyposmia or anosmia, and purulence in nasal cavity on examination. The minor symptoms of CRS include headache, fever, halitosis, fatigue, dental pain, cough, and ear pain, pressure, or fullness.[1]

CRS is one of the most frequently diagnosed chronic diseases affecting up to 16% of American adults.[2] The economic impact of direct treatment of this disease was estimated at $5.8 billion dollars in 1996.[3] Morbidity from CRS symptoms has a substantial adverse effect on mood, physical functioning and social functioning.[4]

### Pathogenesis

CRS is thought to be a multi-factorial disease and although there has been a plethora of research on the disease entity over the last 2 decades, the exact etiology and pathogenesis

of this condition remains elusive.  Factors which have been implicated in the development of CRS include:  super-antigens,[5] abnormalities of the inflammatory cytokine cascade,[6] abnormal cell-mediated immune responses, protracted osteitis of the sinus walls,[7] and the existence of biofilms.[8, 9]

**Bacteria and Chronic Rhinosinusitis**

Bacteria are likely to play a major role in CRS.  The most commonly isolated organisms include *Staphylococcus aureus*, *Staphylococcus epidermidis* and anaerobic gram-negative bacilli.[10]  *S. epidermidis* is generally a low virulence organism and is considered a nasal colonizer.[11]  Gram negative enteric rods including *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Enterobacter* and *Escherichia coli* have also been reported, however, their pathogenicity as primary infective agents in CRS is questionable.[12]

It is believed that CRS may be sequelae of unresolved acute sinusitis.  A theory suggested by Brook states that following an acute episode of sinusitis (usually viral), the environment within the sinus is altered in a way which promotes the growth of other organisms as well as a persistent inflammatory state, which subsequently fosters anaerobic propagation.[12]

***Staphylococcus aureus* and Chronic Rhinosinusitis**

*S. aureus* is a facultatively anaerobic, gram-positive coccus, which are perfectly spherical cells measuring about 1 micrometer in diameter and grow in grape-like clusters. The bacterium is responsible for a wide variety of illnesses in humans, ranging from minor

skin infections to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, Toxic shock syndrome, and septicemia.[13]

*S. aureus* has particular relevance to the probable pathogenesis of CRS for several reasons. Firstly, *S. aureus* is the most common pathogenic aerobic bacteria cultured from with CRS.[14-16] Secondly, Seiberling et al. reported the presence of *Staphylococcal* exotoxins in patients with CRS with nasal polyposis, postulating that these superantigens promote the development of lymphocytic and eosinophilic mucosal infiltrate.[17] Finally, the identification *S. aureus* biofilms on CRS sinus mucosal specimens using fluorescence in-situ hybridization (FISH),[18] and the finding that *S. aureus* isolated from patients with CRS have the capacity to form biofilms,[19] raises the possibility that *Staphylococcal* biofilms are involved in the pathogenesis of CRS.

## Bacterial Biofilms

### Definition

A biofilm is a complex assemblage of microbial cells that are irreversibly attached to a surface and encased within a self produced protective exopolymeric saccharide (EPS) matrix. Biofilms can form on a range of biotic or abiotic surfaces and can comprise of single or multiple microbial species. Bacteria within a biofilm differ genotypically and phenotypically from their planktonic counterparts, are very difficult to culture and are highly resistant to conventional antibiotics.[20]

The phenomenon of a surface attached microbial community was first discovered by the Dutch microscopist Antoni van Leeuwenhoek in 1863 in his description of "living animalcules" from the plaque of his own teeth.[21] However, despite his extraordinary discovery, the fields of microbiology choose to focus primarily on planktonic bacteria. It was not until about 100 years later when Dr. J. William Costerton published his landmark paper[22] that the scientific community began to appreciate the significance of the biofilm mode of existence, and specifically that sessile bacteria may in-fact constitute a major component of the bacteria present in natural environments. There is now a general consensus that in most natural environments, planktonic bacteria exists only transiently, and the majority of the populations are biofilms.[23]

**Why Biofilms Form**

There are several theories pertaining to the reasons why planktonic bacteria form biofilms as a preferred mode of existence. One such theory suggests that bacteria form biofilms as a means of defence, in response to stressful environments such as high shear forces, host defences and nutrient deprivation. Upton entry into a host, certain bacteria are capable of switching on transcription genes responsible for EPS synthesis and thus are able to evade initial host attack.[24] Other theories argue that biofilm formation may be a means, by which bacteria are able to remain in a favorable niche. Bacteria have developed strategies to remain fixed in the human body, such as the expression of an array of adhesion molecules known as microbial surface recognizing adhesive matrix molecules (MSCRAMMs).[25] This allows them to remain in an environment that is stable in terms of nutrient source, water content, oxygen tension and temperature. Bacteria can then

detach once nutrient sources such as glucose have been depleted, in search of an alternative habitat. A third theory suggested that bacteria within biofilms exhibit communal behavior, acting as multi-cellular organisms, thus benefiting from a division of the metabolic burden and horizontal gene transfer. The display of altruistic behavior benefits of the biofilm community as a whole.[26] Finally, the biofilm phenotype may represent the default mode of growth and planktonic counterparts are actually only present when growth conditions are favorable.[26]

**Biofilm Characteristics**

*Extracellular Polymeric Substances (EPS) Matrix*

Biofilm microbes are held together and protected by a complex matrix of excreted polymeric compounds called EPS. This matrix functions mainly to protect the bacteria within, as well as to facilitate intercellular communication via biochemical signaling pathways known as quorum sensing.[20]

Biofilms develop in a vast array of differing environments and thus the structural composition of the biofilm and the EPS will vary accordingly. Polysaccharides comprise up to 90 percent of the EPS matrix.[27] These polysaccharides can either be anionic such as in the case of gram-negative bacteria, neutral or cationic as in the case of some gram-positive bacteria.[28] The ability to incorporate hydrogen bonding makes the EPS a highly hydrated structure. In addition to polysaccharides and water, a wide variety of proteins, glycoproteins, glycolipids and extra-cellular DNA are also present.[27]

## Quorum Sensing

Microbes within a biofilm community actively communicate through a cell-to-cell signaling system known as quorum sensing. There is now evidence that this phenomenon plays a vital role during initial adhesion as well as detachment.[29, 30] It is believed that individual bacterial cells emit chemical signals, when theses signals reach a critical density, the bacteria respond en masse and genes involved in biofilm differentiation are activated.[30] Quorum sensing can occur within a single species as well as between diverse species, and are known to regulate a host of different processes, essentially serving as a simple communication network.[31]

## Antibiotic resistance

Established biofilms can tolerate antimicrobial concentrations of 10-1000 times that needed to kill planktonic counterparts[32] and displays an inherent resistance of phagocytosis.[24] The precise mechanism for antibiotic resistance remains unclear; however, it is likely to be a manifestation of multiple factors. Firtstly, the EPS secreted by biofilm bacteria plays a vital role in restricting the penetration of antimicrobials and antibodies. [33, 34] Furthermore, negatively charged molecules within the matrix are capable of binding to antimicrobial agents.[35] Secondly, bacteria embedded deep within a biofilm exhibit a reduced growth and metabolic rate and thus are less permeable to antibiotics.[36] Thirdly, inactivation of antibiotics can occur either on the biofilm surface or within the matrix itself.[37] [38, 39] Finally, there may be subpopulation of drug resistant, phenotypically and genetically different bacteria within the biofilm, as the close knit community provides the ideal niche for the exchange of extra-chromosomal DNA.[35]

*Biofilm Dispersal*

Detachment of biofilm cells can be caused by either external or internal biofilm factors. External forces include physical shearing or erosion, sloughing and increased flow velocity for biofilms at a liquid interface. Internal biofilm factors are thought to result from reduced nutritional levels or oxygen depletion. These occur via processes such as quorum sensing, endogenous enzymatic degradation, the release of EPS or binding proteins.[20] Dispersal strategies include the shedding of individual daughter cells from a micro-colony, the release of aggregates of biofilm cells or surface dispersal in which cells move across a surface via gliding or twitching motility.[40] According to Costerton, there is a natural pattern of programmed detachment of planktonic cells from biofilms. Thus biofilms may act as "niduses" for recurrent acute infection.[36]


**Microscopic Mucosal Biofilm Detection Techniques**

Researchers have used various microscopic techniques to document the morphology and presence of bacterial biofilms on sinus mucosal surfaces. Initial reports relied upon the use of transmission electron microscopy (TEM) and scanning electron microscopy (SEM). More recently, confocal scanning laser microscopy (CSLM) and fluorescence in situ hybridization (FISH) have been utilized. In one way or another, each technique has its flaws when it comes to interpreting the images they produce. A description/overview of each technique follows.

*Scanning Electron Microscopy (SEM)*

Initial reports of bacterial biofilms on sinus mucosal surfaces utilized SEM and TEM.

The SEM uses a high-energy beam of electrons to scan the surfaces of tissue samples.

Interactions of electrons with atoms on the sample produce signals that contain

information about surface topography, composition and other properties such as electrical

conductivity.[41] SEM can give useful 2-Dimensional morphological information on the

superficial structure of a biofilm. Using SEM, biofilms appear as spherical bodies

resembling bacteria, encased within a matrix and arranged in clusters and towers above a

layer of ciliated epithelium.[42, 43] SEM is a well accepted method of documenting

biofilms; however, this method requires that sample tissue is highly processed through a

series of arduous fixation and dehydration steps, which are likely to result in significant

artifact.[44] Ramadan et al., one of the initial authors describing biofilms in CRS patients,

concedes that although SEM was able to show evidence of biofilm, the technique was

unable to distinguish bacteria within the biofilm mass clearly.[43]


*Transmission Electron Microscopy (TEM)*

Using the same basic principles of the conventional light microscope, the TEM transmits

a beam of electrons through an ultra thin specimen, forming an image as the electrons

pass through the specimen which is subsequently magnified and focused by an objective

lens.[45] TEM analysis of mucosal tissue also requires fixation and dehydration techniques

similar to the SEM, thus are also subject to the same inherent problems of artifact. The

advantage of TEM is that, unlike SEM, it enables high resolution ultrathin two-

dimensional cross-sections to be taken through the biofilm; consequently clusters of

bacteria embedded deep within the EPS can be clearly visualized.[8, 9]


*Confocal Scanning Laser Microscopy (CSLM)*

CSLM is a well recognized method of imaging bacterial biofilms and has been used

extensively in biofilm research.[46, 47] This high tech epifluorescent microscope utilizes

laser light to penetrate and excite fluorescently labeled bio-molecules within the sample.

CSLM microscopy and computer aided analysis allows real-time examination of biofilms

morphology and physiology in four dimensions.[48] Ehrlich et al. used CSLM to document

the presence of viable biofilms in a chinchilla model of otitis media.[49] Sanderson et al.

used CSLM and FISH techniques to identify the presence of *S. aureus*, *S. pneumoniae*

and *H. influenza* biofilms in patients with CRS.[18]


*Fluorescence In situ Hybridization (FISH)*

FISH is a cytogenetic technique used to detect and localize the presence or absence of

specific DNA sequences on chromosomes.[50] When used in combination with CSLM,

fluorescently labeled oligonucleotide probes can allow visualization of species specific

biofilm bacteria and their EPS.[18, 51]


**Biofilms and Chronic Disease**

Until relatively recently, our understanding of microbiology and the treatment of

infections has been based upon the postulates formulated by Robert Koch and Friedrich

Loeffler in 1884 and later refined and published by Koch in 1890.[52] These postulates

were designed to establish a causal relationship between a causative microbe and a disease and stated that: firstly, the microorganism must be detectable in the infected host at every stage of the disease.  Secondly, the microorganism must be isolated from the diseased host and grown in pure culture. Thirdly, when susceptible, healthy animals are infected with pathogens from the pure culture, the specific symptoms of the disease must occur.  Finally, the microorganism must be re-isolated from the diseased animal and correspond to the original microorganism in pure culture.[53]

These principles have resulted in the development of vaccines and antibiotics targeted to the control of these pathogenic organisms and can be credited to the partial eradication of acute epidemic bacterial diseases.  Furthermore, they have provided us with an increasingly accurate understanding of prokaryotic genetics and metabolism and have allowed the isolation and identification of pathogens in a wide variety of diseases.[36]

Advances made in our understanding of acute bacterial infections have been largely based on Koch's principles.  However, there are many diseases seemingly caused by infection which cannot be explained using these hypotheses. Amongst an array of chronic infections, a clear understanding of sinusitis,[42] otitis media,[54] osteomyelitis,[55] cystitis, prostatis,[55] endocarditis,  periodontitis, Cystic fibrosis pneumonia remains elusive.[56] Recent developments in research however have seen the recognition of the association between biofilms and these chronic diseases.  In fact, it is now estimated that more than 65 per cent of chronic infections are caused by bacteria growing in biofilms.[23]

### Biofilms and Chronic Rhinosinusitis (CRS)

The historical perception that pathogenic bacteria were planktonic, isolated free floating organisms was suitable for explaining the pathogenesis of acute sinus infections. However, this model fails to describe the recalcitrant nature of CRS. The very nature of CRS, in which patient's initially respond to a course of antibiotics, only to relapse weeks to months later and the constant inability to culture pathogenic organisms, lends itself to the biofilm hypothesis. There is now mounting evidence of an association between the presence of biofilms and CRS. For example, Cryer et al. initially reported evidence of bacterial biofilms on the mucosal surface of patients with CRS using SEM.[42] Since then there have been a numerous studies suggesting biofilms as an etiologic factor in the pathogenesis of CRS. Human studies using SEM, TEM, CSLM and FISH techniques have reported the incidence of biofilms in 78-100% of CRS patients.[18, 43, 57] Perloff et al. reported biofilms on frontal sinus stents removed from patients who had previously undergone ESS for CRS.[58] The same authors can also be credited with the development of the first animal model demonstrating the presence of biofilms in sinusitis.[59] Furthermore, several *in vitro* studies have demonstrated the ability of microbes isolated from patients with CRS to develop biofilms,[19] the effects of biocidals on CRS biofilms,[60, 61] and an association between biofilm forming capacity of bacteria and poor clinical outcome following surgery.[62]

## *In vitro* Biofilm Models

The extensive body of knowledge pertaining to every aspect of biofilm morphology, physiology and pathology has come about primarily as a result of the *in vitro* examination of this mode of bacterial growth.  As such, there has been a multitude of *in vitro* systems documented for the examination of bacterial biofilms.  Currently, static and dynamic systems are available for examining biofilms and the effects of anti-biofilm therapies. Dynamic models such as a radial flow chamber,[63] parallel plate flow chamber,[64] rotating disc apparatus[65] or Robbins device [66, 67] are intended to grow biofilms under continuous flow conditions. These systems are applicable when studying biofilms in environmental or industrial settings and certain biomedical devices.[68]

### Growing and analyzing Static Biofilms

Numerous methods have been described for the cultivation and quantification of static biofilms *in vitro*.[69-71]  Protocols for static biofilm growth are relatively straightforward and usually can be executed using common laboratory equipment.  Growth parameters such as media used and replacement regimens, incubation time and washing forces can be adjusted readily.[72]  Most static systems however, do not have a continuous supply of fresh medium and are not aerated, thus a limitation of nutrients may result in the inability of some bacteria to form biofilms.[73]

The 96 well microtiter plate assay utilizing Crystal Violet (CV) which was described by Christensen et Al. in 1985[74] and popularized by O'Toole et al. in 1998,[75 76] is among the

most frequently used assays for quantification of biofilms.[77] This well-established high-throughout method uses a surface-associated dye, CV to quantify biofilms mass. CV binds to negatively charged molecules within the biofilms, including nucleic acids and acid polysaccharides.[78] Bacteria are grown on a 96-well microtiter plate for a desired period of time; following which the wells are washed thoroughly to remove all non-adherent planktonic bacteria. The remaining adherent cells are stained with CV which allows visualization of the biofilm mass. A solvent such as ethanol is used to dissolve the surface associated stain, which can then allow a semi-quantitative assessment using a standard laboratory plate reader or spectrophotometer.[75] A detailed overview of this standardized protocol using staphylococci has recently been described by Stepanovic et al.[77] Other dyes such as safarin [79] and a combination of dimethyl methylene blue (DMMB) and resazurin[80] have also been used to stain biofilm and quantify biofilm components.

A direct method to enumerate the number of viable bacteria in a biofilms mass has been described by Phelan.[81] Using a 96 well microtiter plate, biofilms are propagated as above. Wells are then sonicated to remove adherent bacteria. The resulting bacterial suspension is plated on an agar medium to enumerate the bacteria mainly by determining the colony forming units (cfu). This is a common method by which *in vitro* antibiotic biofilm treatments are tested.[82]

Caiazza et al. described an air liquid interface (ALI) assay model which allows for the microscopic analysis of biofilm formation over a time range of 4 to 48 hours.[83] A 24 well

flat-bottom plate is placed at an angle of 45 degrees to horizontal, diluted bacterial

cultures are then inoculated into the wells such that the upper edge of each culture aliquot

is positioned in the centre of a well's bottom. Bacteria are grown for the desired period

of time. The wells can then be washed to remove planktonic bacteria and the remaining

biofilms can be viewed with various microscopy modalities. Many bacteria prefer

aerobic growth and will therefore only form biofilms at the air-liquid interface (ALI).[84] [85]

A colony biofilm system operates by the same basic principles of an ALI. These systems

involve attachment of bacteria to a surface whilst bathed in a nutrient medium. Biofilms

are propagated on a semi-permeable membrane that sits on a nutrient medium of either

agar or nutrient broth, thus allowing a passage for the bacteria to attain nutritional needs,

whilst facilitating waste removal.[86, 87] Supply of nutrients can be changed, or

alternatively drug treatment may be administered without the need to disrupt the biofilms

mass by washing. It is thought that changes in the cell number are more likely

attributable to cell death rather than detachment and thus these systems have been applied

to the assessment of biocides. An adaptation of this model is the application of biological

tissues such as respiratory epithelium above a support semi-permeable membrane. This

membrane rests on a nutrient media and allows the maintenance of viable tissue whilst

allowing biofilm to be propagated on biological surface.[88, 89]

***In vitro* assays in CRS biofilms**

The presence of biofilms on the sinus mucosal surfaces of patients with CRS is now well

established.[90] *In vitro* biofilm studies have enhanced our understanding of possible

disease outcome in CRS patients, bacterial biofilm physiology in the paranasal sinuses and provided researchers an initial platform to trial potential treatment modalities.

Recently, Bendouah et al. described an *in vitro* CV staining method in which isolates of *P. aeruginosa*, *S. aureas* and *coagulase-negative Staphylococcus* from patients with CRS were examined for their biofilms-forming capacity. This showed an association between the biofilm forming capacity of these organisms and a poor clinical evolution in patients who had previously undergone ESS.[62] The same authors then went on to investigate the *in vitro* activity of Moxifloxacin against clinical isolates of *S.aureus* biofilms and concluded that concentrations attainable in topical solutions were capable of killing bacteria in bacterial biofilms.[60] Desrosiers et al. used an *in vitro* assay and measured CFUs to show that the hydrodynamic delivery of citric acid and zwitterionic surfactant disrupted biofilms associated with CRS.[61] Additionally, Woodworth et al. developed an *in vitro* mouse sinus epithelial ALI model to culture biofilms on airway epithelium.[91]

## Animal Sinusitis Models

Animal models allow researchers to investigate disease states in ways which would be inaccessible in a human patient, as such; the short-term objective is to use these animal models in experiments to determine how they respond to treatments. In order to assess the effectiveness of various forms of treatment, a diseased animal model that is similar in etiology and function to the human equivalent must be established.

Various animal models (e.g., Murinae, Canine, Porcine, Rabbit, and Sheep) have been developed to study both the pathophysiology and benefits of potential treatment modalities in CRS; however, few of these are suitable for ESS. These models are described below.

**Murinae Model**

In the past 10 years, genetic knockouts and transgenic mice have revolutionized animal research.[92] Mice are readily available, cost efficient and pathogen free. Jacob et al. has described a mucine model in which they obstructed the maxillary sinus ostium using Merocel nasal packing and injected *Bacteroides fragilis* to cause sinusitis.[92] Mice possess maxillary sinuses and ethmoidal air cells, however, in order to access maxillary the sinuses for inoculation of bacteria they require skin flaps and drilling through bony nasal dorsum. Endoscopic Sinus Surgery (ESS) is not possible.

**Canine**

Several studies were published in the 1950-70s using dog models for the study of frontal sinus disease.[93-95] Dogs posses a similar sinus structure to humans but for ethical reasons they are no longer available.

**Porcine Model**

Porcine models are commonly used in cardiovascular, respiratory, immunological and abdominal surgery.[96-99] However, the sinus alignment and turbinate structure of the pig makes them unsuitable to repeated ESS.[100]

**Rabbit Model**

The rabbit model for the study of sinusitis dates back to the 1950s.[101] Since then, the rabbit model has been used for considerable research in all aspects of sino-nasal disease, including lactic acid accumulation, mucosal blood flow, histochemistry, polyposis and a variety of CRS treatments. [102-109]

With the popularization of endoscopic sinus surgery (ESS), the rabbit model of CRS may now be deemed inappropriate in certain applications. Rabbits have relatively small nasal cavities and sinuses making ESS almost impossible. The frontal sinus of the rabbit is most accessible but requires a trans-cutaneous external approach in which the front wall of the sinus is removed.[59] This approach is not analogous to humans and raises a theoretical risk of disturbing natural sinus function and inadvertently introducing pathogenic contamination. Furthermore*, Pasteurella multocida* , a Gram-negative coccobacillus frequently colonizes the paranasal sinuses of rabbits, causing rhinitis and muco-purulant nasal discharge.[110] Consequently, this may confound results in biofilm studies of the paranasal sinuses.

**Sheep Model**

The use of the sheep model to study sinusitis was first developed by Rajapaksa et al. in 2005.[111] Sheep have a similar spectrum of sinonasal diseases to humans including allergic rhinitis, sinusitis, and nasal polyposis.[112] Its application in previous models to the study sinusitis has been successful for a number of reasons: Firstly, Gardiner et al. showed that the sinus anatomy and orientation of the nasal cavity, turbinates, frontal and

maxillary sinuses are analogous to humans.[113] Secondly, their sinus mucosa has also been shown to be histologically identical to humans [114] and finally, the size and orientation of the sheep's paranasal sinus's makes them amenable to repeated ESS. However, to improve access to the nasal cavity, a middle turbinectomy must be performed.[100] The anterior ethmoid complex can then be seen and removed to allow visualization of the frontal sinus ostium. Superficially, access to the frontal sinus can be attained by making a small skin incision and inserting a small mini-trephine. A mini-trephine can allow manipulation of frontal sinus for investigation of potential treatments.[115]

**An Animal CRS Biofilm Model**

Sinuses are healthy as a result of patent ostia, adequate ventilation, muco-ciliary function and local and systemic host immune defenses. Regardless of the underlying etiology, the most consistent finding in CRS patients appears to be sinus ostial obstruction.[116] Obstruction results in poor ventilation and hypo-oxygenation, subsequent ciliary dysfunction and retention of mucous occur within the sinuses, providing a favorable milieu for bacterial proliferation.[117, 118]

To facilitate pathogenic conditions similar to those seen in human CRS, previous researchers have occluded various sinus ostial openings and introduced bacteria in a variety of ways.[59, 119] Perloff et al. developed a rabbit model of sinusitis in 2005 and used SEM to show evidence of biofilms on the sinus mucosal linings following *P. aeruginosa* propagation of between 1 and 20 days.[59]

The sheep model for sinusitis described by Rajapaksa et al. was based on the observation that sheep infected with *Oestrus ovis* nasal bot fly larvae mounted a similar inflammatory response in the paranasal sinuses as those seen in humans with CRS.[111] *Oestrus ovis* sino-nasal infestation in humans is rarely seen[120] and the presence of *Oestrus ovis* larvae may further confound any results gained from artificially infected sinuses. Therefore such a model may not be suitable for the examination of potential bacterial biofilm treatments. To overcome this however, Meleny et al. argued that the eradication of *Oestrus ovis* is a relatively simple process by which sheep receive an oral drench of a broad-spectrum parasiticide such as Ivermectin 3 months prior to experimentation. This has been shown to be effective in eradicating *Oestrus ovis* infection.[121]

## Potential Biofilm Treatments in CRS

### Arresting biofilm formation

There is a vast amount of research being conducted on the identification of genes required for biofilm formation and the manipulation of different stages of biofilm development. These approaches are primarily based on the utilization of signaling molecules to block adhesion processes and to interfere with biofilm quorum sensing. In 2004, Jefferson et al. presented a detailed summary of the identified genes required for biofilm formation by various bacterial species.[26] Many of these genes have been the target of research into potential biofilm therapies. Researchers have recently succeeded in modulating the quorum sensing activity through the use of QS inhibitors.[122, 123] Ribonucleic-acid-III-inhibiting peptide (RIP) has been shown to have the ability to block

*S.aureus* and *S.epidermidis* biofilm formation.[124, 125] Banin et al. has reported the role of iron uptake genes associated with biofilm formation in *P. aeruginosa*,[126] and Davies et al. has shown that *P. aeruginosa* PAO1 requires the lasI gene product $3OC_{12}$-HSLR to develop normal biofilms.[30]


**Removal of established biofilms**

The increased antimicrobial resistance encountered in biofilm bacteria may potentially be overcome by increasing local antibiotic concentrations.[127] Topical application of therapies to sinus mucosal membranes by either nasal irrigation or nebulizers enables the delivery of high local concentrations of antibiotics, with the potential advantage of low serum levels and thus reduced side effects. [128, 129] Desrosiers et al. has shown that high concentrations of Moxifloxicin, obtainable in topical solutions were effective in killing biofilm bacteria *in vitro*.[60]


Additionally, the mechanical hydrodynamic disruption of biofilm matrix via the use of saline irrigation, sprays and douching of biofilm may have role in biofilm removal.[51] Desrosiers et al. has demonstrated that the addition of additives such as a soap-like surfactant and a calcium-ion sequestering agent may also be effective.[61]

# CHAPTER 3


# *In vitro* activity of mupirocin on clinical isolates of Staphylococcus aureus and its potential implications in Chronic rhinosinusitis

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**In vitro activity of mupirocin on clinical isolates of Staphylococcus aureus and its potential implications in Chronic rhinosinusitis**

**The Laryngoscope 2008 March;118:535-540**

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## Abstract

**Background**

It has been postulated that bacterial biofilms are involved in the pathogenesis of chronic rhinosinusitis.  Biofilms present on sinus mucosa are difficult to eradicate with conventional antibiotic therapy and are thought to provide a nidus for recurrent infection. Topical delivery of antibiotics via nasal irrigation may present a way of delivering high concentrations of anti-biofilm agents with potentially low systemic absorption and side effects.  This study investigates the effectiveness of mupirocin and two other antibiotics, ciprofloxacin and vancomycin on established *in vitro* biofilms of *S.aureus* isolated from patients with CRS.

**Methods**

*S.aureus* ATCC25923 and 12 clinical isolates were investigated for their ability to form biofilms in an *in vitro* setting, using a 96 well microtitre crystal violet (CV) plate assay and confocal scanning laser microscopy analysis (CSLM).  Antimicrobial susceptibility tests to determine minimum inhibitory concentrations (MICs) were performed on planktonic and biofilm forming strains. Additionally, established biofilms were subjected to the antimicrobial agents at a series of doubling dilutions over a range of concentrations.  A CV analysis of biofilm mass was performed following 1 hour and 24 hours of treatment and minimum biofilm inhibition concentrations at 50 percent (MIB50) and 90 percent (MIB90) biofilm inhibition were recorded.

**Results**

Using a 96 well microtitre plate CV assay, 8 of the 12 clinical isolates formed mature biofilms following 8 days of culture. These results correlated with findings from CSLM analysis of *in vitro* biofilms grown on permanox chamber slides. Increased antimicrobial resistance was observed in the biofilm isolates when compared with planktonic counterparts. Mupirocin was capable of reducing biofilm mass by greater than 90 per cent at concentrations of 125_g/ml or less in all *S.aureus* isolates. Ciprofloxacin and vancomycin were largely ineffective in attaining MIB90 concentrations within safe dosage ranges.

**Conclusions**

The topical application of mupirocin via nasal irrigation may be useful in eliminating *S.aureus* biofilms present on the sinus mucosa of patients with CRS and may offer an additional treatment to patients with recalcitrant sinusitis.

**Introduction**

Chronic rhinosinusitis is a common and debilitating condition. Despite extensive medical and surgical treatment, some patients continue to suffer from recurrent relapses of this disease. Ostial obstruction and secondary bacterial infections are thought to be one of the primary etiologic factors involved in sinusitis,[130] with *Staphylococcus aureus* being one of the most commonly isolated organism.[131, 132]

Recently, a number of papers have described the presence of bacterial biofilms on the sinus mucosa of patients with CRS, [8, 18, 57] leading some researchers to postulate that these structures may be involved in the pathogenesis of this condition. The biofilm bacteria exist within structured communities and are irreversibly attached to the sinus mucosal surface. The bacteria produce and encase themselves within a protective exopolysaccharide matrix and are extremely resistant to conventional antibiotic treatment.

The precise mechanism for biofilm antibiotic resistance remains unclear, but possible hypotheses include: restricted antibiotic penetration through the biofilm EPS matrix, chemical alterations in microenvironments within the biofilm, and sub-populations of phenotypically and genetically different bacteria.[133]

The advent of topical application of antibiotics to sinus mucosal membranes by either nasal irrigation or nebulisers enables high local concentrations of antibiotic at the site of

biofilm infection with the potential advantage of low serum levels and therefore reduced side effects. [128, 129] Desrosiers et. al.[60] postulated that topical antibiotic therapy may have a role in the treatment of CRS and showed that increased antibiotic concentrations were effective in killing bacteria existing within biofilms.

Recent research by Bendouah et. al.[62] has demonstrated an association between the *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms and worse outcomes following endoscopic sinus surgery, further implicating the role of biofilms in CRS. Although P. *aeruginosa* is frequently isolated from patients with CRS, S.*aureus* remains the predominant pathogenic organism.[132] Mupirocin has been shown previously to reduce P.*aeruginosa* biofilms,[134] however, its activity on established biofilms of *S.aureus* have not been reported. The aim of this study was to investigate the *in vitro* susceptibilities of well established S. *aureus* biofilms grown from planktonic isolates of patients with CRS, to mupirocin, ciprofloxacin and vancomycin.

## Materials and Methods

**Bacterial strains, inoculum and antibacterial agents**

*Staphylococcus aureus* reference strain ATCC 25923, and 12 clinical isolates of S.*aureus* were used in this study. Clinical strains were all recently isolated from the nasal passages of patients diagnosed with Chronic Rhinosinusitis from different tertiary care centres in South Australia, Australia and were provided by the Institute of Medical and Veterinary

Science, South Australia (IMVS). Patients were diagnosed with CRS according to the criteria previously outlined by the Chronic Rhinosinusitis Task Force in 2003.[1]

All bacterial strains used were tested for their ability to form biofilms by the means of a 96 well microtitre plate crystal violet (CV) assay and a 4 chamber slide analysis using confocal scanning laser microscopy (CSLM). Prior to inoculation, all strains were transferred from stock cultures to Columbia horse blood agar (Oxoid, Thebarton, South Australia). Following incubation overnight at $37^{\circ}C$, 1-2 single colonies were inoculated into 2mls of 0.45% normal saline and adjusted to turbidity of 0.5MacFarland opacity standard. Each bacterial solution was then transferred to a sterile test tube for subsequent use.

Mupirocin was obtained from GlaxcoSmithKline, Victoria, Australia. Ciprofloxacin was obtained from Aspen Pharmacare, NSW, Australia and Vancomycin from Mayne Pharma, Vic, Australia.

**Quantification of Biofilm Formation**

***96 well microtitre plate CV assay***

Biofilm production was determined by a microtitre plate adherence assay as described by O'Toole and Kolter,[75] with modifications. An initial 96 well microtiter plate (Nunc, Roskilde, Denmark) experiment using S.*aureus* reference strain ATCC 25923 was performed to document biofilm growth conditions under static conditions. 10µl of bacterial inoculum was added to wells containing 190µl of CSF broth (Oxoid, Thebarton, South Australia). Wells containing 200µl of CSF broth alone were used as negative

controls. Microtitre plates were incubated at 37$^{\circ}$C for between 1 to 10 days. Fresh media changes of 50µl, 100µl, 150µl or 200µl were performed on either a daily basis or every second day.

Biofilm forming ability was also examined for each clinical isolate.  Isolates were inoculated into 96 well microtitre plates and examined daily for 8 days. 50µl of media was changed every second day.  Following incubation at given time points, the supernatants were aspirated and wells washed twice with sterile phosphate buffered solution (PBS) to remove any planktonic bacteria (pH 7.3). Adherent bacteria were then fixed with methanol for 15mins. Methanol was removed and plates air dried for a further 15mins. Adherent bacteria were then stained with 200µl of 0.1% crystal violet violet solution for 5mins. The crystal violet solution was aspirated and the remaining wells washed three times with sterile MQ water to remove excess stain. Adherent material was then solubilised by incubation with 250µl of 95% ethanol for 1hr on a rocker.  Finally, the optical density of each well was measured at 595nm (OD$_{595}$) using a Biorad Microplate reader to quantify biofilm growth.

***Permanox chamber slide CSLM analysis***

The biofilm forming ability of S.*aureus* strains was also investigated using CSLM. Using 4 chamber Lab-Tek Permanox chamber slides (Nunc, Roskilde, Denmark), 20µl of bacterial inoculum was added to wells containing 580µl of CSF broth (one strain per slide). Chamber slides were incubated at 37$^{\circ}$C for 8 days, with 150µl of fresh media changed every 48hrs.  Well contents were aspirated and 1ml of sterile MQ water

containing 1.5 μl aliquots of component A (Syto 9) and component B (Propidium Iodide) of BacLight LIVE/DEAD kit (Invitrogen, Molecular Probes) was added to each well. Chamber slides were then incubated in this solution in darkness at room temperature for 15 minutes. Following incubation, the chambers were removed and slides washed thoroughly in 3 separate beakers of sterile MQ water to remove any planktonic bacteria. Cover slips were applied and slides examined for the presence of biofilm structures using a Leica TCS SP5 spectral scanning confocal microscope (Leica Microsystems, Exton, Pa).

**Antibiotic susceptibility testing**

The minimum inhibitory concentration (MIC) for planktonic bacteria was determined with a micro-dilution method as described by Nishimura et. al,[135] with modifications. Briefly, 100μl of CSF broth supplemented with antibiotic was added to each well of a 96well microtitre plate at a 2-fold serial dilution (concentration range of 0.125 to 16μg/ml). The plates were then seeded with 5μl of bacterial inocullum and incubated for 24hrs. For determination of biofilm MIC (BMIC), bacterial strains were grown as biofilms for 8 days as previously described. Wells were then washed twice with PBS and antibiotic media added (concentration range of 7.18 to 1000μg/ml). The MIC was defined as the lowest concentration that yielded no visible growth following incubation.

**Antimicrobial activity against formed biofilms.**

Biofilms of ATCC 25923 and 8 biofilm forming isolates were grown on 96 well microtitre plates for 8 days at 37°C with 50uls of media changed every second day.

Following incubation, wells were washed twice with sterile PBS to remove non-adherent bacteria. 200μl of media supplemented with antibiotic was added to each well at a 2-fold serial dilution and a concentration range of 7.81 to 1000μg/ml. Wells with media alone served as negative controls. The plates were incubated for either 1hr or 24hrs and analysed using a crystal violet biofilm adherence assay as described previously.

The relative inhibition of biofilm (expressed as a mean percentage) was calculated as follows: Percentage of Biofilm Inhibition $= 100 - [(OD_{595}$ of treatment well$/ OD_{595}$ of positive control well$) \times 100)]$. The Minimum Inhibition Biofilm concentration at 50 per cent inhibition ($MIB_{50}$) and at 90 per cent inhibition ($MIB_{90}$) was determined for each antibiotic.

In all experiments, wells with media alone served as negative controls. All bacterial strains were studied in triplicate and experiments repeated twice. The mean percentage of biofilm inhibition and standard deviation were considered.

**Statistical Analysis**

The mean and standard deviation was considered when comparing results. Statistical analysis was performed using Graph Pad Prism 2.01 for Windows. A one-way analysis of variance (ANOVA) and multiple comparisons used Dunnet's multiple comparison post-test.

## Results

### Biofilm formation

Using a 96 well microtitre plate assay optimal biofilm growth conditions were studied, biofilm formation of S.*aureus* ATCC 25923 was measured from day 1 until day 10. Other variables such as time and amount of media change were also measured. Biofilm density was greatest at day 8 (mean $OD_{595}$ 0.13). No significant difference was observed in biofilm growth when media was changed every 24hrs or 48hrs ($P > 0.05$). When nutrient media was changed every 48hrs, it was found that a media change of 50ul yielded the greatest growth (data not shown).

Biofilm forming capacity of all clinical isolates were also investigated. Not all strains formed biofilms equally. Using a 96 well crystal violet assay, isolates with $OD_{595}$ reading less than 20 percent of OD for ATCC 25923 ($OD_{595}$ <0.026) following 8 days of growth were considered as non-biofilm forming strains (isolate 1002, 1005, 1013, and 1068) (see figure 1). Mean $OD_{595}$ of 0.025 was observed in negative controls and thus used as a cut-off levels for non biofilm formers. Of the isolates that did form biofilms, there was a trend for minimal growth until day 4 (mean $OD_{595}$ 0.85), a steady increase in biofilm mass between until day 8 to 9, where the biofilm achieved maximal growth and then plateaued (mean $OD_{595}$ 0.21 and 0.17).
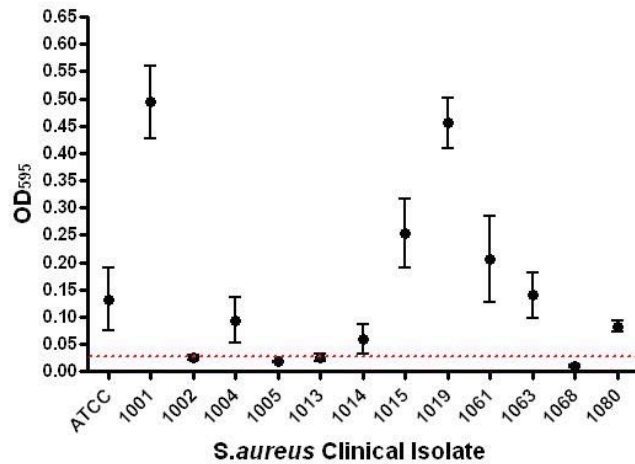
**Figure 1.**
*In vitro* biofilm formation of S.*aureus* ATCC 25923 and Clinical Isolates after 8 days
Non-biofilm forming strains = <20 percent mean $OD_{595}$ of ATCC reference strain ($OD_{595}$ 0.026, indicated by the dotted line).

The results obtained by analysis of biofilms under CSLM almost paralleled those of the

96 well CV assay. Immotile, irreversibly attached, live bacteria, in characteristic clusters

and towers of micro-colonies were found in varying degrees of density throughout the

slides (see figure 2). Control slides and Isolates 1005 and 1068 showed no evidence of

biofilm.  Isolates 1002 and 1013 demonstrated only several scattered low-density biofilm

colonies.  No attempt was made to quantify the amount of biofilms present.
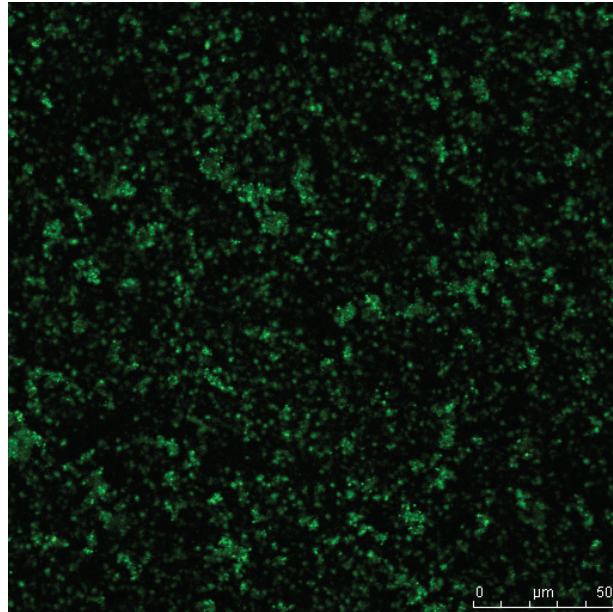
**Figure 2.**
Confocal scanning laser microscopy image at 20x magnification of *S.aureus* isolate 1019 day 8 biofilm. The biofilm is comprised of many intensely fluorescing live bacteria organised in clusters and towers.

**Antibiotic Susceptibilities**

Quality controls in the microtitre plates with S.*aureus* ATCC25923 were within the

defined CLSI quality control range. All planktonic isolates fell below the CLSI-defined

S.*aureus* susceptibility breakpoints.[136] The MICs and BMICs for each isolate are shown

on table 1.

**Table 1.**

Minimum Inhibitory Concentrations for Planktonic and Biofilm

| Isolate Strain | Mupirocin | | Ciprofloxacin | | Vancomycin | |
|---|---|---|---|---|---|---|
| | planktonic | biofilm | planktonic | Biofilm | Planktonic | biofilm |
| ATCC | 0.25 | ≤7.81 | 0.5 | 15 | 2 | 31.25 |
| 1001 | 0.5 | ≤7.81 | 0.5 | ≤7.81 | 2 | 15.62 |
| 1004 | 0.25 | ≤7.81 | 0.5 | ≤7.81 | 1 | 31.25 |
| 1014 | 0.25 | ≤7.81 | 0.5 | ≤7.81 | 2 | 15.62 |
| 1015 | 0.25 | ≤7.81 | 0.5 | ≤7.81 | 2 | 62.5 |
| 1019 | 0.25 | 15.62 | 1 | 15 | 1 | 15.62 |
| 1061 | 0.25 | ≥1000 | 0.5 | >1000 | 1 | >1000 |
| 1063 | 0.25 | ≤7.81 | 0.5 | ≤7.81 | 1 | ≤7.81 |
| 1080 | 0.25 | ≤7.81 | 0.5 | >1000 | 2 | 31.25 |

Values are numbers of micrograms per milliliter

Biofilms were challenged with antimicrobial agents for 1 hour and 24 hours at concentrations ranging from 7.81 to 1000μl/ml. Table 2 and 3 show the $MIB_{50}$ and $MIB_{90}$ for each strain. With the exception of isolate 1004, Ciprofloxacin was unable to reduce established biofilms by more than 90 percent in the concentration ranges measured. Following 24hrs of treatment, mupirocin was effective in reducing biofilms in all isolates by 90 percent or greater at concentrations ranging from 7.81 to 125μg/ml. However, at 1 hour only 50 percent of all biofilms were eliminated within this concentration range. The $MIB_{90}$ at 24hours for vancomycin was greater than 500μg/ml for ATCC25923 and isolates 1004, 1015, 1019 and 1063, with similar results observed at 1hour.

**Table 2.**

Susceptibility of S. *aureus* isolates to antibiotics following 1 hour incubation.

| Isolate Strain | Mupirocin | | Ciprofloxacin | | Vancomycin | |
|---|---|---|---|---|---|---|
| | $MIB_{50}$ | $MIB_{90}$ | $MIB_{50}$ | $MIB_{90}$ | $MIB_{50}$ | $MIB_{90}$ |
| ATCC | $\leq 7.81$ | $\geq 1000$ | $\leq 7.81$ | $\geq 1000$ | 125 | $\geq 1000$ |
| 1001 | 15.62 | 62.5 | $\geq 1000$ | $\geq 1000$ | $\leq 7.81$ | 62.5 |
| 1004 | $\leq 7.81$ | $\geq 1000$ | $\leq 7.81$ | $\geq 1000$ | $\leq 7.81$ | 500 |
| 1014 | $\leq 7.81$ | $\geq 1000$ | $\leq 7.81$ | $\geq 1000$ | $\leq 7.81$ | 500 |
| 1015 | $\leq 7.81$ | 250 | $\geq 1000$ | $\geq 1000$ | 250 | $\geq 1000$ |
| 1019 | $\leq 7.81$ | 15.62 | 31.25 | $\geq 1000$ | 15.62 | $\geq 1000$ |
| 1061 | $\leq 7.81$ | $\geq 1000$ | $\geq 1000$ | $\geq 1000$ | $\leq 7.81$ | 250 |
| 1063 | $\leq 7.81$ | 125 | 62.5 | $\geq 1000$ | 250 | 500 |
| 1080 | $\leq 7.81$ | $\geq 1000$ | $\geq 1000$ | $\geq 1000$ | $\leq 7.81$ | 125 |

Values are numbers of micrograms per milliliter.

**Table 3.**

Susceptibility of S. *aureus* isolates to antibiotics following 24 hours incubation.

| Isolate Strain | Mupirocin | | Ciprofloxacin | | Vancomycin | |
|---|---|---|---|---|---|---|
| | $MIB_{50}$ | $MIB_{90}$ | $MIB_{50}$ | $MIB_{90}$ | $MIB_{50}$ | $MIB_{90}$ |
| ATCC | $\leq 7.81$ | $\leq 7.81$ | $\leq 7.81$ | $\geq 1000$ | 500 | $\geq 1000$ |
| 1001 | $\leq 7.81$ | $\leq 7.81$ | 15.62 | $\geq 1000$ | 15.62 | 31.25 |
| 1004 | 31.25 | 125 | $\leq 7.81$ | $\geq 1000$ | $\leq 7.81$ | 500 |
| 1014 | $\leq 7.81$ | 62.5 | $\leq 7.81$ | $\geq 1000$ | $\leq 7.81$ | $\leq 7.81$ |
| 1015 | $\leq 7.81$ | 15.62 | 15.62 | $\geq 1000$ | $\leq 7.81$ | $\geq 1000$ |
| 1019 | 15.62 | 62.5 | $\leq 7.81$ | $\geq 1000$ | 15.62 | $\geq 1000$ |
| 1061 | 15.62 | 31.25 | $\leq 7.81$ | $\geq 1000$ | $\leq 7.81$ | 250 |
| 1063 | $\leq 7.81$ | $\leq 7.81$ | $\leq 7.81$ | $\geq 1000$ | 250 | 500 |
| 1080 | $\leq 7.81$ | 62.5 | $\leq 7.81$ | $\geq 1000$ | $\leq 7.81$ | 125 |

Values are numbers of micrograms per milliliter.

## Discussion

The findings of this study suggest that mupirocin was effective in reducing mature, well established S.*aureus* biofilms *in vitro*. Concentrations equal to or lower than 125μg/ml were capable of reducing biofilm mass by over 90 per cent in all S.*aureus* isolates tested. Mupirocin is a unique antibiotic, exerting its antibiotic effect by interfering with the action of isoleucyl-transfer RNA synthetase.[137, 138] Its antimicrobial activity against a wide spectrum of gram positive and gram negative bacteria makes it a suitable agent for eliminating bacteria found the nasal sinuses of CRS patients. It has been shown that topical application allows high concentrations of mupirocin to be delivered directly to the site of mucosal biofilms, with minimal systemic absorption and side effects.[138-140]

Although all planktonic isolates were sensitive to ciprofloxacin, this antibiotic was largely ineffective in reducing biofilms beyond 90 percent within the concentration ranges tested.  50 percent biofilm inhibition was achieved for all isolates following 24 hours treatment (ranges ≤7.81 to 15.62μg/ml).  These findings are similar to those in previous studies.[141]  Vancomycin was capable of greater than 90 percent biofilms inhibition in 6 of the 9 strains tested after 24 hours, however, the concentrations required were considerably higher than those required for mupirocin (range ≤7.81 – 500μg/ml). Significant systemic absorption from the topical application of vancomycin has been reported previously.[138] The effective topical dose required for nasal biofilm therapy would lead to serum levels well above the recommended target range (20-40μg/ml)[142] and thus preclude the use of vancomycin due the potential for toxicity.

A well-established method of quantifying biofilms using crystal violet was used in this study. Crystal violet binds to negatively charged molecules within the biofilm, including nucleic acids and acid polysaccharides and thus represents an overall measure of biofilm mass.[78] The optimal conditions for *in vitro* biofilm growth were determined by preliminary studies using S.*aureus* ATCC 25923, a known biofilm forming reference strain,[135] and these findings were applied to subsequent biofilm assays. Of the 12 clinical isolates tested, only 8 (66%) were capable of forming biofilms using the CV biofilm model. These results correlate well with the findings from CSLM examination for biofilms using the Invitrogen BacLight kit, and are consistent with previous studies, which have shown that not all S.*aureus* isolates form biofilms.[143, 144]

In accordance with findings of previous studies,[141, 145] S. *aureus* biofilms were more resistant to antimicrobial therapy when compared with their planktonic counterparts. Although we cannot make definitive conclusions for isolates with BMICs less than 7.18μg/ml, the results suggest that BMICs for mupirocin were relatively low when compared with the other two antibiotics.

The presence of biofilms on the mucosal surfaces of patients with CRS is now established. However, the precise role of biofilms in the pathogenesis of this condition remains unclear. Research by Bendouah et al has indicated that the presence of bacterial biofilms may predispose patients to worse outcomes following endoscopic sinus surgery,[62] It is postulated that biofilms may be a primary causal factor in patients with CRS recalcitrant to medical and surgical therapy. If this is in fact the case, then there is a

need for the development of therapy targeting the elimination of biofilms. Topical anti-biofilm agents such as mupirocin applied in nasal irrigations may be beneficial in eradicating biofilms left behind following surgery, and offer a potential treatment for recalcitrant CRS.

From our knowledge, this is the first study to describe the *in vitro* effects of mupirocin on S.*aureus* biofilms. Our department is currently in the process of conducting *invivo* animal and human trials to investigate the efficacy of mupirocin in the treatment recalcitrant chronic rhinosinusitis.

## Conclusion

In conclusion, we have used an *in vitro* crystal violet biofilm reduction assay to investigate the effect of mupirocin, ciprofloxacin and vancomycin on S.*aureus* clinical isolates from patients with CRS. Our results suggest that topical mupirocin may be useful in eliminating *S.aureus* biofilms present on the sinus mucosa of patients with CRS.

# CHAPTER 4

# A Sheep Model for the Study of Biofilms in Rhinosinusitis

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**Am J Rhinol. 2007 May-Jun;21(3):339-45.**

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**A Sheep Model for the Study of Biofilms in Rhinosinusitis**

**Am J Rhinol. 2007 May-Jun;21(3):339-45.**

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# Abstract

**Background**

Bacterial biofilms have been demonstrated in chronic diseases such as cystic fibrosis, cholesteatoma and otitis media with effusion. More recently their detection on the mucosal tissue of sinusitis patients has implicated them in the pathogenesis of this condition. We present an animal model using sheep experimentally infected with Staphylococcus aureus to study the possible association between biofilm and sinusitis.

**Method**

24 sheep underwent bilateral endoscopic sinus surgery to identify their frontal ostia. The frontal sinuses were treated in one of the following ways according to pre-operative randomization: (1) ostium left patent (2) ostium left patent and bacteria instilled (3) ostium occluded (4) ostium occluded and bacteria instilled. The frontal mucosa was harvested at day 7 and examined for biofilm presence using confocal scanning laser microscopy as well as scanning and transmission electron microscopy.

**Results**

All three modalities demonstrated different rates of biofilm detection. Three dimensional structures that could be interpreted as biofilms were documented in 86% (n=36) of the sinuses analyzed using SEM. These structures were seen in all four study groups. The detection rate using the other two modalities was much lower with CSLM demonstrating biofilms in 48% (n=20) and TEM in only 29%. (n=12) of the sinuses analyzed . Unlike

SEM, these two modalities only detected bacterial biofilms in sinuses randomized to bacterial instillation.

**Conclusion**

The demonstration of bacterial biofilms in this animal model of sinusitis further supports the hypotheses that biofilms may play a role in the pathogenesis of this condition. There is an obvious discrepancy in the sensitivity and specificity of biofilm detection using the three modalities mentioned. CSLM appears to be the most objective technique. The inherent flaws, sampling error and subjectivity involved in SEM and TEM make these less reliable in documenting biofilm existence.

Ha, K.R., Psaltis, A.J, Tan, Lorwai and Wormald, P-J. (2007)  A sheep model for the study of biofilms in Rhinosinustis.
*American Journal of Rhinology, v. 21 (3), pp. 339-345, May/June 2007*
(*Erratum: American Journal of Rhinology, v.21 (4), pp. 519, July/August 2007)*

NOTE:  This publication is included on pages 57-79 in the print copy of the thesis held in the University of Adelaide Library.

# CHAPTER 5

# Confocal Scanning Laser Microscopy Evidence of Biofilms in Patients with Chronic Rhinosinusitis

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**Confocal Scanning Laser Microscopy Evidence of Biofilms in Patients with Chronic Rhinosinusitis**

**The Laryngoscope; 2007 July: 117:1302-1306.**

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**Confocal Scanning Laser Microscopy Evidence of Biofilms in Patients with Chronic Rhinosinusitis**

**The Laryngoscope; 2007 July: 117:1302-1306.**

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# Abstract

## Objectives

The recent detection of bacterial biofilms on the sinus mucosa of patients with chronic rhinosinusitis (CRS) has implicated them in the pathogenesis of this condition. Electron microscopy has been the main modality used to document the presence of biofilms on sinus tissue, however, it has inherent problems associated with tissue preparation and sampling. Recently, Confocal Scanning Laser Micrsocopy (CSLM) has emerged as a non-invasive, non-destructive technique for the analysis of biofilms. This study used CSLM as the means of investigating biofilm presence in CRS patients.

## Study Design and Methods

A prospective study comparing the presence of bacterial biofilms on the sinus mucosa of CRS and control patients was conducted using CSLM. 38 CRS patients undergoing endoscopic sinus surgery and 9 control patients were enrolled in this study. Demographic and clinical information was recorded from each patient and intra-operatively, sinus culture specimens and mucosal samples were obtained for microbiological and microscopic examination.

## Results

Using previously documented CSLM criteria, bacterial biofilms were found in 17 (44%) of the 38 CRS patients. No biofilm structures were evident in any of the controls. Patients having undergone previous sinus surgery seemed to have a higher incidence of biofilms

compared to those undergoing their first procedure. The difference however was not statistically significant. No correlation between positive bacterial cultures and biofilm presence was observed.

**Conclusions**

The CSLM detection of biofilms in CRS patients and their absence in controls further supports the hypothesis that biofilms may play a role in the pathogenesis of CRS. This study's lower reported incidence of biofilms compared to previous studies may reflect the increased accuracy of biofilm detection with CSLM.

Key Words:

Chronic Rhinosinusitis, CRS, bacterial biofilms, sinus mucosa, confocal, CSLM.

# Introduction

Rhinosinusitis can have a chronic, recalcitrant course often requiring multiple courses of antibiotics and/ or surgical procedures. Despite its high prevalence and the extreme socio-economic burden it places on society, its pathogenesis still remains unclear. Current hypotheses include the role of fungus, staphylococcal super antigens,[5] abnormalities of the inflammatory cytokine cascade,[6] abnormal cell-mediated immune responses, protracted osteitis of the sinus walls[7] and the presence of bioflms.[8, 9]

Biofilms are structured communities of bacterial cells encased in a self-produced exopolymeric matrix and are irreversibly attached to an inert or living surface. 99% of bacteria adopt this protective mode of growth and differ from their planktonic counterparts with respect to the genes they transcribe and phenotype they exhibit. They are very difficult to culture using standard techniques, and are extremely resistant to host defences and conventional antibiotic therapy. A recent study by Bendouah et al, has also demonstrated that biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa* is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis.[62] These characteristics have made the implication of biofilms in the pathogenesis of chronic diseases both attractive and plausible. Chronic diseases in which biofilms have been implicated include otitis media with effusion,[54] cystic fibrosis,[56] cholesteatoma[146] and chronic tonsillitis.[147] They have also been isolated on prosthetic devices such as central venous catheter tips, urinary catheters, orthopaedic prostheses and tympanostomy tubes.[32]

Recently bacterial biofilms have also been detected on sinus specimens from patients with CRS. Unfortunately the vast majority of the research in this area has utilised electron microscopy to document the presence of these structures.[8, 9, 42] This paper describes the use of confocal scanning laser microscopy (CSLM), for the study of biofilms in CRS patients. Unlike other imaging modalities CSLM allows the non-invasive and non-disruptive imaging of living biofilms, and it is the opinion of the authors that it may allow more accurate determination of biofilm presence.

## METHODS

### Patients and Tissue Collection

A prospective study of thirty-eight consecutive patients undergoing endoscopic sinus surgery (ESS) was conducted in hospitals of a tertiary rhinological practice in Adelaide, South Australia, Australia. All patients were diagnosed with chronic rhinosinusitis according to the criteria previously outlined by the Chronic Rhinosinusitis Task Force in 2003.[1] Tissue from nine patients undergoing endoscopic trans-sphenoid resection of pituitary adenomas was used as the control group. The study was approved by the hospital based Ethics of Human Research Committee with all patients providing informed consent before enrolment.

Clinical data, including demographical information, relevant past medical and surgical history, asthma, allergy and smoking status were all recorded. Each patient was asked

specifically to indicate the severity (score 0-5) of the following 5 sinusitis symptoms: nasal obstruction, rhinorrhea, post nasal drip, headache/facial pain, and loss of smell. Radio-allergo-sorbent testing (RAST) to 4 common allergens and total IgE levels were also performed prior to the individual's operation. If taken, the results of intra-operative bacterial and fungal sinus cultures were also recorded.

**CRS Patient Group**

Two random mucosal samples, ranging from 5 -10mm2 were taken from the middle meatus and ethmoid cavity and stored immediately in Dulbecco's modified eagle medium (Gibco, Invitrogen Corporation, Grand Island, NY) on ice. The samples were transferred for same day analysis at Adelaide Microscopy. Patients on concurrent antibiotic or steroid therapy were not included in the study

**Control Group**

Nine patients undergoing endoscopic trans-sphenoidal procedures for benign pituitary tumours were used as controls. None of these patients had symptoms suggestive of rhinosinusitis and recorded no previous rhinological history. Posterior ethmoid tissue harvested during superior turbinectomies of these patients were used for this study. Samples were stored and processed in an identical fashion to that of the patient group. All 9 patients denied using antibiotics or steroids prior to their procedure

**Tissue Preparation and analysis**

The sinus specimens obtained from each patient was processed and analysed within two hours of collection. Each sample was washed thoroughly in 3 separate beakers of sterile MQ water to remove any planktonic bacteria. The sample was then immersed in 1ml of sterile MQ water, to which 1.5 ul aliquots of component A (Syto 9) and component B (Propidium Iodide) of BacLight LIVE/DEAD kit (Invitrogen, Molecular Probes) were added. Samples were incubated in this solution in darkness at room temperature for 15 minutes. When used in combination Syto 9 preferentially stains live cells green and Propidium iodide stains damaged or dead cells red. Following incubation, each sample was rinsed in sterile MQ water to remove excess Baclight and mounted on cover slips for analysis with a Leica TCS SP5 spectral scanning confocal microscope (Leica Microsystems, Exton, Pa). The entire area of each specimen was scanned for the presence of biofilm structures using a water immersion lens at both 20x and a 63x magnification.

**Biofilm Criteria**

The investigator examining the sample was blinded as to the disease state of the patient. Samples were assessed for bacterial biofilms as determined by the presence of immobile, irreversibly attached, live bacteria of appropriate size (0.5 - 2um diameter) and morphology, existing in characteristic clusters and towers of micro-colonies.[155] Using the Leica Application Suite Advanced Fluorescence Software (LAS AF), the entire depth of the mucosal surface was imaged by means of z-stacks with slices taken at 0.5um thickness. This was performed to ensure that biofilms lying deep within the mucosa were not missed.

**Statistical Analysis**

Statistical analysis was performed using SPSS(r) 11.0. All parametric data were characterized by mean ± standard deviation. Analysis of the 47 patients was performed per-protocol and inter-observer variability tested using 12 independent observers. For all statistical tests employed, a = 5%, ß = 20% and P was considered to be significant at a p value = 0.05.

Differences between the 2 patient groups, as well as between CRS patients with and without biofilms were analysed using the following statistical tests: a Chi-Square or Fisher's exact test for dichotomous data, a Mann-Whitney-U-Test for ordinal data and a Student's T-test for continuous data.

To evaluate inter-observer reliability, the average measure of intra-class coefficient (ICC) was calculated using a two-way random model with absolute agreement. To account for chance in our set of dichotomous data, Fleiss' kappa was calculated as a measure of inter-observer agreement.

## RESULTS

**Patient Demographics**

47 patients were analysed for the presence of biofilms. The control group consisted of 9 patients (5 females, 4 males), with a mean age of 51.7 ± 16.3. The CRS patient group included 38 patients (13 females, 25 males), with a mean age of 53.1 ± 16.2 (table 5).

**Table 5.**
Comparison of CRS and control groups

| Data | CRS Patients | Controls |
|---|---|---|
| Number analysed | 38 | 9 |
| Age (mean ± s.d) | 53.1 ± 16.2 [48 to 62.9]* | 51.7 ±16.3 [44.6 to 58.9]* |
| Gender | Female 13   Male 25 | Female 5    Male 4 |
| Symptom Score (max.=25) | 16.1 ± 3.3 [15.5 to 18.1]* | 0 |
| Previous Sinus Surgery | 30 | 0 |
| Presence of Biofilm | 17 | 0 |
| Positive Bacterial Culture | 22 of 31 | N/A |
| Positive Fungal Culture | 6 of 21 | N/A |

*95% Confidence Interval

**Clinical Data**

Consistent with the inclusion criteria, no controls had any symptoms suggestive of

sinusitis.  Conversely, the average symptom score for the CRS patient group was 16.1 ±

3.3 (table 5).  The most debilitating symptom recorded was nasal obstruction with an

average subjective severity of 3.6/5. The majority of patients reported a symptom

duration of greater than 5 years.

Approximately half of the CRS patients had medical co-morbities ranging from asthma,

gastro-oesophageal reflux and diabetes to bronchiectasis and immunoglobiln deficiency.

79% (30/38) of CRS patients had undergone previous ESS with 47% (14/30) of these

patients having had more than one procedure. 12 patients demonstrated allergy to

common environmental allergens, with house dust mite being the most common allergen.

Most patients showed pan-sinusitis on CT imaging.

Intra- operative sinus swabs for bacteriology were taken from 31/38 CRS patients, with

22 yielding a positive culture. Staphylococcus aureus was the most commonly isolated

organism (16/22, table 6). Based on clinical index of suspicion, fungal cultures were

performed in 21 patients however only 6 recorded a positive growth.

**Table 6.**
Organisms isolated from CRS patients
Number of patients culturing the organism specified

| | Organism Isolated | Number of patients |
|---|---|---|
| **Bacteria** | Staphylococcus aureus | 16 |
| | Escherichia coli | 3 |
| | Mixed aerobes | 2 |
| | Pseudomonas aeruginosa | 2 |
| | Streptococcus pneumoniae | 2 |
| | Proteus mirabilus | 1 |
| | Haemophyllis Influenza | 1 |
| **Fungus** | Aspergillus | 3 |
| | Alterneria sp. | 2 |
| | Curvularia sp. | 1 |
| | Epicoccum nigrum | 1 |
| | Rhizobus sp. | 1 |
| | Ulocladium sp. | 1 |

**CSLM Findings**

According to previously documented criteria, all 47 samples were analysed by the

authors (AJP and KRH) for the presence of biofilms. To test inter-observer reliability12

independent observers also analysed randomly ordered CSLM z-stacks of each sample.

All observers received a standardized tutorial regarding the CSLM features of bacterial

biofilms prior to their participation in this study.  The frequency of biofilms in patients

was 44.7% (n=17) and 0% for controls (Table 5). This difference was statistically

significant (Chi-square test, p < 0.006). Figure 12 demonstrates the CSLM appearance of

a healthy control specimen. Figure 13 highlights the contrasting appearance of a chronic

rhinosinusitis patient with a biofilm.
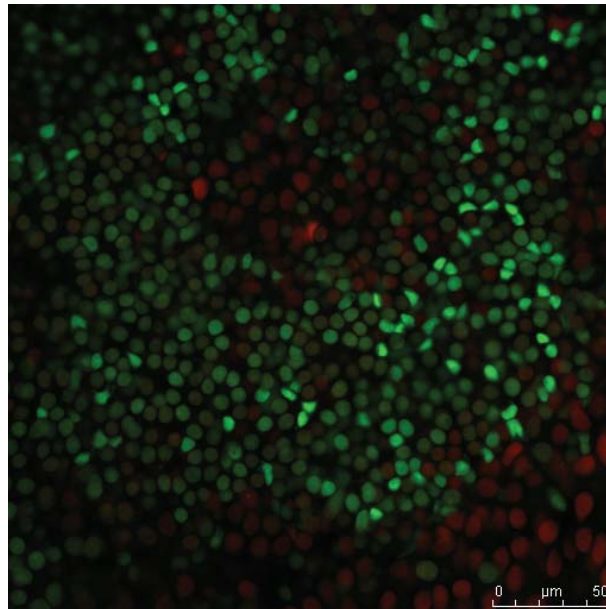


**Figure 12.**
Confocal scanning laser microscopy image (63x magnification) of sinus tissue from a
control specimen. Note the presence of both live (green) and damaged or dead (red)
epithelial cells. No bacterial biofilm is seen.
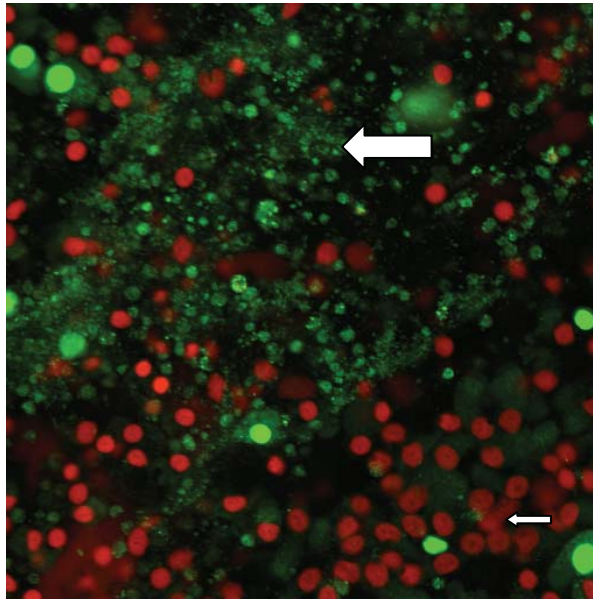
**Figure 13.**
Confocal scanning laser microscopy image (63x magnification) of a CRS patient demonstrating a bacterial biofilm. The biofilm is comprised of many intensely fluorescing live bacteria organized in clusters (large arrow). Note the much larger live and dead epithelial cells (small arrow).

The ICC for inter-observer reliability of determining the presence or absence of a biofilm in z-stack images obtained using CSLM was 0.97 (95% CI: 0.96-0.98). As there exists a 50% chance of achieving ratings when analysing any dichotomous data, a Fleiss' kappa value was calculated to correct for this possibility. The Fleiss' kappa was 0.729.

# DISCUSSION

Using confocal scanning laser microscopy, bacterial biofilms were detected on the sinus mucosa of 17 of 38 (44%) with chronic rhinosinusitis. No control specimens demonstrated any evidence of biofilms (p < 0.01). The frequency of observed biofilms in this study was however considerably lower than previously published studies employing other modalities. Using scanning electron microscopy, Sanclement et.al. reported bacterial biofilms in 24 of 30 (80%) CRS patients.[9] More recently, Sanderson et. al. used fluorescent in situ hybridization in their study of 18 patients with CRS. This group detected bacterial biofilms in 14 of 18 (78%) patients, and in 2 of 5 controls. No statistically significant difference between the 2 groups for the presence of biofilms was observed.[18]

The discrepancy between the results of this study and those previously published might actually exist or alternatively could be as a result of the different detection modalities used, differences in the patient population studied or sampling/ analytical error. Regardless of this, the consistent demonstration of biofilms on the sinus tissue of CRS patients and their relative absence on control specimens leads one to believe that these structures may play a role in either the pathogenesis or persistence of chronic rhinosinusitis. The often recalcitrant nature and extreme resistance of CRS to antibiotics, as well as the difficulty in consistently culturing pathogens all suit the biofilm paradigm.

Of the patients analysed in this study, 30 of 38 (79%) had undergone previous sinus surgery reflecting the tertiary nature of this rhinological practice. 50 % of these revision cases demonstrated biofilms under CSLM examination.  The high incidence of biofilms in patients with CRS refractory to surgical intervention further implicates biofilms as a contributing factor in the persistence of this condition. Furthermore it is important to note that  of the small number of patients undergoing their first surgical intervention, only 2 of 8 (25%)  showed biofilms. This observed difference was not statistically significant (p=0.19) however due to the small number undergoing primary surgery.

Bacterial cultures were taken from 31 CRS patients. In 7 patients, specimens were either not sent or not available.  No statistically significant difference was seen between patients with evidence of biofilms and patients without biofilms with respect to the presence of a positive bacterial culture (p>0.05). (see table 7 for raw data)  This finding also supports the biofilm model, whereby bacteria adopting this form exhibit a reduced growth and metabolic rate, making them difficult to culture using the standard techniques. These current culture techniques only reliably detect planktonic bacteria. Sinus swabs were not taken from the control group, and so comparison between culture rates with the CRS group could not be made.

**Table 7.**
Comparison of CRS patients with biofilms and CRS patients without biofilms.

| Data | CRS patients with Biofilms | CRS patients without Biofilms |
|---|---|---|
| Number analysed | 17 | 21 |
| Age (mean ± std) | 53.7 ± 16.7 [41.1 to 66.7]* | 50.6 ± 16.2 [46 to 66.9]* |
| Gender | Female 6  Male11 | Female 7  Male 14 |
| Symptom Score (max.=25) | 16.1 ± 3.1 [13.9 to 18.6]* | 16 ± 3.6 [15.3 to 19]* |
| Lund McKay Score (median) | 21 (range 5-24)** | 15 (range 3-24)** |
| Previous Sinus Surgery | 15 | 15 |
| Positive Bacterial Culture | 8 of 12 | 14 of 19 |
| Positive Fungal Culture | 4 of 8 | 2 of 13 |

*95% Confidence Interval

**Statstically significant difference (P<0.05 Mann Whitney U score 76.50)

Table 7 highlights comparisons made between CRS patients in whom biofilms were detected and CRS patients in which no such structures were demonstrated. The groups were similar with respect to demographics and showed no significant difference in symptom scores (p=0.8606). Interestingly enough however, there was a statistically significant difference in the Lund McKay radiological stage between those patients with biofilms and those without (P<0.05). This may reflect what is already believed by some researchers that patients with biofilms have a propensity for more severe sinus disease. The lack of correlation between symptom score and radiological stage is not surprising with the vast majority of papers published to date showing poor correlation between the two. With regards to previous surgery, 88% (15 of 17) of patients demonstrating biofilms

had undergone more than one procedure in comparison to 71% (15 of 21) of CRS patients who did not show biofilms. The extremely high percentage of biofilm positive patients undergoing multiple procedures may again reflect the difficulty in completely eradicating the biofilm nidus with surgery and may explain the tendency for the sinus disease to recur in this group of patients.

The recent development of multi-photon laser microscopy, fluorescently labelled rRNA-targeted oligonucleotides, and confocal scanning laser microscopy have not only allowed researchers to image biofilms in a non-invasive and non-destructive manner, but have also provided more detailed information on their entire structure. Despite the advent of such technology, most of the research involving biofilms in sinusitis has relied upon electron microscopy. We have previously shown in our animal model that CSLM offers many advantages over both scanning and transmission electron microscopy in the study of biofilms on sinus tissue.[155] This technique avoids many of the inherent flaws of tissue preparation, orientation and analysis associated with both scanning and transmission electron microscopy.

Tissue preparation for CSLM takes no longer than 20 minutes, and is processed fresh thus circumventing the need for any use of fixatives or other dehydrating agents. Samples analysed can be much larger than their electron microscopic counterparts making orientation simpler and more reliable. The CSLM technique in this study employed the use of nucleic acid probes. These probes specifically stain the cellular structures and the exopolymeric saccharide matrix making them easily distinguishable from mucus.

Bacteria can be readily differentiated from other cells by their smaller size and more intense fluorescence. As a result biofilms can be more reliably detected using this technique.

To test the inter-observer reliability of biofilm detection using CSLM, 12 independent observers were asked to examine a series of images obtained from each patient. The ICC score of 0.97 achieved here, is regarded as "outstanding"[156] and the weighted kappa value of 0.73 assures that this inter-observer reliability was not simply due to chance.[157]

## CONCLUSION

Although the role of biofilms in CRS remains unclear, this paper presents further evidence of their existence in this condition. Their presence alone does not implicate them in the pathogenesis of CRS, but may partly explain its recalcitrant nature and its resistance to standard antibiotic therapy. The fact that biofilms were present in only 44% of CRS patients, suggests that their pathogenesis of CRS is multi-factorial.

# CHAPTER 6

# SUMMARY, CONCLUSIONS
# & REFERENCES

# Summary

This thesis consists of a number of separate studies. The results of each study were designed to help provide an evolution of knowledge that could be applied to our subsequent investigations on the topic of bacterial biofilms and chronic rhinosinusitis. Initial *in vitro* experiments were conducted to determine the growth characteristics and the optimal conditions required for *S.aureus* reference strain ATCC 25923 to form biofilms. CSLM was employed to confirm *in vitro* biofilm growth as well as to provide a platform of knowledge with regard to the methodology of detecting and analyzing bacterial biofilms on mucosal surfaces. An animal model using sheep was then developed as a tool for further investigating the role of bacterial biofilms in CRS and additionally, to evaluate the use of SEM, TEM, and CSLM in bacterial biofilm detection on sinus mucosal surfaces. The next step was to use CSLM to document the incidence of bacterial biofilms in patients with CRS. Finally, the *in vitro* biofilm model was revisited to determine the biofilm forming capacity of *S.aureus* strains isolated from patients with CRS, and to investigate the efficacy of Mupirocin, Ciprofloxacin and Vancomycin on biofilm reduction.

*S.aureus* was chosen as the microbe of choice in our studies as it is the most commonly isolated organism in patients with CRS and is likely to play a role in pathogenesis of this condition.[12] *S.aureus* is an accepted biofilm forming organism and has been studied extensively in the scientific literature. Reference strain ATCC25923 was used as this is a well documented biofilm forming strain.[149]

*In vitro* biofilms were investigated by means of a 96-well microtiter crystal violet plate assay based on the model described by O'Toole and Kolter.[75] This model has been used extensively in the literature as a means of propagating and investigating bacterial biofilms.[77] It is a high through-put system that provides a suitable tool for screening large numbers of bacterial strains and easily allows adjustment of growth variables. Using these methods, the optimal growth parameters were determined for of *S.aureus* reference strain ATCC 25923 and these conditions were then replicated for the propagation of biofilm on Permanox chamber slides and subsequent CSLM examination. Using specific nucleic acid stains Syto 9 and propidium iodide and techniques based on the work of Ehrlich et al.,[49] CSLM examination of *S.aureus* biofilms revealed intensely fluorescing, immobile bacteria arranged in towers and clusters. This allowed biofilm criteria to be defined and these criteria to be used on subsequent studies to identify biofilms in the sheep model and human study.

There are obvious limitations in *in vitro* studies in terms of the accurate representation of the natural biological and pathological processes that occur in humans. The development of a reliable animal model to study biofilms in CRS will allow further investigation into the role of biofilms in CRS as well as an avenue by which potential treatment modalities can be trialed *in vivo*. A previous animal model established for the study of biofilms in CRS utilized New Zealand white rabbits.[59] This model has a number of inherent problems including the requirement for external surgical access, the inability to perform ESS, and confounding *Pasteurella* colonization. Sheep are a more suitable animal model for the study of CRS for the reasons that, they have a similar spectrum of disease, have

comparable histological and anatomical sinus architectures, can accommodate repeated ESS and have easily accessible frontal sinuses.

To replicate findings often seen in human CRS, sheep underwent preparatory ESS to expose frontal sinuses ostia.  These openings were then experimentally occluded, and bacteria introduced into the sinus through a frontal mini-trephine.  Based on the findings of our previous *in vitro* studies, potential *S.aureus* biofilms were propagated for 7 days and mucosal specimens subsequently removed for examination of bacterial biofilms. CSLM, SEM and TEM were used to evaluate the efficacy of these microscopy modalities in the detection of biofilms on sinus mucosa.  The CSLM biofilm detection rate was 100% in obstructed sinuses with bacteria introduced, whereas TEM detected only 66%. Both these objective measures failed to identify biofilms in control groups.  SEM found biofilms in all experimental groups including controls.  The traditional electron microscopic techniques used to identify biofilm on sinus mucosal surfaces is capable of providing high resolution 2-dimensional images of mucosal surfaces.  These methods however, require preparation techniques which use fixatives and dehydrating agents, which inevitably results in dehydration of EPS matrix and distortion of biofilm structure. Analysis of SEM images for biofilm is additionally complicated by the difficulty differentiating normal mucosal mucus from biofilm matrix.  This is likely to result in erroneous over-detection bacterial biofilms.  Conversely, TEM only allows examination of micro-thin sections of sample mucosal tissue, thus can potentially overlook the presence of biofilms. CSLM has various advantages over electron microscopy, mucosal tissue can be analyzed fresh and in real-time; avoiding artifact and distortion associated

SEM/TEM, viable adherent bacteria and a haze of EPS can be objectively visualized in a 3-dimensional view and at various levels if required. Finally, large surface areas can be scanned making this a highly specific modality.

The animal model developed will enable further investigation into the role and treatment of biofilms in sinusitis; however, it is important to recognize that this model has a number of clear limitations. Although we believe that the sinuses of sheep closely resemble that of humans, it would be neglectful to assume that it is an accurate representation of normal human sinus physiology, immunological function, and response to treatment. An artificial state of sinus infection and inflammation is created in the assumption that this occurs as a pathological process of CRS in humans. CRS is a multi-factorial disease and it is unlikely that all cases involve bacteria. Additionally, this model propagates biofilms for 1 week and the chronic changes present at this time do not necessarily represent the clinical and histological picture at 12weeks. Finally, we have used a laboratory reference strain of *S.aureus* in our studies as we believe this is a likely pathogen in CRS. The human sinus is colonized with a multitude of aerobic and anaerobic organisms,[10] any of which may be responsible for an acute or chronic infection, and any of which may form biofilms or indeed, a multi-species biofilms.

The vast majority of research to date has utilized SEM and TEM to document biofilms in CRS, with a reported incidence of up to 80 per cent.[9] From the findings of our animal model, these results may be misleading. Using CSLM, we detected biofilms in only 44 per cent of patients with CRS. This discrepancy may represent differences in population,

sampling error or analytical errors. The morphology of biofilms on CRS patient specimens varied considerably between patients and when compared to biofilms on sheep mucosal specimens. This may represent differences in host physiological or immunological processes, bacterial strains or species or the diversity of potential multi-species biofilms in CRS. The additional use of rRNA-targeted oligonucleotides in FISH would allow further characterization of specific bacterial species within mucosal biofilms; however, the intention of this particular study was purely to determine the incidence of biofilms in CRS.

*In vitro* biofilm methods were revisited with the aim of documenting the biofilm forming capacity of *S.aureus* strains isolated from patients with CRS and to examine the potential efficacy of anti-microbial treatments. Of the 12 clinical isolates tested, only 8 (66 percent) formed biofilms, a finding consistent with previous studies which have shown that not all *S.aureus* isolates form biofilms. [143, 158] Subsequently, three antimicrobials with known planktonic anti-staphylococcal activity were tested for their capacity to reduce biofilm mass in the clinical isolates. Mupirocin, Ciprofloxacin and Vancomycin were chosen as these medications could potentially be applied directly to sinus mucosal surfaces via nasal irrigation. Mupirocin was the only antimicrobial capable of reducing mature, well established biofilms of all isolates at safe concentrations, and concentrations easily attainable with nasal irrigation washes.

The application of Mupirocin to nasal irrigation therapies may provide an effective means of treating *S.aureus* biofilms if present on CRS sinus mucosal surfaces. Topical therapy

enables the delivery of high local concentrations of Mupirocin with the added benefit of minimal systemic absorption. Moreover, Mupirocin nasal nasal lavage has recently been shown to be well tolerated and effective therapy for patients with CRS recalcitrant to ESS.[159]

The *in vitro* microtiter plate format used in these experiments allows a screening of multiple strains or species and multiple anti-biofilm treatments. Reproducibility is high as multiple biofilms are grown on the same plate at the same time. The model can also accommodate the combination of several anti-microbials with a different mechanism of action for additive or synergistic anti-biofilm potential. This type of biofilm model, however, has a number of inherent limitations. Crystal violet stains both bacterial cells and the nucleic components within the EPS matrix and hence may not be a true representation of the number of adherent bacteria. Nevertheless, when bacteria are propagated in a plate assay, it is generally expected to see increased adherent biomass over a period of time, followed by a decrease as the bacteria detach or die. The methods instituted to evaluate the effectiveness of antibiotics involved calculating the amount of biofilm reduction following treatment. It is expected that this correlates with the biofilm killing capabilities of these anti-microbials; however, definitive conclusions cannot be made. An alternative is to use a sonicator to dislodge adherent bacteria followed by plating and counting viable bacteria, this though is a time consuming tedious task that can be complicated by further variables.[68] Furthermore, this study measured antibiotic concentrations required to reduce biofilm mass by a standard 90 percent, this does not necessarily ensure the eradication of all biofilm bacteria at these doses. It is accepted that

any remaining biofilm cells could potentially act as a nidus for further biofilm growth. Finally, planktonic *S.aureus* isolates were obtained from cultures of patients with CRS. It is presumed that these planktonic bacteria originate from biofilms within the sinus, though it is recognized that they may not be representative of the true biofilm community *in vivo*.

# Conclusions

Bacterial biofilms are present in many cases of chronic rhinosinusitis, though their precise role in the pathogenesis of this condition remains to be defined. Our *in vitro* biofilm studies provide further evidence that bacteria isolated from CRS sinuses have the capacity to form biofilms. A reliable animal model has been developed to further investigate the role of biofilms in CRS and potential treatments *in vivo*. This model has also provided validation that confocal scanning laser microscopy is superior to traditional electron microscopy for investigating the presence of biofilms on sinus mucosal surfaces. The incidence of biofilms in CRS patients is likely to be considerably lower than previous reports, suggesting that the pathogenesis of CRS is a multi-factorial. Topical Mupirocin may be useful in the treatment biofilm related CRS however further *in vitro* and *in vivo* studies are required.

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