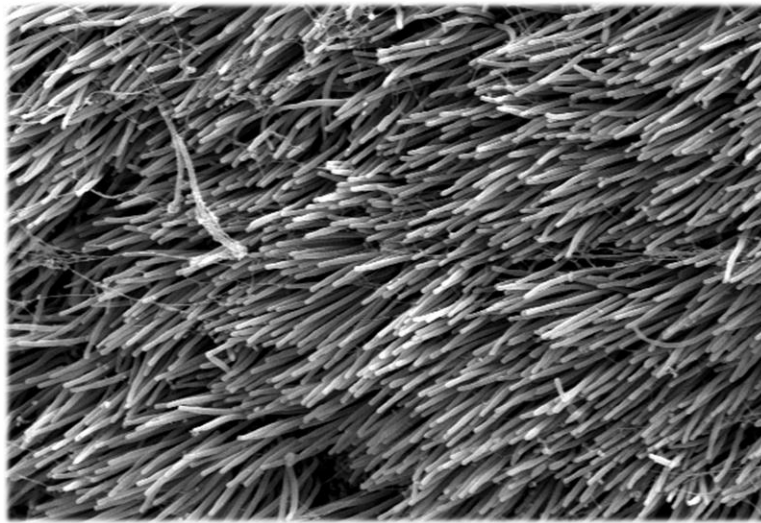


Bacteriophage therapy for application against
Staphylococcus aureus infection and biofilm
in chronic rhinosinusitis



Amanda Jane Drilling

Faculty of Health Sciences

School of Medicine

Discipline of Surgery

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The enemy of my enemy is my friend

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I. Abstract

Chronic rhinosinusitis (CRS) is a debilitating condition characterised by critical inflammation of the mucosa of the nose and paranasal sinuses. Affecting up to 14% of the world's population CRS severely impacts a patient's quality of life. The aetiology of CRS is complex and relatively undefined encompassing a multitude of contributing factors. Bacterial infection is one factor thought to play a role in the pathogenesis of CRS. More specifically biofilm forms of the bacterial species *Staphylococcus aureus* have been shown to negatively influence post-operative progression. Current practice treatment strategies often fail to remove biofilms from the mucosa of the nose. It is therefore of import to develop novel anti-biofilm therapeutics. Our understanding of the epidemiology of *S. aureus* infections and biofilms in CRS is also limited. Increasing our epidemiological knowledge would help in the development of effective treatment strategies against recurrent infections.

Investigation into the epidemiology of *S. aureus* infections was undertaken by collecting *S. aureus* isolates from mucous and biofilm structures of CRS patients. The clonal type of each isolate was then compared to the other isolates using pulse field gel-electrophoresis. Results of this study indicated that the majority of patients experiencing recurrent infections maintained the same clonal type. Furthermore the study suggested that long-term antibiotic therapy in some patients can lead to the development of bacterial antibiotic resistance. Therefore development of a novel antibacterial therapy outside of antibiotics is required.

A potential anti-biofilm therapy both eliminating and preventative in nature is the application of bacteriophage. Bacteriophage (phage) are viruses that specifically target, infect and destroy bacterial cells. Initially *in vitro* study was undertaken to assess the anti-biofilm activity of a phage cocktail specific for *S. aureus* (CT-SA) using a minimal biofilm eradication assay plate. *S. aureus* isolates from CRS patients were grown to mature

biofilm form and treated with CT-SA for 48hrs. Following treatment biofilm biomass was determined by staining bacteria with a Live/Dead BacLight stain, imaging the biofilm using confocal scanning laser microscopy and determining biofilm biomass using software COMSTAT2. Results showed CT-SA significantly reduced *S. aureus* biofilms of susceptible strains. Results also indicated that a cocktail of phage was superior to use of a single phage as it reduced the frequency of bacterial resistant to the phage treatment.

Following on from *in vitro* work, the safety and efficacy of CT-SA was assessed *in vivo* using a sheep model of frontal sinusitis associated with *S. aureus* infections. CT-SA was also combined with ethylenediaminetetraaceticacid (EDTA) to observe if these therapies would synergise. Results indicated both CT-SA and EDTA were safe for short term topical application to the sinus regions. Furthermore both CT-SA and EDTA individually significantly reduced *S. aureus* biofilm levels in the frontal sinus, but were not seen to synergise.

Work conducted in this thesis has helped lead towards development of a novel anti-*S. aureus* biofilm agent. Future translation of CT-SA to a clinical trial setting may not only reduce or remove *S. aureus* biofilm from CRS patient noses but also improve their symptomatology and quality of life.

II. 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 to Amanda J Drilling 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.

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IV. Presentations and Awards Arising from this thesis

Presentations:

Basil Hetzel Institute post-graduate presentation, Adelaide, Australia, **Bacteriophage as a Novel Treatment of Recalcitrant Chronic Rhinosinusitis**, August 2011

Australian Society of Otolaryngology Head & Neck Surgery Annual Scientific Meeting, Adelaide, Australia, **'Bacteriophage as a Novel Treatment for Staphylococcus aureus Biofilm,'** April 2012

Basil Hetzel Institute post-graduate presentation, Adelaide, Australia, **'Bacteriophage treatment of biofilm in an in vivo sheep model of sinusitis,'** August 2012

TQEH Research Foundation Research Day (Poster presentation), Adelaide, Australia, **'Bacteriophage reduces biofilm of *Staphylococcus aureus* ex vivo isolates from chronic rhinosinusitis patients,'** October 2012

Australian microbiological Society Microbiological updates seminar, Adelaide, Australia, **'Can Stalin's forgotten cure be used to treat sinusitis?'** October 2012

University Engagement and the Florey Medical Research Foundation Friends & Benefactors presentation, Adelaide, Australia, **'Bacterial Therapeutics: The enemy of my enemy is my friend,'** July 2013

Australian Microbiology Society Conference, Adelaide, Australia, **'Bacterial Therapeutics: The enemy of my enemy is my friend'** July 2013, invited speaker.

Basil Hetzel Institute post-graduate presentation, Adelaide, Australia, **'Cousins, siblings or copies: the genomics of recurrent *Staphylococcus aureus* infections in chronic rhinosinusitis,'** August 2013

American Rhinologic Society Annual Meeting, ‘**Safety and efficacy of topical bacteriophage and EDTA treatment of *Staphylococcus aureus* infection in a sheep model of sinusitis,**’ Vancouver, Canada, October 2013

TQEH Research Foundation Research Day (Poster presentation), Adelaide, Australia, ‘**The enemy of my enemy is my friend: Assessing bacteriophage treatment of *S. aureus* biofilm in vivo,**’ October 2012

Awards:

Finalist in Poster Presentation,

TQEH Research Foundation Research Day, Adelaide Australia, 2012

Best Presentation Senior PhD researchers

TQEH Research Foundation Research Day, Adelaide Australia, 2013

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VII. Abbreviations

Abbreviation	Description
AR	Acute rhinosinusitis
ATCC	American type culture collection
BIM	Bacteriophage insensitive mutant
C	Confluent
CI	Clinical isolate
CPC	cetylpyridinium chloride
CRS	Chronic rhinosinusitis
CRSwNP	CRS with nasal polyps
CRSsNP	CRS without nasal polyps
CT4	Cocktail of <i>Staphylococcus aureus</i> specific phage concentration 6×10^4 PFU/mL
CT6	Cocktail of <i>Staphylococcus aureus</i> specific phage concentration 6×10^6 PFU/mL
CT8	Cocktail of <i>Staphylococcus aureus</i> specific phage concentration 6×10^8 PFU/mL
CT _{hi}	Cocktail of <i>Staphylococcus aureus</i> specific phage (heat inactivated)
CT-SA	Cocktail of <i>Staphylococcus aureus</i> specific phage
CTSA-EDTA	Combination of cocktail of <i>Staphylococcus aureus</i> specific phage and ethylenediaminetetraaceticacid
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraaceticacid
EPOS	European position paper on rhinosinusitis and nasal polyps committee
EPS	Extracellular polymeric substances

FESS	Function endoscopic sinus surgery
IgE	Immunoglobulin type E
MRSA	Methicillin resistance <i>Staphylococcus aureus</i>
NP	Nasal polyps
NT	No treatment
PFGE	Pulse Field gel electrophoresis
PFU	Plaque forming units
O	Opaque
QOL	Quality of life
SAGs	Superantigens
SEB	Staphylococcal enterotoxin B
SEM	Scanning electron microscopy
SC	Semi-confluent
TEM	Transmission electron microscopy

1 Systematic review of the Literature

1.1 Rhinosinusitis

The Sinus and Allergy Health Partnership Task Force for Rhinosinusitis define rhinosinusitis as ‘a group of disorders characterized by inflammation of the mucosa of the nose and the paranasal sinuses.’¹ Comparison of computed tomography scans taken of healthy sinuses and rhinosinusitis afflicted sinuses emphasises the gross inflammation often observed in the paranasal sinus region of an individual with rhinosinusitis (Figure 1.1). Previously known as sinusitis, rhinosinusitis has become the prevalent term used to describe this condition as sinusitis is often associated with rhinitis symptoms and frequently preceded by rhinitis associated symptoms¹.

A number of factors are known to contribute to rhinosinusitis. These commonly include inflammation (allergic and nonallergic), microorganism presence and/or colonisation, and a number of other non-infectious and non-immunologic causes. Quality of life is shown to be significantly impaired by rhinosinusitis. Sufferers describe interference with sleep, reduced concentration due to headaches, disruption to an individual’s daily routine. Such symptomology may lead to emotion dysfunction². Patients with rhinosinusitis have also registered significantly lower quality of life scores in regards to bodily pain and social functioning compared to other individuals including those experiencing angina, chronic obstructive pulmonary disease, congestive heart failure and back pain³.

This section of the introduction will provide a holistic background to CRS. It will consider various classifications of the condition, the prevalence and incidence stratified geographically and discuss some of the common factors contributing to the disease and current practice methods to manage the condition.



Figure 1.1: Computed tomography scan of paranasal sinus region. This figure highlights the differences seen in computed tomography scans of a (A) healthy and (B) rhinosinusitis afflicted paranasal sinus region. (A) The healthy sinus region is shown to be clear from obstruction, and has minimal inflammation within the sinonasal region. This contrasts with the image of the (B) rhinosinusitis afflicted nasal region showing high levels of mucosal inflammation disrupting normal functioning of the nasal cavity.

1.1.1 Acute and Chronic Rhinosinusitis

Rhinosinusitis is classified as either an acute or chronic style of infection dependent upon symptom duration. A number of different committees and taskforces have reviewed and defined aspects (definition, diagnosis and treatment) of rhinosinusitis. These taskforces have noted several discrepancies (Table 1.1). The European position paper on rhinosinusitis and nasal polyps committee (EPOS) define acute rhinosinusitis (AR) as an infection lasting for <12 weeks, and chronic rhinosinusitis (CRS) as >12 weeks in duration⁴. Both the American Rhinologic Society¹ and British Society for Allergy & Clinical Immunology⁵ designate AR as <4 weeks symptom duration and CRS as >12 weeks duration. A fourth committee, the American Academy of Otolaryngology–Head and Neck Surgery propose a third distinction of subacute rhinosinusitis characterised by 4-12 weeks symptomology. The committee also characterises AR as <4 weeks and CRS as

>12 weeks⁶. Finally, a fifth panel, the Joint Task Force on Practice Parameters define acute as symptoms lasting <4 weeks, subacute between 4-8 weeks and chronic >8 weeks⁷.

Further, many bodies include the classification of recurrent rhinosinusitis. Recurrent rhinosinusitis is distinct from CRS and refers to an acute style of disease that occurs more than 3⁷ or 4⁶ times a year. The 5 committee papers have slight variations in what should be considered when diagnosing CRS; however, there is significant overlap of a number of symptoms. In considering all bodies' classifications, two are more major symptoms should be present for the diagnosis of CRS. These include:

- Nasal congestion/obstruction.
- Nasal discharge (posterior and anterior).
- Facial pain or pressure.
- Reduced or loss of sense of smell.

Minor symptoms include:

- Fatigue.
- Fever.
- Halitosis.
- Sore throat.
- Malaise.

Whilst the presence of such symptoms may suggest CRS, clinical pathophysiology plays an important role in any diagnosis of the condition. This may involve nasal endoscopy to identify the presence of mucosal swelling, nasal polyps and purulent discharge. Such investigations may be combined with CT imaging to identify thickening of the sinus mucosa, changes in the bone structure as well as assessment of the air-fluid interface⁴.

The current study is focused on a chronic disease state, and hence the review of literature will be focus on chronic infections.

Table 1.1: Sample of 5 rhinosinusitis committees' definitions and diagnosis of CRS.

Committee	Classifications	Diagnostic symptoms
BSACI ⁵	ARS: <4 wks CRS: >12 wks	Evidence of 2 of the following: -nasal congestion or obstruction - anterior or posterior nasal discharge ± facial pain or pressure ±reduction in sense of smell
ARG ¹	ARS: <4 wks CRS: >12 wks	Evidence of ≥2 symptoms: -nasal obstruction -facial pain or pressure -reduction in sense of smell, -purulent anterior discharge/postnasal drainage.
EPOS ⁴	ARS: <12 wks CRS: >12 wks	Evidence of ≥2 symptoms: -nasal congestion or obstruction -purulent anterior discharge, -postnasal drainage ±facial pain, ±decreased sense of smell.
AAO-HNS ⁶	ARS: <4 wks Sub-ARS: 4-12 wks CRS: >12 wks	Evidence of inflammation in the paranasal sinuses. Evidence of ≥2 symptoms: -purulent anterior discharge -nasal congestion -postnasal drainage -facial pain or pressure -reduced sense of smell
JTFPP ⁷	ARS: <4 wks Sub-ARS: 4-8 wks CRS: >8 wks	Evidence of major symptoms: -purulent anterior discharge -congestion, -facial or dental pain -postnasal drainage -headache -cough. Less frequent symptoms: -nausea, fatigue, fever, halitosis, sore throat or malaise

Note: BSACI = British Society for Allergy & Clinical Immunology; ARG = American Rhinologic Society; EPOS = European position paper on rhinosinusitis and nasal polyps; AAO-HNS = American Academy of Otolaryngology–Head and Neck Surgery; JTFPP = Joint Task Force on Practice Parameters; ARS = acute rhinosinusitis ; sub-ARS = subacute rhinosinusitis; CRS = chronic rhinosinusitis

1.1.2 Prevalence of Chronic rhinosinusitis

Inhitherto, the prevalence of CRS has only been investigated in a limited number of studies (Table 1.2). Studies have found varying prevalence rates with results from 1-27%. The lowest prevalence was reported as being 1% in a study conducted in Korea⁸ and the highest prevalence was found by researchers in Portugal who found prevalence of, 27%⁹.

Interestingly CRS prevalence differs between studies conducted in the same country such as Korea (1-7%)^{8, 10, 11} and the United States (2-14%)¹²⁻¹⁴. A consideration of the Korean studies suggests that the prevalence of CRS may have increased over time in this country. The study reporting the lowest rates of CRS (1%) was conducted in 1991 whereas those reporting rates of approximately 7% were conducted in 2008. Furthermore, differences in sampling methodology between the studies may have contributed to this variation. The study with lower prevalence excluded patients who had undergone previous sinus surgery possibly eliminating individuals who, despite previous surgery, were still experiencing the CRS condition⁸.

The varying results found in the U.S appear to be most readily explained by the sampling methods utilised by the researchers. The Minnesota study¹⁴ undertook a review of medical records rather than a survey style used in the other two studies^{12, 13}. There was also only approximately 2000 people participating in the Minnesota study whereas in the two other nationwide studies around 300,000 people were surveyed. The lower sample size may have contributed to the variation seen between studies.

Common factors that were shown to increase the risk of CRS included allergy or rhinitis^{8, 11, 15-18}, female gender^{9, 16}, cigarette smokers^{9, 16, 18}, older ages^{10, 11}, asthma^{15, 16, 18} and a low income^{16, 17}. Despite these commonalities some results indicated the opposite. One study

suggested males were significantly more likely to develop CRS¹¹ whilst a European study suggested younger demographics were, in some cases, more at risk⁹. Interestingly, one Korean study found that undergoing an influenza vaccination appeared to increase the risk of CRS¹¹.

It is difficult to assess whether geographical location alters the risk of an individual contracting CRS due to the varying sampling methodologies employed. However, within studies variation has been noted in data from the USA¹⁹, Canada¹⁶, Korea⁸ and the European Union⁹. Further, one Korean study found people living in crowded areas had a larger risk of developing CRS⁸. This may suggest that people who live in large cities have a greater risk of developing CRS.

Table 1.2: Summary of Literature discussing the Prevalence of CRS

Study	Country, year	Prevalence of CRS (%)	Sampling method	At risk populations
Adams 1999 ¹²	USA, 1996	14.1	National Health Survey	undefined
AIHW 2010 ¹⁵	Australia, 2004	9.2	National Health Survey.	Allergy, Asthma
Benson & Marano 1998 ¹³	USA, 1995	12.5	National Health Survey	undefined
Chen et. al. 2003 ¹⁶	Canada, 1996	4	National Health Survey	Older age, Smoking, Low income earners, Allergy, Asthma, COPD, Females
Cho et. al., 2012	Korea, 2008	7.12	Patient reported symptoms, endoscopic exam.	Older age
Hastan et. al. 2011 ⁹	European average, 2008	10.9	Postal questionnaire	Females, Younger age, Smoking
	Sweden	8.65		
	Finland	6.9		
	Denmark	7.9		
	Poland	17.13		
	UK	10.6		
	Netherlands	14.3		
	Belgium	18.8		
	Germany	10.5		
	France	13.3		
	Macedonia	8.2		
Italy	10.9			
Portugal	27.1			
Kim et. al. 2011 ¹¹	Korea, 2008	6.95	Physical examination and questionnaire	Males, Older age, Heavy stress, Allergy, Septal deviation, Influenza vaccination
Min et. al. 1996 ⁸	Korea, 1991	1.01	Review of medical history and physical examination	Crowding, Allergy
Pilan et. al. 2012 ¹⁷	Brazil, 2011-2011	5.5	Personal interviews/questionnaire	Asthma, Allergy, Low income earners
Shashy, 2004 ¹⁴	USA, 2000	1.96	Review of medical records	undefined
Thisling et. al. 2012 ¹⁸	Denmark, 2008	7.9	Postal questionnaire	Smoking, Asthma, Allergy, Cleaning occupations

Note: USA = United States of America, UK = United Kingdom

Only one study has investigated the incidence of CRS²⁰. This study found the average incidence to be 83 ± 13 cases per 100,000 person-years for CRS with nasal polyps (CRSwNP) and 1048 ± 78 per 100,000 person-years for CRS without nasal polyps (CRSsNP). Furthermore, this study found that patients with CRS attended more outpatient visits and were more likely to be prescribed antibiotics compared to control subjects. Premorbid conditions noted to increase the risk of CRS development included asthma, otitis media, gastroesophageal reflux disease and allergic rhinitis for both CRSwNP and CRSsNP. Further, number of different infections including pneumonia, bronchitis, influenza and conjunctivitis were associated with CRSsNP diagnosis. Whilst more studies are required to investigate incidence, findings in this study support a unified airway hypothesis where asthma and CRS are strongly linked.

Considering all these studies as a whole, the average percentage of individuals experiencing CRS around the globe equates to 7.4%. With a global population of 7.2 billion²¹, a prevalence of 7.4% would suggest approximately 534 million people would be experiencing CRS. With a possibility of such a large number of people being affected by this condition, it is imperative we find effective strategies to manage CRS to perhaps reduce the numbers afflicted with this condition.

1.1.3 Economic burden of the CRS condition

High rates of CRS contribute to increased costs due to greater treatment and management. The cost of treating and managing chronic rhinosinusitis patients in the USA was estimated at \$4.3 billion in 1994²², \$5.8 billion in 1996²³ and \$8.6 billion in 2007²⁴. Costs are attributed to direct expenses including to medical services such as clinical assessments, diagnostics and surgical intervention and indirect expenses including days off work and reduced productivity²⁵. Such data indicates the high economic burden CRS treatment and

suggests measures need to be taken to reduce prevalence of CRS and thus the associated costs. A study by Bhattacharyya et. al. 2011 assessed the associated patient costs to the year before, during and two years following CRS surgical intervention. Results showed expenses in the year prior to surgery of \$2,449, the year of surgery, 45 days post-operatively of \$7,726, in the year following surgery \$885, and two years after surgery of \$446²⁶. Such figures suggest that surgery may significantly reduce the economic burden of CRS. However, if we could achieve a similar reduction with a medical therapy rather than requiring a high-cost surgical procedure, this may further reduce the economic burden of CRS. One way of developing strong medical alternatives is to enhance our understanding of CRS aetiology.

1.1.4 Factors contributing to CRS

CRS is a multi-factorial condition, with a complex pathophysiology. A number of factors have been proposed to contribute to the pathogenesis of CRS including bacterial²⁷ and fungal infection²⁸, allergy²⁹, nasal polyposis³⁰, genetic disorders³¹, environmental factors (eg. Tobacco smoke or pollution)³², *Staphylococcus aureus*³³, and bacterial biofilm formation³⁴ (Figure 1.2). This section will briefly review factors proposed to contribute to the CRS condition. As this thesis is focused towards *S. aureus* infection and biofilm formation, these factors will be discussed further in Sections 1.2 and 1.3 respectively.

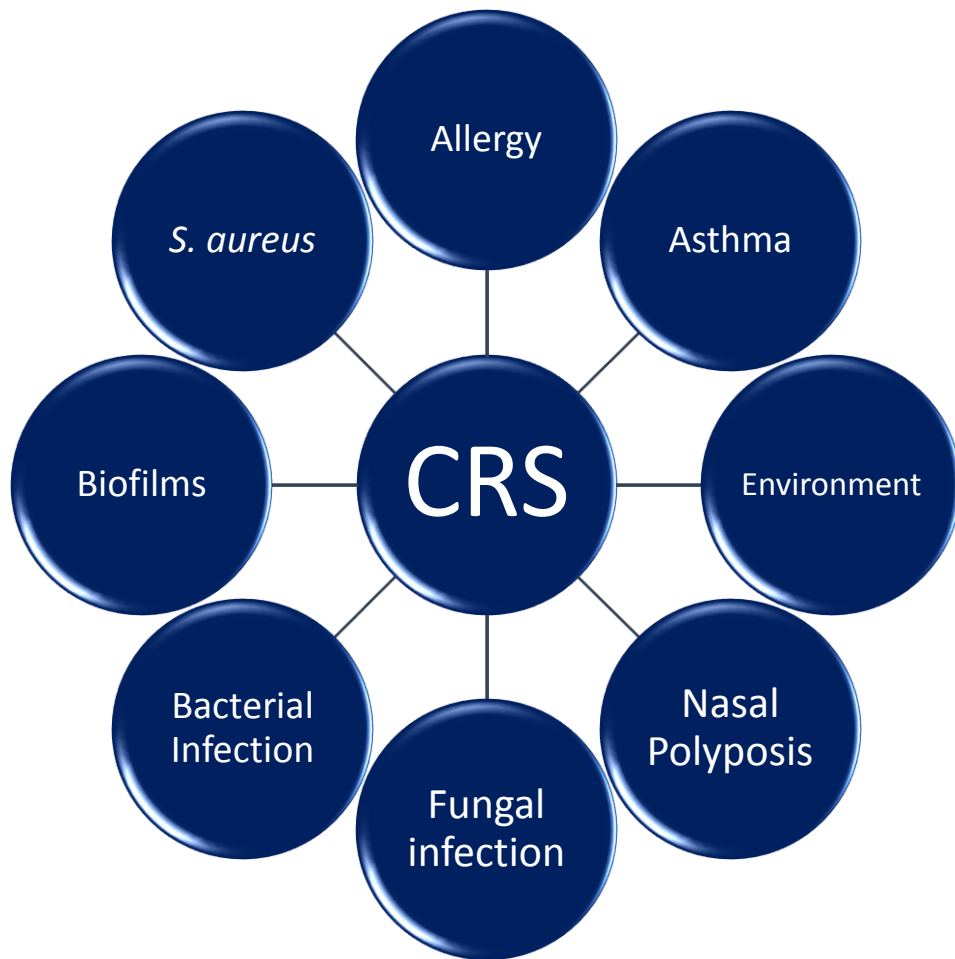


Figure 1.2: Factors contributing to the pathogenesis of Chronic Rhinosinusitis. Being multifactorial in nature, a number of diverse factors contribute to the CRS condition. Such a diverse range of aetiology contributors makes understanding, and effectively treating CRS a complex process.

1.1.4.1 Nasal polyposis

Nasal polyps (NP) in the context of CRS are defined as ‘benign nongranulomatous inflammatory tissue projection with an epithelial lining within the sinonasal cavity’³⁵.

Visually NPs are round, grape-like protrusions of nasal mucosa. They are normally composed of edema fluid, inflammatory cells, connective tissue, and some mucus glands and capillaries that are mainly covered in pseudo-stratified epithelial cells³⁶. NPs are significantly more common in CRS populations (20% compared to 1-4% general

population).³⁷ It could therefore be postulated that the underlying causes for nasal polyp development may contribute to the pathogenesis of the CRS condition. Due to the high rate of CRS associated with nasal polyposis and the differences noted between patients with polyps and without the literature distinguishes these two disease states and CRS is classified as CRS with and without polyps.

Comparing these two CRS subsets allows investigation of the effect that nasal polyps have on the CRS condition⁴. Whilst CRSwNP patients often manifest differential expression of inflammatory markers and inflammatory cell load compared to CRSsNP patients³⁸⁻⁴⁰, it is important to assess the clinical effect of polyps in CRS. CRSwNP patients have been noted to experience a worse condition than CRSsNP⁴¹⁻⁴³ and a greater risk of disease recurrence⁴⁴ and revision surgery⁴¹. Conversely, others have demonstrated minimal clinical differences in terms of disease burden or symptom scores between CRSwNP and CRSsNP⁴⁵⁻⁴⁷ as well as medical and surgical outcomes^{48,49}. Such discrepancy between studies confound any definite conclusions about the causes of nasal polyposis in CRS. However, further research is warranted to determine the causes of nasal polyposis in CRS as this may identify more effective treatment strategies in NP laden patients.

1.1.4.2 Asthma

The 'Unified Airway Model' suggests a link between the upper and lower respiratory tracts⁵⁰. In fact, some researchers have demonstrated that challenging an area of the respiratory tract stimulates changes in distant areas of the same respiratory tract^{51,52}. Such findings may explain why CRS and asthma are often co-morbid conditions. A large volume of literature indicates a link between the two conditions. Asthma has been strongly correlated with CRS^{20,53} and present at a high rate in CRS patients (18-48%)^{20,54-56}.

However, some results diverge from these findings. A study undertaken in China found

only 2-3% prevalence in CRS populations. This may be due to the lower prevalence of asthma seen in the Chinese populations; however, this requires further elucidation⁵⁷. The nasal airway has been noted to have increased levels of mucosal inflammation in asthmatics^{58, 59}, which may suggest that asthma exacerbates an already inflamed CRS mucosa. This is supported by findings that the presence of asthma increases the severity of the disease^{54, 56, 60}, and may also increase the risk of surgical complications⁶¹. However, such conclusions are not settled and others report opposite findings^{62, 63}. This discrepancy between studies may be due to differences in asthma severity seen within the asthmatic group, as one study has noted, as the severity of asthma increases, so too does the severity of CRS⁶⁴. Work has also considered patients with allergic rhinosinusitis over a period of 7 years and it was found that patients with asthma had a quicker progression to CRS compared with non-asthmatics⁶⁵.

The literature suggests that asthma may, in some cases, lead to a more severe style of CRS. A systematic review and meta-analysis of literature assessing patients asthma condition following functional endoscopic sinus surgery showed that patients subjective asthma scores improved following sinus surgery⁶⁶. It may follow that treatment of asthma alongside CRS treatment may improve CRS patient outcomes. Further research is needed to confirm this; however, current findings suggest that clinicians must carefully assess whether CRS is present concurrently with asthma⁵⁰ to ensure both conditions are appropriately managed. Such appropriate treatment is likely to improve medical and surgical outcomes.

1.1.4.3 Allergy

As noted in section 1.1.2, allergy is often associated with CRS. Atopy or allergy is defined as an increased tendency to produce immunoglobulin type E (IgE) upon exposure to an

allergen⁶⁷. Mast cells possess high-affinity IgE receptors, which bind to these IgE's released upon allergen encounter. Following secondary interaction with an allergin the surface bound IgE triggers a signal cascade within the mast cells which subsequently releases a number mediators that act in pro-inflammatory, nociceptive and vasoactive ways⁶⁸. The prevalence of allergy (for example allergies to dust mites or moulds) has been assessed in CRS populations. Findings suggest allergies present in around 29-84% of CRS populations^{29, 56, 69-75}. Allergies may impact the CRS condition by creating a low grade chronic inflammatory response which may predispose the patient to developing CRS once the patient is exposed to CRS etiologic factors. Such hypothesis have been proposed by a number of radiological studies.^{76, 77}

Some studies indicate allergy presence increases CRS disease burden^{56, 75, 78}. A possible explanation offered for this correlation is increased inflammatory related genes and greater numbers of inflammatory cell types in some patients with allergies⁷⁹⁻⁸³. Conversely a subset of studies suggest that allergy has little bearing on a patient's CRS condition.^{42, 49, 84-87}. Some studies have even noted non-allergic patients to have an increased disease severity^{88, 89}. When reviewing this conflicting literature it becomes difficult to ascertain what role, if any allergy plays in CRS. However, discovery of the mechanisms of allergy, particularly in regards to inflammation, may assist in development of new treatments for CRS. One study has already exploited the knowledge of IgEs role in inflammation and allergy. The researchers demonstrated that upon application of omalizumab, an anti-IgE antibody, quality of life scores were significantly improved for both allergic and non-allergic CRSwNP patients⁹⁰. Therefore, resolving the conflict of whether allergy contributes to the disease as well as exploiting our knowledge of inflammatory mechanism would aid in our understanding of the condition and help lead to production of effective anti-inflammatory therapies. In any event, even if there is no causative relationship

between the two conditions, the literature clearly demonstrates that they are often co-morbid conditions. Therefore, treatments that improve symptomology associated with both or either conditions will undoubtedly be of great benefit to patients whether such treatment is implemented under a treatment regime for CRS or allergic condition.

1.1.4.4 Bacterial and fungal infection

Whether bacterial and fungal infection play a role in the pathogenesis of CRS is highly controversial. Studies assessing the function of bacterial and fungal infection in CRS patients are complicated by the presence of bacteria⁹¹⁻⁹³ and fungus^{94, 95} in healthy patients.

With regard to the bacteriology of CRS, most studies have implemented culture dependent techniques. More recently, there has been a shift to the application of culture independent techniques provides a more nuanced understanding of the bacteriology of a patient's sinus⁹⁶. Culture independent techniques, for example, 16S ribosomal RNA sequencing⁹⁷, utilise the genetics of microorganisms to identify the species present. Culture dependent techniques have identified a variety of bacteria in the sinuses of patients with CRS including *S. aureus*, *Pseudomonas aeruginosa*, *Haemophilus spp.*, *Streptococcus pneumoniae*, and *Staphylococcus epidermidis*^{91, 98-101}. Similarly, culture-independent techniques have identified large number of bacteria species residing in CRS patients' noses¹⁰²⁻¹⁰⁵. Whilst it is important to identify the composition of bacteria in CRS patients' noses, it is perhaps more important to compare these compositions to healthy individuals, as well as stratifying these results with CRS severity, to identify individual species or communities that may exacerbate the CRS condition. As the implications of *S. aureus* and biofilms will be discussed in detail in later chapters this section will focus on other bacterial species and genera. The presence of *P. aeruginosa* has been identified as a factor influencing CRS. Patients harbouring this pathogen often have a more severe CRS

condition and an increased risk of revision surgery¹⁰⁶. Excluding *S. aureus*, the majority of recent microbial studies comparing CRS to control patients have thus far failed to identify an individual bacterial species playing a significant role in CRS. Rather, such studies suggest that the complexity of the microbiome in the nose may confound any accurate identification of a specific pathogen.

In some cases, researchers have discovered similar bacterial compositions both in CRS and control cohorts' sinuses^{103, 105, 107, 108}. However, within these studies the amount of bacteria was increased in CRS sufferers¹⁰³ and the presence of pathogenic bacteria was associated with a more severe patient condition¹⁰⁸. Furthermore, CRS may experience a hypersensitive response to bacterial and fungal communities when assessed *in vitro*¹⁰⁵, and bacterial infection has been noted to correlate with enhanced immune response¹⁰⁹ and increased risk of subepithelial fibrosis¹¹⁰. Such studies suggest bacteria plays a role in the CRS condition, but exactly what this role is requires further research.

Similarly to bacterial hypotheses, the role of fungus in CRS is a contentious subject. A number of studies report that fungus plays a role in CRS^{95, 111}, some studies even suggesting it is the cause of all CRS conditions¹¹². However, other studies have produced opposite results^{113, 114}. Interestingly, a meta-analysis of the literature assessing the treatment of CRS patients using anti-fungals found that there was no benefit of anti-fungal treatment when compared to placebo¹¹⁵. Such analysis obviously confounds the finding that all cases of CRS are exacerbated by fungal presence¹¹⁵.

Whether fungus is a main contributor or cause of CRS is unclear; however, fungal infection both invasive and non-invasive has been noted in CRS⁴. Fungal balls are one type of non-invasive fungal infection, commonly cause by *Aspergillus*, which can cause blockages in the sinuses, and are removed using surgical techniques¹¹⁶. Invasive fungal

rhinosinusitis occurs in immunocompromised patients and can be life threatening. Urgent removal of infected and dead tissue and systemic anti-fungals are warranted to treat these patients⁴. Whilst it is difficult to prove all cases of CRS are exacerbated by the presence of fungus, fungal sinusitis can be classified as either allergic fungal sinusitis or non-allergic fungal sinusitis depending on the eosinophilic response of the patient.

An interesting link between bacterial infection and fungal infection is found in one study, suggesting that damage caused by bacterial infections of the nose can lead to the development of fungal infection¹¹⁷. This highlights again the complexity of the flora of the nose, and suggests that there is not only a complex interplay between bacterial species, but also bacterial and fungal species. With such conflicting research it is difficult to ascertain the role of fungus and bacteria in CRS, and further research is required to aid in our understanding. With the new approach of using culture-independent techniques it is likely that we will begin to quickly expand our knowledge of the bacterial and fungal species residing in the nose, and help lead to deciphering their contribution in CRS.

1.1.4.5 Genetics

When considering the variation in CRS prevalence both geographically and ethnically, it may be suggested that genetic susceptibilities play a role. Genetic predisposition to CRS is of increasing interest to researchers with a large volume of publications being released in the last few years.

In the earlier literature associations of a number of gene variants and CRS were discovered. Cystic fibrosis attributed to dysfunction of the Cystic Fibrosis Transmembrane Conductance Regulator due to genetic mutation is known to be associated with increased CRS prevalence^{31, 118}.

Prevalence of a specific antigen gene *HLA-B54* was found to be increased in CRS patients in Japan. Whilst the reason for this association has not been fully considered, the authors surmise that it may contribute to different host responses to immune modulating compounds increasing inflammation and immune response in the patients and subsequently stimulating CRS ¹¹⁹.

Mutation in the *interleukin 1Ra* gene, reducing its ability to inhibit inflammatory responses mediated by interleukin-1 is also noted to be increased in CRS populations¹²⁰. Further, a mutation in the *interleukin 1A* gene is observed at a higher frequency in nasal polyp patients compared with control populations¹²¹. This may indicate an important role of interleukin-1 in CRS development.

Another polymorphism thought to increase the risk of CRS involves the gene *tumour necrosis factor B* which, similar to interleukin 1Ra protein, is involved in inhibition of inflammatory processes. This polymorphism is thought to reduce the capacity of this protein to inhibit inflammation, hence potentially leading to an increase in inflammatory processes in these individuals¹²².

Recently an investigation of bitter taste receptors has revealed an important role of the bitter taste receptor T2R38. This receptor is involved in recognition of gram negative bacterial infection in the upper respiratory tract. It is postulated that genetic variation of this gene produces differential responses to these infections¹²³. Preliminary data suggests that mutations in the taste receptor T2R38 that reduce the receptors functional ability may increase the risk of CRS¹²⁴.

In addition to this earlier research, an explosion of CRS genetic studies has occurred over the last two years linking polymorphisms of genes including *tumor necrosis factor alpha*¹²⁵, *cytochrome c oxidase-2 and met proto-oncogene*¹²⁶, *osteoblast specific factor-2 and lactoferine*¹²⁷, *Cluster of Differentiation 8a*¹²⁸, *Interleukin-4*¹²⁹, *Interleukin-6*¹³⁰, *forkhead box P3 and Epstein-Barr virus induced gene 3*¹³¹ to an increased risk of developing CRS.

As many of these studies are preliminary in nature it is important that more in-depth studies are conducted on these genes to explicate their impact on the CRS condition. However, this emerging area of research may help to increase our knowledge of the condition and may eventually allow clinicians to accurately identify patients at risk of developing severe forms of CRS and potentially allow tailored CRS treatments.

1.1.4.6 Environmental factors

A number of environmental factors such as pollutants, occupational exposure to inhalant chemicals and tobacco smoke may influence a person's likelihood of contracting CRS or their experience of the condition. The sinonasal mucosa is often the first line of defence against many of these chemical irritants¹³² and when it is damaged this may increase susceptibility to infection, cause inflammation or increase other variables effecting the condition. When reviewing the literature on chemical irritants and CRS, it is apparent that these harmful chemical exposures exacerbate the CRS condition.

Exposure to both primary and second hand tobacco smoke has been linked to increased severity CRS^{27, 32, 133-137}. It has been observed that individuals who smoke have a slower mucociliary clearance when compared to non-smokers^{138, 139} and cigarette smoke has been shown to inhibit cell growth and trigger apoptosis in normal healthy nasal epithelial cells¹⁴⁰. This cell death and reduced mucocilliary clearance may cause a reduction in

clearing of bacteria, other chemical irritants or the like from the nose which may in turn damage the region. Cigarette smoking is also often linked to poorer surgical outcomes¹⁴¹,¹⁴² and a shorter time to revision surgery¹⁴³.

Pollutants in the environment such as sulphur dioxide have also been attributed to CRS exacerbation. Studies have shown a correlation between increasing levels of pollutants and respiratory problems¹⁴⁴ such as CRS^{8, 145}, although some correlations are relatively weak¹⁴⁶. Assessing the effect of pollutants on CRS in the community is difficult as it is not possible to precisely measure the intake of such components; hence, most of the data involves circumstantial evidence correlating geographical location and the associated prevalence of CRS. Overcoming this limitation, one study directly exposed a murine model to diesel exhaust particles. Nasal mucosa inflammation was increased when exposed to diesel exhaust particles¹⁴⁷ suggesting that such a pollutant can influence inflammation in the nasal cavity and potentially lead to a rhinosinusitis condition. Although current studies suggest a link, further research is required to allow accurate correlations between CRS and environmental pollutants.

Exposure to allergens and chemical irritants in industry can contribute to a condition referred to as occupational rhinosinusitis which has the potential to lead to a chronic condition¹⁴⁸. Occupational rhinosinusitis is associated with non-infectious inflammation in the upper air tract initiating symptoms of nasal secretion and blockage as well as sneezing. Literature in this area is sparse due to the expense and difficulties in assessment of exposure volumes¹⁴⁹. However, occupational rhinitis has been found in the health care professionals^{150, 151}, hairdressers¹⁵², animal care workers^{153, 154}, newspaper factory employees¹⁵⁵, pharmaceutical production workers¹⁵⁶ and cleaners^{151, 157}. Considering

specifically occupations increasing the risk of rhinosinusitis, it is not surprising that exposure to inhalant gases, fumes or chemicals increases the risk of developing CRS^{18, 148}.

Much of the data about chemical pollutants and their contribution to CRS problems is circumstantial, as it is impossible to measure the levels of pollutants and chemicals to which an individual is exposed. However, the data discussed above suggests exposure to irritants that stimulate sinonasal inflammation can contribute to CRS conditions.

Appropriate methods of exposure reduction such as the prohibition of smoking in public areas or provision of adequate personal protective equipment at work may lead to a reduction in the prevalence of CRS as a result of such chemical exposures.

1.1.5 Medical management of CRS

In the medical management of CRS, there are a number of medical mainstays that are employed to try and resolve the condition, so patients do not progress to surgical intervention. The 2012 EPOS guidelines recommend the two main medical management techniques: antibiotics and steroids⁴. Additionally, there are a number of novel treatments in development which are becoming recognised as appropriate treatments in the management of CRS. As this study focuses on development of a novel treatment for *S. aureus* biofilms in CRS, this literature review will focus on novel treatments of *S. aureus* biofilm which will be discussed in section 1.3.5. In this section we will discuss current practice medical management of steroids and antibiotic therapies.

1.1.5.1 Systemic and topical steroids

AS CRS involves chronic inflammation, steroids are often trialled to reduce this inflammation prior to surgery. Steroids may be used either orally, topically or in combination.

Oral or systemic steroids are primarily indicated for treatment of CRSwNP and there is a considerable lack of evidence for use of systemic steroids to treat CRSsNP^{158, 159}. With regards to CRSwNP, a number of placebo-controlled studies have demonstrated a significantly beneficial effect on symptom and endoscopy score as well as inflammatory load and ostial patency when treating patients with systemic steroids¹⁶⁰⁻¹⁶⁴. System steroid treatment has also been trialled in children with CRS. It was found that they are both well tolerated and effective¹⁶⁵. However, oral steroids are often only applied only in the short term and therefore in a chronic condition this can mean that improvements are only short lived^{4, 160}. This problem has been considered⁴ by a number of studies by continuing to manage inflammation following system steroid cessation using topical steroid sprays¹⁶¹⁻¹⁶³.

Topical steroids are often employed in CRS and have been noted to be quite effective¹⁶⁶. Breaking CRS down into CRSsNP and CRSwNP, a number of studies have indicated some benefit of topical steroids over placebo in the treatment of CRSsNP¹⁶⁶⁻¹⁷⁰, whilst some did not show difference from placebo^{171, 172}. More commonly, topical steroids are employed for the treatment of CRSwNP and many studies have assessed topical steroid efficacy in a randomised controlled trial setting. A rigorous meta-analysis of studies conducted by the EPOS2012 review found topical nasal steroids reduced nasal polyp size, recurrence rate and increased nasal air flow⁴. Unfortunately, current data is limited to one study that assessed topical steroids effect on quality of life (QOL) scores. This study noted no difference between the placebo group and the topical steroid treatment group¹⁷³.

Taken together, available research appears to suggest that steroid treatment can be beneficial for patients in reducing inflammation relating to their CRS condition. However

more rigorous study is required before it can be confirmed that steroids have a beneficial role both in controlling CRS and also improving patient quality of life.

1.1.5.2 Antibiotics

Bacterial infection is known to play a role in the pathogenesis of CRS. In fact, CRS is the fifth leading diagnosis for which antibiotics are prescribed¹⁷⁴, and commonly employed as a treatment in CRS^{175, 176}. Whilst short term treatment is necessary for acute exacerbations of bacterial infections, long-term antibiotic therapies are often more relevant in the CRS condition⁴.

Thus far only one study has found significant benefit of short term doxycycline treatment following surgery indicating reduced polyp size and fewer inflammatory markers¹⁶⁰.

Review of the literature by Soler et. al. 2013 found that in terms of antibiotic type, oral antibiotics and prolonged macrolide antibiotics are potential therapeutic option in CRS treatments whilst other options such as intravenous antibiotics, topical antibiotic and antifungals are contraindicated¹⁷⁷. However, whilst long term antibiotics are a common therapy in the treatment of CRS, and are a suggested therapeutic option there is limited clinical evidence supporting their use.

Looking first at long term macrolide therapy, two studies assessed the use of long-term macrolides in CRS populations with mixed results, one showed significant improvement in symptoms and endoscopy score¹⁷⁸, whilst the other showed no improvement¹⁷⁹. Due to the limited number of placebo controlled trials and small patient cohorts, further analysis of the effect of long term macrolide treatment is required to make any form of conclusion.

When assessing systemic antibiotic use only one study has thus far compared treatment with a control using a macrolide antibiotic and found significant benefits to SNOT-22

score and nasal endoscopy¹⁷⁸. Investigation of topical antibiotic use reveals that the therapy was effective in the short term at improving patient condition, however, long term results suggested this improvement was not maintained.¹⁸⁰

There is certainly cause for use of antibiotics in CRS, particularly during acute sinonasal bacterial infections where antibiotics are often seen to be effective¹⁷⁷. However, to determine whether long-term antibiotic therapy should be indicated for all CRS patients without strong evidence of an infection present would require an increased number of randomised controlled clinical trials. Long term efficacy of such treatments are of particular concern as new findings suggest that long term clarithromycin may increase the risk of cardiovascular events in patients¹⁸¹.

1.1.6 Surgical management of CRS

For patients that fail maximal medical therapy clinicians may resort to functional endoscopic sinus surgery (FESS). FESS or ESS (endoscopic sinus surgery) involves the removal of polyps and polypoid mucosa and inflammatory or diseased tissue in an effort to open the sinus drainage pathways and improve nasal aeration and decrease disease load by removal of diseased tissue⁴. Patients failing medical therapy who subsequently progress to surgery are often seen to have significant improvement in symptom scores^{49, 182-189}. In fact, a systematic review of the literature and meta-analysis showed a significant improvement in QOL scores of all symptoms, with nasal obstruction showing the most improvement¹⁹⁰. Further to this, a systematic review of the paediatric FESS literature assessing 15 studies found 13 of these studies indicated a positive outcome following paediatric application of FESS in 71-100% of patients and 5 studies showing significant improvement in QOL scores¹⁹¹. Another benefit of FESS is that it achieves an increase in sinus accessibility for medical therapies^{192, 193}.

There are however a population of patients who fail both medical and FESS intervention,. For these patients an endoscopic modified Lothrop procedure or Draf 3 is available. This advanced surgical procedure involves creating a large opening into both frontal sinus regions.¹⁹⁴ The procedure has been noted to be effective in the majority of patients who fail FESS¹⁹⁵⁻¹⁹⁷.

Whilst sinus surgery is quite effective, it is not free of complications including haemorrhage, orbital complications and CSF leak¹⁹⁸. Therefore, whilst surgery is an option in the treatment of CRS, attempts to manage a patient medically prior to the implementation is necessary due to the risks involved in undergoing a surgical procedure. Whilst maximal medical and surgical intervention is successful at improving and maintaining improved CRS patient condition, there remains a population of patients that are recalcitrant to these conditions. Rates of medical and surgical recalcitrance range between 10-26% of CRS patients¹⁹⁹. Due to the multi-factorial nature of the condition it is difficult to fully ascertain exactly why this is the case; however, evidence of the contribution of *S. aureus* infection and bacterial biofilm formation may play a role, which will be discussed in detail in the following sections.

1.2 *Staphylococcus aureus*

A member of the Micrococcaceae family, *S. aureus* is a gram positive, aerobic bacteria, with a cocci appearance²⁰⁰ (Figure 1.3). *S. aureus* has a circular chromosome composed of approximately 2.8 Kb²⁰¹ with additional extrachromosomal elements including plasmids harbouring antibiotic resistance genes²⁰². This chromosome is encompassed by a cell wall comprised mainly of peptidoglycan to which teichoic acid is anchored. Proteins including receptors are also constituents of the cell wall. These surface proteins can be anchored to the surface or traverse the membrane and protrude into the cytoplasm²⁰⁰. The majority of *S. aureus* strains also possess a polysaccharide capsule layer external to the cell wall which helps prevent phagocytosis of the bacteria during infection²⁰³. In this section will include discussion on the opportunistic nature of *S. aureus*, its virulence factors, and how these factors may contribute to CRS.

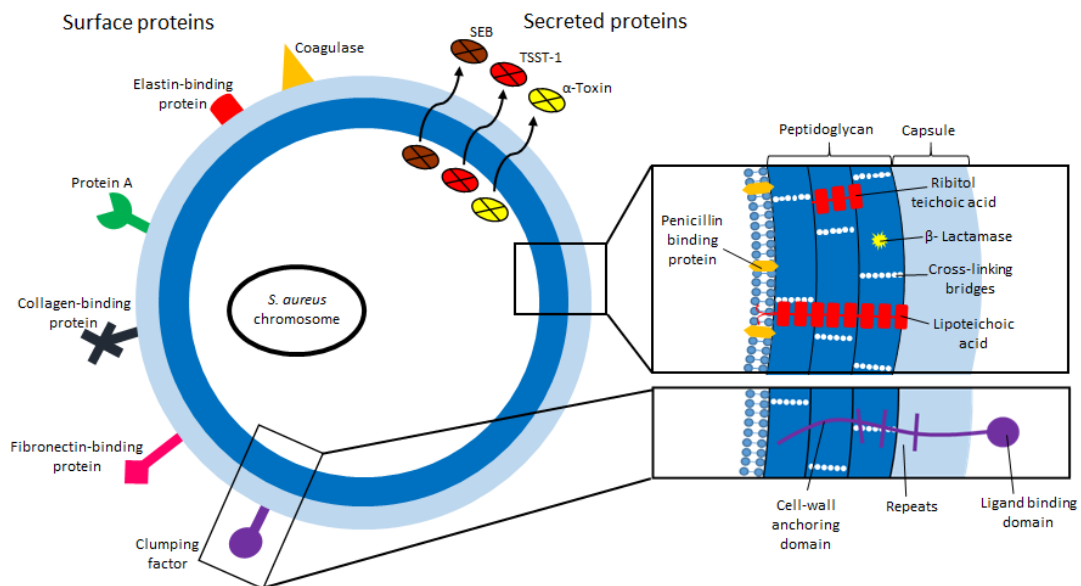


Figure 1.3: Structure of *Staphylococcus aureus* cells. Figure adapted from article:

Lowy, 1998²⁰⁰. *S. aureus* is a gram positive, cocci shaped cell. A number of well-characterised proteins (eg. Protein A), both attached to and secreted by *S. aureus* contribute to the survival and virulence of the bacteria. Close up view of the cell wall shows the primarily peptidoglycan cell wall intersected by teichoic acids and cell-wall anchored proteins. The cell wall also houses antibiotic resistance contributors including β -Lactamase. Abbreviations: SEB = Staphylococcal enterotoxin B, TSST-1 Toxic Shock Syndrome toxin 1.

1.2.1 Friend or Foe?

Staphylococcus aureus is a highly complex and successful pathogen. Recognised as a cause of abscesses in 1880 by Alexander Ogston²⁰⁴, it was not until the late 1930's when people started to recognise that *S. aureus* could also be recovered from the skin and anterior nares of healthy individuals²⁰⁵. Such a finding indicated that *S. aureus* is found not only in times of infection, but is also carried by healthy individuals. It is now established that there are three types of healthy *S. aureus* carriers. The first is persistent *S. aureus* carriers who harbour the bacteria in a primarily innocuous manner. Approximately

20-25%²⁰⁶⁻²⁰⁸ of the population fall within this group. The second group are termed intermittent carriers and constitute approximately 60% of the population. As the name suggests, this cohort intermittently harbour the pathogen^{205, 208}. The remaining 15-20% of the population are ‘non-carriers’ and almost never carry the pathogen^{205, 208}.

Although *S. aureus* is often an innocuous commensal, it may also behave in a highly pathogenic manner. It is noted to cause a multitude of infections of the skin, bones, kidney, lungs, bacteremia and endocarditis²⁰⁰. Such infections can be fatal and *S. aureus* was documented to contribute in 30 000 deaths in the US in 2005²⁰⁹.

As *S. aureus* may be both pathogenic and commensal it may be termed a ‘pathobiont’: a commensal bacteria that can switch to pathogenic characteristics under appropriate conditions²¹⁰. The conditions required to facilitate this change are as yet unknown. Given the rate of *S. aureus* mediated mortality it is paramount that this bacteria be understood and therapies be developed to reduce *S. aureus* infection. Furthermore, *S. aureus* has many implications for morbidity and it has been demonstrated to aggravate or contribute to various other conditions. Of importance to this thesis, *S. aureus* is a known contributor to the pathogenicity of CRS^{33, 211, 212}. Therefore, understanding the role of *S. aureus* virulence in CRS is of great importance.

1.2.2 Epidemiology and Prevalence of *S. aureus* in CRS

S. aureus has been found in 15-61%^{91, 93, 98, 103, 104, 107, 213, 214} of patients with CRS. CRS patients who have undergone more than one FESS are often found to have an increased prevalence of *S. aureus* infection in the paranasal region⁹⁸. Research has indicated that *S. aureus* plays an important role in the pathophysiology of CRS^{33, 211, 215, 216}, particularly

when in biofilm form²¹¹. Patients harbouring intracellular and biofilm forms of *S. aureus* are at greater risk of microbiological relapse post-surgery, as well as recalcitrance^{211, 212}.

The *S. aureus* strain or clonal type that causes the majority of *S. aureus* CRS infections is unknown. Furthermore, only limited study has investigated whether patients experiencing multiple or ongoing *S. aureus* infections are experiencing a persistence of the same *S. aureus* clonal type. Thus far only one study has considered the clonal relation of *S. aureus*. These researchers obtained swabs from the same patient 1 year apart. Of the 8 patients assessed, each bacterial pair were the same clonal type²¹⁷. The implications of this study are restricted as all patients were positive for intracellular *S. aureus*. It would therefore be of interest to conduct similar work on larger patient cohorts with inclusion of intracellular negative patients. This would help to expand our knowledge of clonal relationships between recurrent nasal infections.

Although it is relatively unknown which strains are predominantly causing *S. aureus* infection in CRS patients, a number of studies have assessed the contribution of a variety of *S. aureus* virulence strategies. In the coming section virulence factors contributing or potentially impacting on disease exacerbation will be discussed.

1.2.3 *S. aureus* Virulence Factors and their role in CRS

A number of factors contribute to *S. aureus* virulence. These include toxin production, superantigen production, immune evasion, antibiotic resistance, and biofilm formation. This section will review literature on the influence of these factors in CRS. We will also discuss recently discovered virulence mechanisms of *S. aureus* that is yet to be investigated in CRS that may contribute to the etiopathogenesis of the condition.

1.2.3.1 Superantigens

Superantigens (SAGs) are produced primarily by gram positive bacteria including *S. aureus*²¹⁸. Classically an antigen will stimulate a small percentage of the T-cell population (0.01%). A SAG however can stimulate up to 25% of the T-cell population by binding to the outside of the MHCII receptors. In addition to T-cells, the pro-inflammatory action of SAGs stimulates a multitude of immune cells including eosinophils, mast cells and macrophages²¹⁹ triggering massive cytokine and pro-inflammatory gene production. Such excessive cytokine production may cause a patient significant harm²²⁰.

An example of a *S. aureus* SAG is enterotoxin A. Enterotoxin A was isolated and characterised in 1966²²¹ and found to induce severe vomiting similar to cases of food poisoning. SAGs produced by *S. aureus* have also been identified as bearing potential implications in Sudden Infant Death Syndrome, Staphylococcal Extreme Pyrexia Syndrome, Pneumonia and, Toxic Shock Syndrome²¹⁸.

Research into the role of SAGs in CRS has noted that CRSwNP patients possess SAG proteins in the mucosa and polyp regions, whilst mucosa of healthy patients were devoid of such toxins^{222, 223}. Some findings have suggested that CRSwNP are more often associated with the presence of SAGs when compared to CRSsNP patients^{223, 224} although these toxins can also be found in a small number of CRSsNP patients^{224, 225}. Higher levels of SAG specific IgE are noted in CRSwNP compared to controls^{226, 227}.

Despite findings that SAG levels are increased in CRS patients, the direct effect of SAGs on the mucosa or local immune system of the paranasal sinus region has not been investigated in depth. As SAGs stimulate high levels of immune response, SAGs may act primarily by stimulating a self-damaging hyper-immune response. Toxins, particularly SEB, upregulate

the expression pro-inflammatory markers interferon gamma and interleukin-5²²⁸⁻²³⁰, and SEB has been noted to increase endoplasmic reticulum stress responses known to be related to increased inflammation in CRS²³¹. Furthermore, some correlation between SAg presence and higher levels of eosinophils has been noted²³². Another factor may be the ability of SAg to manipulate the expression of glucocorticoid receptors. Studies have linked SAg presence with an increased level of glucocorticoid insensitivity related to glucocorticoid receptor expression in CRSwNP^{25, 26}. Therefore, SAg may also reduce the ability of corticosteroids to reduce nasal inflammation and potentially lead to a failure of maximal medical intervention.

The majority of studies support the idea that SAg contribute to the inflammatory processes in CRS, particularly when they are associated with NPs. As CRS is defined as chronic nose and paranasal sinus inflammation¹, compounding inflammatory processes would likely increase condition severity. Reduction in SAg burden in CRS patients could potentially reduce inflammatory load and steroid insensitivity. With this in mind developing effective treatment and management strategies against *S. aureus* nasal infection is paramount.

1.2.3.2 Enzymes and non-superantigen toxins

In addition to SAg, *S. aureus* possesses a large arsenal of other toxins and enzymes that may be detrimental to humans and enhance *S. aureus* virulence²³³. One such toxin is α -toxin, a potent secreted toxin with a highly lethal nature. This toxin has the ability to act on numerous cell types, including epithelial cells leading to pro-inflammatory processes as well as cell death²³⁴. Additionally, β -toxin has been noted to stimulate neutrophil recruitment through chemotactic processes in lungs, enhancing inflammation²³⁵, and delta-

toxin also stimulates pro-inflammatory processes²³⁶. As CRS is an inflammatory condition, such toxins may exacerbate the inflammatory processes in the nose.

Apart from toxins, damaging enzymes are also produced by *S. aureus* which may increase its virulence²³³. Such enzymes encompass an arsenal of proteases, lipases and hyaluronidases²⁰⁰. Two studies have investigated the effect of proteases in CRS with both finding that *S. aureus* serine proteases increase inflammation^{237, 238}, compounding the already inflamed mucosal environment.

Another protein with possible protease activity is *S. aureus* produced 'protein A'. Protein A has been found to contribute to degradation of epithelial cell tight junctions, leading to a leaky epithelial layer and allowing invasion of *S. aureus* into sub-mucosal tissue as well as impairing cilia functioning²³⁹. This may also lead to increased CRS severity by damaging important barrier and clearance functions in the nose and paranasal sinus regions and therefore should be investigated in the context of CRS.

This section describes a limited number of known virulence factors produced by *S. aureus*. Furthermore, many of these proteins are still not well understood and many more may be yet to be discovered. Given the inflammatory nature of CRS, it is important to investigate the effects of these virulence proteins in CRS due to their abilities to enhance inflammatory processes, damage epithelial cells and impair epithelial barrier functions. Research has only just begun to investigate the complex role that *S. aureus* virulence proteins play in disease exacerbation. More research is needed to paint a clear picture of the contribution of *S. aureus* in CRS.

1.2.3.3 Antibiotic Resistance

Antibiotic resistance is a mechanism by which bacteria avoid antibiotic therapy thus allowing infection to persist in a patient²⁴⁰. *S. aureus* antibiotic resistance emerged after the application of penicillin to treat infections. Just six years after widespread implementation of penicillin therapy approximately 60% of *S. aureus* isolates were found to be resistant to penicillin²⁴¹ with even higher rates reported in hospital settings²⁴². Although many new classes of antibiotics have been developed bacterial resistance has developed against each new class²⁴³.

There are a number of mechanisms that bacteria may use to resist antibiotic therapy (Figure 1.4) including efflux pumps, antibiotic-modifying enzymes, and alteration of target sites²⁴⁴. As previously mentioned, antibiotic treatment is commonly prescribed to treat CRS¹⁷⁴, and it is likely that where antibiotics are constantly applied, antibiotic resistance may occur. There is evidence that erythromycin resistance in *S. aureus* is beginning to increase in CRS patients²⁴⁵. Interestingly methicillin resistance *S. aureus* (MRSA) does not seem to be commonly isolated in CRS patients^{245, 246}. However, research suggests that antibiotic usage rates in CRS may be contributing to an increase in MRSA prevalence²⁴⁷.

Comparing the bacteria present in CRS patients undergoing either primary or revision surgery^{51, 52, 100}, antibiotic resistance rates are increased in revision patients compared to primary patients. The above research taken together suggests that patients with long standing CRS or patients who are treated extensively with antibiotics have a higher rate of antibiotic resistance. However, antibiotic resistance (excluding biofilms) does not appear to be of a significant complication in the treatment of CRS. This is perhaps due to the current practice of determining the antibiotic susceptibility profile of the causative bacteria present in infections²⁴⁸. This allows the application of culture directed antibiotics, leading

to successful antibiotic therapies and reducing the risk of bacteria persisting and developing further antibiotic resistance mechanisms²⁴⁸. Despite this, one study found that even when culture directed therapy was employed in recalcitrant CRS associated with *S. aureus* infection, antibiotic therapy failed to resolve the infection¹⁸⁰. This may be due to bacteria residing in intracellular or biofilm forms which will be discussed in section 1.2.3.4 and 1.3 respectively. Whilst antibiotic resistance is not yet a significant burden in CRS, it is of great importance to employ antibiotics in an appropriate manner. Misuse or overuse of these therapies is the main cause of antibiotic resistance generation²⁴⁹.

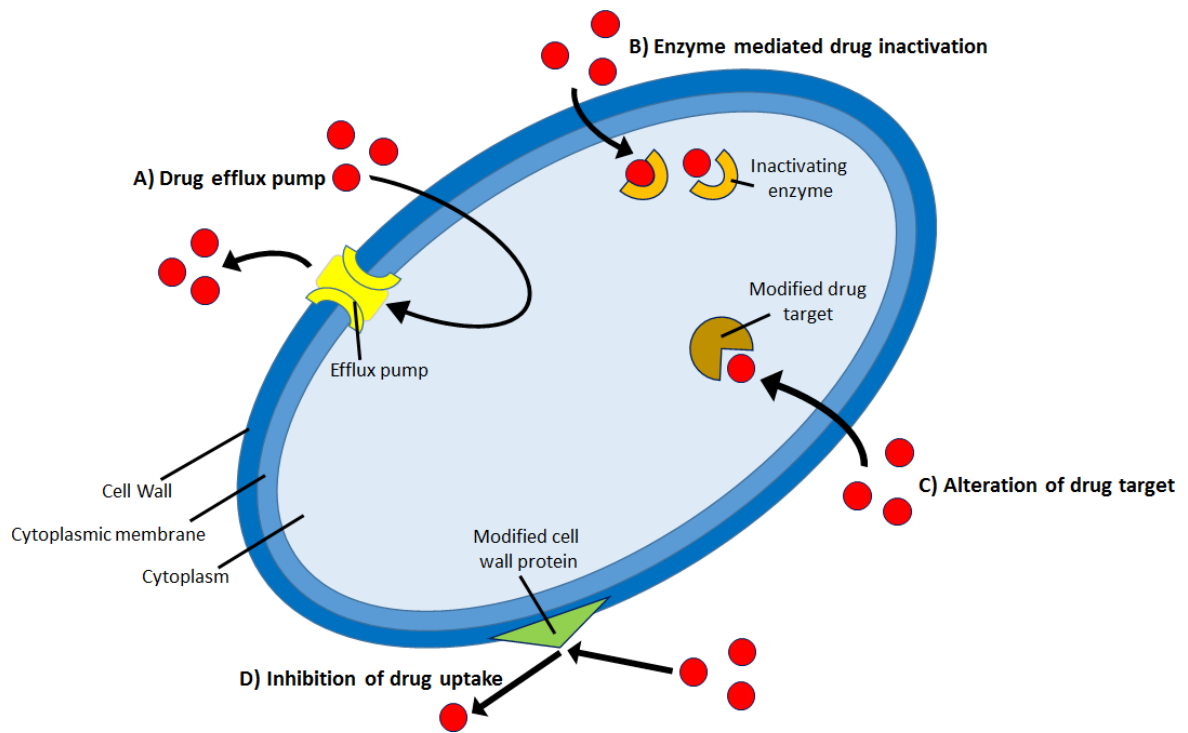


Figure 1.4: Mechanism of bacterial resistance to antibiotics. Figure adapted from Encyclopedia Britannica, 2009²⁵⁰. Four different mechanisms of bacterial resistance to antibiotics have been thus far discovered and described. A) Drug efflux pumps act to actively expel antibiotics from the cytoplasm of the cell once they have been internalised. B) Enzymes are produced by the bacteria that bind to the antibiotic and modify them in such a way that they become inactivated. C) Mutation can arise in the protein of which the antibiotic targets, causing the antibiotic to no longer be effective against the target bacteria. D) Cell wall proteins can be modified in two ways. Firstly their expression can be down-regulated and are therefore not available for the antibiotic to recognise. Secondly, mutations can arise in the targeted cell-wall proteins rendering the antibiotic ineffective as it can no longer recognise the protein.

1.2.3.4 Immune Evasion

Another important characteristic of *S. aureus* infection persistence is immune evasion. This can occur at a gene/protein level where the bacteria produce proteins to inhibit immune responses, or a mechanical level where the bacteria hide in intracellular or biofilm forms to escape the immune system.

In regards to proteins that aid in immune system evasion, there are a myriad of proteins capable of disturbing immune responses to *S. aureus* infection. These proteins act to disrupt neutrophil and leukocyte migration²⁵¹⁻²⁵³, lyse neutrophils²⁵⁴⁻²⁵⁶, inactivate complement^{253, 257}, degrade immunoglobins^{257, 258}, and defend against antimicrobial peptides^{259, 260}. Immune evasion proteins and their role of *S. aureus* in paranasal sinuses of CRS patients has yet to be investigated.

Investigations in the CRS field have focussed on physical or mechanical styles of evasion including intracellular *S. aureus*. Intracellular *S. aureus* is an emerging area of investigation in CRS and thus far have been identified in 39%-62%^{212, 217, 261, 262} of patients. Intracellular residency appears to provide an infection reservoir, leading to recurrent *S. aureus* infection in CRS patients^{217, 263}. This suggests that *S. aureus* is avoiding extracellular components of the immune system in an intracellular form, and may escape from cells when conditions are favourable to recolonise the sinus region. Discovery of this evasion strategy in nasal tissues suggests that focus must shift to development of treatments not only targeting biofilms or extracellular *S. aureus* infection but treatments targeting these intracellular forms. Such targeted treatments may help to effectively manage these infection in CRS and prevent harmful re-infections particularly in the post-operative period.

1.2.4 Summary of the contribution of *S. aureus* in CRS

The complexity of the pathobiont *S. aureus* makes it difficult to determine what exact role it is playing in CRS. With a number of virulence factors over-stimulating the immune system, damaging cells, and evading the immune response it is difficult to both understand and treat this bacteria effectively. Continued research into mechanisms of virulence and development of novel therapies to treat *S. aureus* are paramount to manage this bacteria in the nose and reduce its contribution to CRS etiopathogenesis. A final important characteristic of *S. aureus* is their propensity to form biofilms on the mucosa of the nose and paranasal sinus regions. These biofilms have been shown to exacerbate the CRS condition and are perhaps the most well studied virulence mechanisms of *S. aureus* in the context of CRS. Biofilms and their contribution in CRS will be discussed in the next section.

1.3 Biofilms

Biofilms are thought to be the state in which 99% of all bacteria exist²⁶⁴. Biofilms are bacterial cells attached to a surface, composed of either one or multiple bacterial species, encased in a matrix^{265, 266}. In the biofilm state, bacteria have increased resistance to antibiotic therapies and the host immune system²⁶⁶. Biofilms also act as a survival mechanism protecting bacteria from harmful environments such as nutrient depleted surroundings²⁶⁶. Biofilms have been found to be the source of persistent infections in numerous disease states²⁶⁷⁻²⁷¹. As such, development of efficacious anti-biofilm treatments is becoming a rapidly expanding area of study. This section shall discuss biofilm formation and structure, mechanisms of therapeutic resistance, and current research into anti-biofilm treatments with a focus of *S. aureus* biofilms.

1.3.1 Universal biofilm structure

As mentioned above, biofilms are composed of bacterial cells encased within a matrix. This matrix makes up approximately 90% of biofilm structure and is primarily comprised of polysaccharides²⁷². In addition, other extracellular polymeric substances (EPS) including protein and extracellular DNA may be present within the matrix²⁷³.

It is difficult to discuss biofilms in a general context, as previous work has noted that every microbial biofilm consortium is unique in architecture²⁷⁴; however, some global concepts can be described. Biofilm structures are heterogeneous in nature, and are often observed to be comprised of microcolonies of matrix bound cells intersected or separated by water channels (Figure 1.5). Formation of the microcolonies within the biofilm often begins with the formation of cone shaped towers, which as the biofilm develops, eventually form mushroom like protrusions²⁷⁵. The water channels or spaces within the biofilm are thought to act akin to a rudimentary circulatory system, delivering nutrients and expelling toxic metabolic by-products²⁷⁵. These microcolony towers and water channels are observed in

all biofilms but the building blocks that help form these structures is where the uniqueness begins.

Biofilm 'starting material' is often dependent on environmental conditions and may even incorporate host factors²⁷⁶. Furthermore, each bacterial species develops biofilms in a unique way, and despite the abovementioned general biofilm concepts, no two biofilms are the same. Therefore, this introductory section shall focus primarily on *S. aureus* biofilms: the primary focus of this research project.

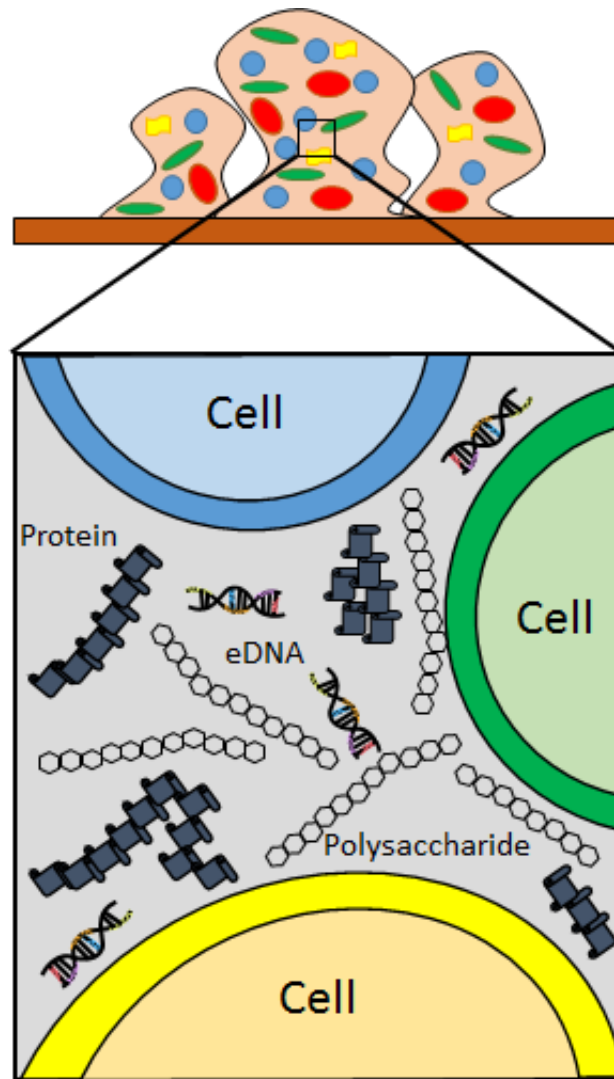


Figure 1.5: Generalised Biofilm Structure. Figure adapted from Flemming & Wingender, 2010²⁷⁷. Mature biofilms are found to appear mushroom-like in structure and in the environment will often be composed of mixed cell populations. Upon closer inspection of the matrix, cells within the biofilm are held tightly together by a mixture of protein, polysaccharides and extracellular DNA (eDNA).

1.3.2 Formation of Bacterial Biofilms

Review of the biofilm based literature reveals the complexity of biofilm formation. No one protein has been identified as the primary initiator of biofilm formation²⁷⁸. Differences in biofilm formation are noted not only between bacterial species but also within bacterial species. An example of this is ‘biofilm associated protein’²⁷⁹. This protein has been noted

to be important for biofilm formation in *S. aureus* cultured from ruminant mastitis infections but has never been identified in the genome of isolates cultured from human based infections²⁷⁹. Despite each colony's differences, biofilms form generally by first attaching to a surface, produce matrix elements, continue growing and producing matrix until a mature mushroom like biofilm structure forms. Cells are then dispersed as seeds to colonise a new area with biofilm or re-infect an area when conditions are favourable (Figure 1.6).

The first stage of biofilm formation is cell-surface attachment. This occurs through Brownian motion and environmental hydrodynamic and gravitational forces²⁷². This process may be influenced by the roughness and physiochemistry of the substratum and by the composition and hydrodynamics of the surrounding aqueous environment²⁷².

Once in contact with a surface a number of proteins and polysaccharides may be implicated in early attachment events. The main polysaccharide involved in *S. aureus* biofilm formation is polysaccharide intercellular antigen ("PIA")²⁸⁰ which is comprised of beta-1,6-linked glucosaminoglycan residues²⁸¹. The intercellular adhesion locus controls the production of PIA²⁸² which is regulated by a myriad of genes²⁸³⁻²⁸⁷. This complex regulatory process is likely due to biofilm formation being stimulated by multiple environmental conditions²⁸⁸. Biofilm formation independent of PIA has also been recognised^{289, 290}. Mutant non-PIA producing *S. aureus* strains have allowed identification of PIA replacements including Protein A²⁹¹, fibronectin binding proteins²⁹², and biofilm associated protein^{279, 293}.

Further, extracellular DNA (eDNA) is heavily involved in biofilm formation²⁸⁰. This eDNA appears to be an important structural component of biofilm, and is added to the

biofilm often through bacterial cell lysis events^{294, 295}. Other proteins are also stimulated to assist in biofilm formation upon eDNA release. An example is beta-toxin which, in the presence of eDNA, is shown to oligomerise, likely becoming a component of the matrix²⁹⁶.

Such findings highlight the difficulty in developing biofilm treatments targeting matrix proteins. Biofilm components are unique to each individual biofilm both between bacterial species and even within a species. This makes it extremely difficult to develop a treatment that will prove effective against all biofilms. Despite this great variation in composition, a biofilm state consistently allows for increased protection from environmental stresses, and confer bacterial persistence in harsh conditions.

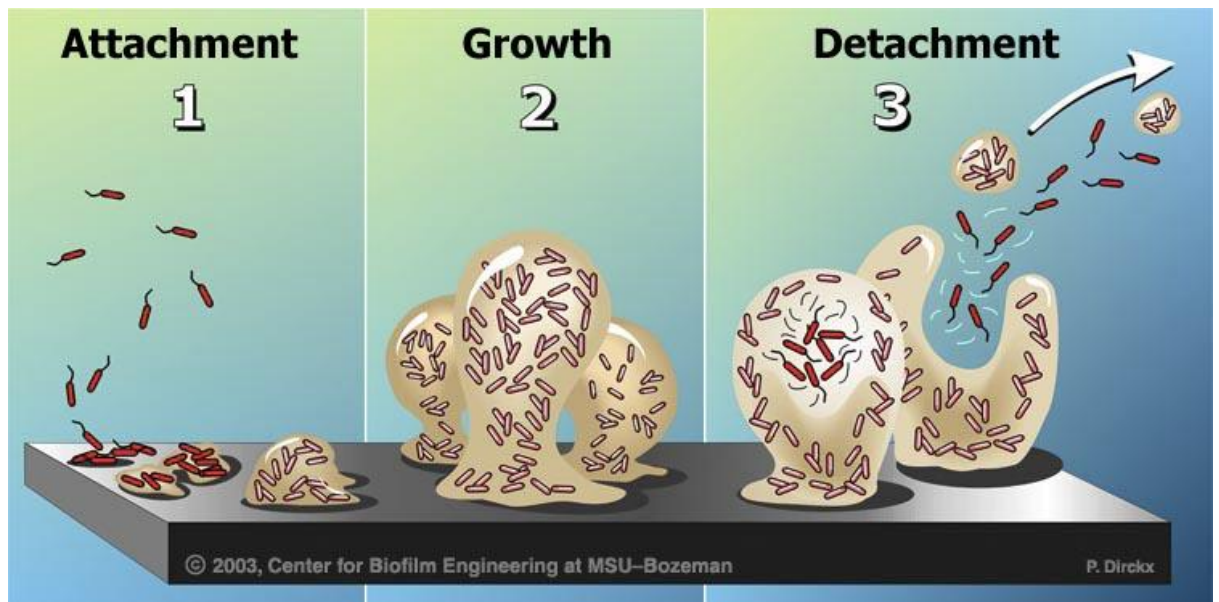


Figure 1.6: General, simplified stages of biofilm development. Image credited to Paul Stooley (2003), permission granted for use of image. Simplistically speaking there are 3 main stages of biofilm development. 1) Attachment of planktonic cells to a surface where they begin to produce matrix components when through quorum sensing they determine that there are enough bacteria present to form a biofilm. 2) Growth of the cells within the biofilm in parallel with continued matrix production leading to a mature biofilm form complete with water channels as a rudimentary circulatory system. 3) When conditions are favourable bacteria or chunks of biofilm can detach to seed new surface areas with biofilm, or to re-infect an environment.

1.3.3 Mechanism of resistance

Biofilms are a nidus for infection. They cause approximately 65% of chronic infections²⁹⁷.

Ability to persist, particularly in humans, is enhanced by biofilms' apparent resistance to both antimicrobial therapies and the host immune response²⁹⁸⁻³⁰⁰. There are a number of biofilm characteristics thought to contribute to this resistance which shall be discussed briefly in this section.

1.3.3.1 Biofilm resistance to antibiotics

One important biofilm survival mechanism is enhanced resistance to antibiotics³⁰¹⁻³⁰³.

There are a number of ways in which biofilms have been reported to resist antibiotics (Figure 1.7). Antibiotics reduced efficacy has been observed to be caused by their inability to efficiently penetrate biofilms. This was mainly observed with beta-lactams and aminoglycosides and may be a result of the antibiotics inability to access some bacterial cells sheltered within the biofilm matrix^{299, 304-307}.

Reduced penetration through the biofilm may also lead to a reduction in diffusion speed. A reduction in diffusion speed may increase the efficiency of enzymes such as beta-lactamase in inactivating the antibiotics before they are able to diffuse through the entirety of the biofilm thus protecting the internal cells^{307, 308}. Antibiotic directed efflux pumps are also important in biofilm resistance^{309, 310} including biofilms of *S. aureus*³¹¹. As some antibiotics are able to diffuse rapidly through the biofilm matrix, efflux pumps allow biofilm bound cells to expel antibiotics from their cytoplasm and thereby resist antibiotic mediated death³¹². Quorum sensing molecules produced by bacteria have also been noted to increase antibiotic resistance possibly by stimulating gene expression changes in an effort to overcome antibiotic attack^{313, 314}.

In the environment bacteria often exist in biofilms composed of more than one species of bacteria. It has been noted in the literature that these more complex biofilms have a greater resistance to antibiotics³¹⁵. This suggests that when we assess treatments against biofilms we need not only to assess them *in vitro* in single species culture, but also *in vivo* where they will form more complex biofilms intertwined with other bacterial species.

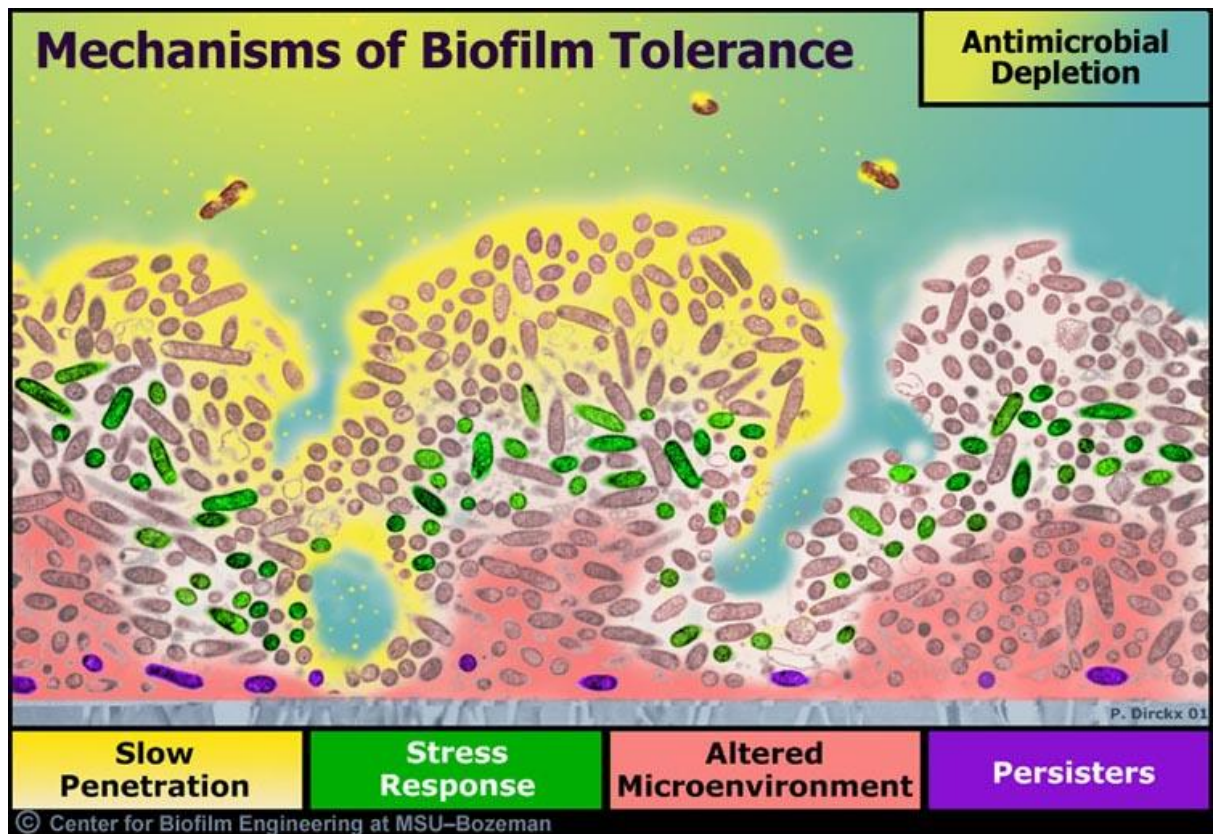


Figure 1.7: Mechanisms of antimicrobial resistance in Biofilms. Image credited to Phil Stewart & Peg Dirckx (2001), permission granted for use of image. Biofilms have been shown to resist antimicrobial therapies in a number of ways. 1) Slow penetration: The biofilm matrix often reduces or slows penetration of things such as antibiotics through biofilms, reducing the potential of the antimicrobial to contact or kill all bacteria present in the biofilm. 2) Stress response: Some bacteria within the biofilm can be primed to respond to the therapeutic stress imposed. This can work in combination with slow penetration to eliminate the environmental threat before it diffuses through the entirety of the biofilm. 3) Altered Microenvironment: some cells towards the lower sections of the biofilm will have for example a reduced metabolism which in some cases causes them to become resistant to antimicrobial stressors. 4) Persisters: These are cells often at the very bottom of the biofilm that are the most protected from antimicrobial attack and will often persist to re-colonise an environment under favourable conditions.

1.3.3.2 *Biofilm resistance to host immune response*

Biofilm resistance against the immune system is thought to involve similar mechanisms to those observed in antibiotic protection mechanisms. This includes limited penetration³¹⁶, gene expression alterations^{317, 318} including quorum sensing^{313, 319}, and inactivation of immune system components^{320, 321}.

S. aureus biofilms have been shown to interact differentially with the immune system compared to their planktonic counterparts. *S. aureus* biofilms have been observed with some reduced pro-inflammatory processes³²² and macrophage growth and phagocytosis^{322, 323}. Genes involved in metabolism and cell growth have been noted to be down regulated when a *S. aureus* biofilm encounters macrophages *in vitro*. This suggests that *S. aureus* is tempering the immune response. However, research has found the immune system is still activated by the presence of biofilms.

Leukocyte interaction with biofilms indicates ample biofilm penetration³²⁴, but inhibited bacterial phagocytosis³²¹. Interestingly, opsonisation of biofilms with antibodies triggered an increased oxygen radical production³²⁵. Furthermore, *in vivo S. aureus* biofilms are noted to trigger a Th1 and Th17 style reaction, producing cytokine and other pro-inflammatory factors³²⁶. In summary, it appears that whilst biofilms resist immune responses, they may still trigger production of cytokines and pro-inflammatory elements. These attacks from the immune system do not seem to be capable of complete biofilm eradication and may even lead to damaging responses and harm surrounding tissue allowing infection to progress and persist^{325, 326}.

Further investigation of biofilm resistance to antibiotics and the immune system will allow us to understand biofilms better and enhance our development of effective treatments. Alarming bacteria, particularly *S. aureus*, in biofilm form are seen to have a higher rate of mutagenesis compared to planktonic counterparts³²⁷. Furthermore, biofilm bound bacteria have an enhanced rate of horizontal exchange of plasmids³²⁸. Both these characteristics may increase the frequency of bacteria developing new mechanisms of resistance. Therefore, it is important that we enhance our knowledge of biofilm resistance mechanisms, and develop a diverse range of treatment strategies. This would afford us the ability to modulate our treatment to overcome these diverse biofilm resistance methods.

1.3.4 Biofilms in CRS

Biofilms were first identified in the sinonasal mucosa of CRS patients by Cryer et. al. 2004²⁶⁷. Since this initial identification numerous studies have investigated the presence of biofilms in CRS and, more recently, the implications of these biofilms. Biofilms have been identified on the mucosa of the nose and paranasal sinus regions in 41-100% of CRS patients^{213, 329-338}. A number of bacterial species have been implicated in these biofilm formations including *Moraxella catarrhalis*, *S. aureus*, *S. pneumoniae*, *P. aeruginosa* and *Haemophilus influenzae*. Patients with bacterial biofilms in their nose have a greater degree of osteitis³³⁹, increased epithelial damage and mucosal metaplasia³⁴⁰, an increased number of active dendritic cells³³⁵, and are often found to generally have a poorer prognosis^{336, 341}.

Focusing on *S. aureus*, between 9 and 50% of CRS patients have been found to harbour *S. aureus* biofilms^{100, 103, 212, 342-348}. This suggests a possible influence in the pathogenesis. Research has identified that sinonasal *S. aureus* biofilms negatively affect post-operative outcomes and patient quality of life^{211, 213, 349}. Further, an enhanced capacity of *S. aureus* to form biofilm *ex vivo* is indicative of poorer post-operative patient outcomes²¹⁶. As biofilms have been identified as a nidus of persistent infection it is important that we

develop effective treatment strategies to combat them. Their inherent resistance to antibiotic treatment and host immune systems makes the development of novel treatment of *S. aureus* biofilm an imperative area of research in the context of CRS.

1.3.5 Novel anti-biofilm therapies targeting *Staphylococcus aureus*

Development and discovery of novel anti-biofilm therapies is an ever-expanding area of study. Numerous *in vitro* work has described the efficacy of agents against *S. aureus* biofilms including antibiotic cationic peptides³⁵⁰, ethylenediaminetetraacetic acid (EDTA)³⁵¹⁻³⁵³, nebulised antibiotics³⁵⁴, dispersin B and triclosan³⁵⁵, quorum sensing inhibitor RIP³⁵⁶, varidase³⁵⁷, manuka honey³⁵⁸ and DNase1L2³⁵⁹ and maggot secretion³⁶⁰. Further studies have identified a novel bacterial deoxyribonuclease as well as nitric oxide³⁶¹ are both effective treatments, particularly against *S. aureus* biofilms of strains isolated from CRS patient's noses³⁶². Photodynamic therapy has also been identified as an effective model mimicking the architecture of the human nasal passage³⁶³.

Whilst it is important that anti-biofilm agents be assessed for efficacy *in vitro*, biofilms grown in a laboratory may diverge from what is noted *in vivo*^{364, 365}. Therefore, it is important to extrapolate *in vitro* research into an *in vivo* context. A number of compounds have been noted to maintain anti-biofilm effects when translated from the laboratory to an animal model. These includes the *P. aeruginosa* derived antibiotic mupirocin^{366 367}. A recent clinical study also found that mupirocin improved CRS patients symptomatology and endoscopy score in the short term¹⁸⁰. Bakkiyaraj & Pandian 2010 also showed efficacy of coral associated actinomycete against *S. aureus* biofilms both in the laboratory and in an animal model³⁶⁸. Work has indicated that a surfactant citric acid/zwitterionic surfactant when delivered in combination with hydrodynamic force is effective *in vitro*³⁶⁹ and *in vivo*³⁶⁷. However, this finding is most likely only applicable to industrial applications due to the cilia toxicity observed during mucosal application.³⁷⁰

There is a clear need for effective anti-biofilm therapies that are safe for clinical applications. However, as yet there has been no treatment accepted as a clinical anti-biofilm therapy. Investigation into the literature has identified a recently rediscovered antibacterial, bacteriophage: viruses that target and kill bacteria. Many studies have documented the efficacy of bacteriophage against biofilms, including *S. aureus*^{371, 372}. It is therefore the aim of this study to assess the efficacy of bacteriophage against *S. aureus* biofilms in an effort to develop an effective clinical anti-biofilm therapy.

1.4 Bacteriophages

Bacteriophages (“phage”) are viruses that infect bacteria. The majority of discovered phages are highly specific in host range and infect only one host species³⁷³. Phages have been employed extensively in the scientific community in phage display technology helping to discover disease diagnostic tools³⁷⁴, in vaccine development³⁷⁵⁻³⁷⁷, and the identification of tumour targeting agents³⁷⁸. Phage typing technology has also been employed to differentiate bacterial strains and investigate epidemiology for bacteria such as *Salmonella*³⁷⁹⁻³⁸¹, *Escherichia Coli*³⁸², *Campylobacter*^{383, 384}, *Vibrio cholera*^{385, 386} and *S. aureus*^{387, 388}. Phages have also been implicated as potential non-antibiotic treatments of bacterial infections. This study is focused on developing a therapy for *S. aureus* infection in CRS patients using phages. This section shall review the extensive literature related to basic phage biology and phage therapy.

1.4.1 History of Bacteriophage

In 1886, Ernest Hankin observed antibacterial activity against the bacterial pathogen *Vibrio cholerae*, in the waters of two Indian rivers: the Ganges and Jumna. He described that an undiscovered, filterable, heat labile material was responsible for the antibacterial characteristics³⁸⁹. Despite Hankin’s discovery the scientific community did not properly investigate phages until some 30 years later³⁹⁰. Frederick Twort became interested in phage in the early 20th century. Twort observed bacteriophage whilst working with bacteria of the *Micrococci* genus. He observed that *Micrococci* cultures of a white colour would be killed on contact with ‘clear’ *Micrococci* cultures. Unfortunately due to monetary constraints Twort was unable to further his research into this phenomenon³⁸⁹ coming to the conclusion that the occurrence could be due to either an ultra-microscopic virus or a self-destructive enzyme produced by the *Micrococci* itself³⁹⁰.

Just two years later Felix d'Herelle presented work at the 1917 Academy of Sciences meeting describing an 'invisible microbe endowed with antagonistic properties' of which he believed was a virus that targeted bacteria³⁹¹. Much debate continued into the 1920's about whether the antibacterial effect (later to be discovered to be bacteriophage) was viral or enzymatic in nature, mainly between d'Herelle (who argued the viral side) and an immunologist Jules Bordet³⁹². Eventually a consensus was reached with the viral argument victorious. Following this research into phage as a treatment of bacterial infection began. In the 1940's, however, due to the Second World War and the widespread employment of penicillin, the use of phage as a treatment method faded into the background in western society³⁹³.

Although western countries trended away from the use of phage, research continued in eastern Europe³⁸⁹. In some Eastern European countries findings showed great promise of exploiting the naturally derived viruses and, as such, phages are still clinically employed.

However, interest in phage as a treatment for bacterial infections in western countries is re-emerging. Overuse of antibiotics has triggered an increased prevalence of antibiotic resistant bacterial species. Potential emergence of strains resistant to all available antibiotics is of real concern³⁹⁴. Due to the enormity of this problem and success of eastern European bacteriophage therapy many Western institutions have begun to investigate the potential of these viruses to treat bacterial diseases³⁸⁹.

1.4.2 Bacteriophage Taxonomy

The International Committee on Taxonomy of Viruses (ICTV) provides a comprehensive summary of the discovered viruses and their classifications. Table 1.3 summarises the

information currently available from the ICTV^{395, 396}. Further to the available information characterisation of 5 archaeal and 4 bacterial viruses are still pending³⁹⁷.

The bacteriophage to be used in this study have been identified using transmission electron microscopy to be of the *Myoviridae* family (data not published). Therefore, this literature review will focus on examples of structure and mechanism of action to the *Myoviridae* family.

Table 1.3: Summary of the taxonomy of bacterial and/or archaeal targeting viruses based on current information from the International Committee on Taxonomy of Viruses.

Genome	Order	Family (*)	Subfamilies	Number of Genus's (^)	hosts
dsDNA	Caudovirales	Myoviridae	Peduovirinae, spounavirinae, tevenivirinae	6 (12)	B, Ar
		Podoviridae	Autographivirinae, Picovirinae	5 (6)	B
		Siphoviridae	-	10	B, Ar
	Ligamenvirales	Lipothrixviridae	-	4	Ar
		Rudiviridae	-	1	Ar
	UnAssigned	Ampullaviridae	-	1	Ar
		Bicaudaviridae	-	1	Ar
		Corticoviridae	-	1	B
		Fuselloviridae	-	2	Ar
		Globuloviridae	-	1	Ar
		Guttaviridae	-	2	Ar
		Plasmaviridae	-	1	B
		Tectiviridae	-	1	B
		Unassigned (Salterprovirus)	-	-	Ar
ssDNA	UnAssigned	Inoviridae	-	2	B
		Microviridae	Gokushoviridae	3(1)	B
dsRNA	UnAssigned	cystoviridae	-	1	B
positive sense ssRNA	UnAssigned	Leviviridae	-	2	B

* Genus if unassigned to a family, ^ when subfamilies are present represents the number of genus's unassigned to a subfamily. ssDNA/RNA = single stranded DNA/RNA; ds DNA/RNA = double stranded DNA/RNA; B = bacteria; Ar = archae.

1.4.3 Myoviridae family

Myoviridae phage are classified as such mainly due to their structure³⁹⁸. Such structural elements include:

- 1) Large head structures with higher particle and DNA weights than other tailed phage.
- 2) Central core structure containing stacked rings, encompassed by a contractile sheath with helical shape.
- 3) Long, reasonably thick contractile tail.

These phage have also been noted to be more sensitive to freezing and thawing as well as osmotic shock in comparison to tailed phage of other families³⁹⁸.

There are 3 subfamilies of the myoviridae family. Each contain 2 genera and a further 13 genera that have not been classified into a subfamily³⁹⁶. Elements such as genome organisation, DNA replication, and packaging mechanisms are used to segregate phage in this family into their different subfamilies and genera.

The phages that shall be investigated and employed in this study have been previously investigated using transmission electron microscopy (TEM). TEM analysis has shown the four phages present in the phage cocktail to be of the *Myoviridae* family similar in sequence to the staphylococcal phage K (unpublished work).

1.4.3.1 *Structure of Phage K*

Elucidation of the structure of phage K began in the late 1940's. It is described as possessing a round head, long tail and a 'bob' or circular structure at the tail end of the phage structure (Figure 1.8).

More recent research employing higher resolution technology has found that phage K has an icosahedral head structure attached to a long and thin contractile tail, and culminating in a complex baseplate structure³⁹⁹. The structure of the base plate has not been completely elucidated; however, researchers have noted that it has small knob like structures protruding from the base plate⁴⁰⁰ (Figure 1.9).

Each of these individual components of the bacteriophage's structure play important roles in the phage infection, replication, and release from host cells. Therefore it is not only important to discuss the general structure of the phage, but also the role each element plays in the phage lifecycle.

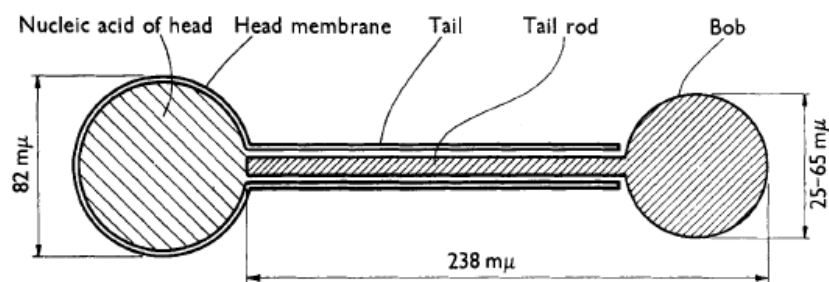


Figure 1.8: Diagram of Phage K structure proposed in 1954. Image C credit to Hotchin, 1954. C) Using the electron microscopy technology available at the time, this image shows a diagram of the proposed structure of phage K.

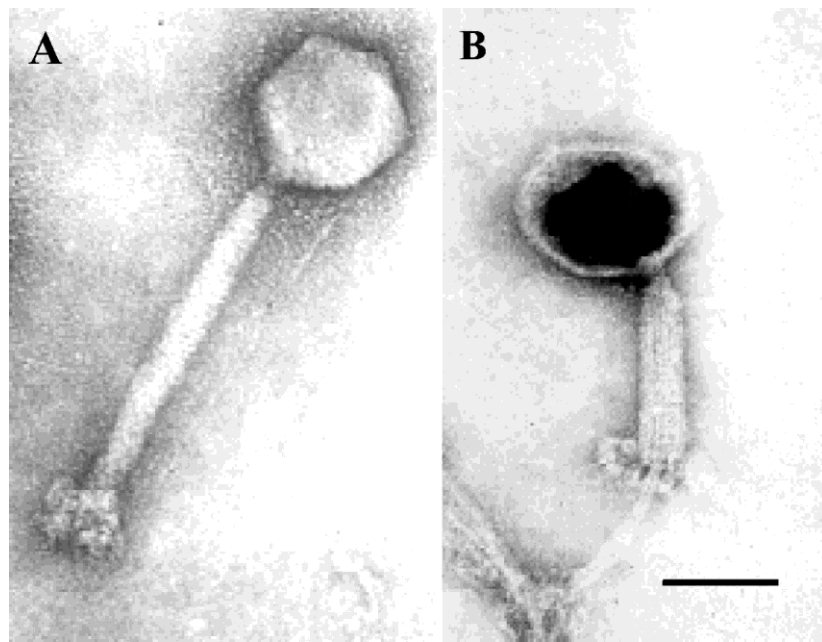


Figure 1.9: Electron Microscopy image of Phage K. Image A/B credited to O’Flaherty et. al. 2005. Electron microscopy imaging of Phage K, showing both an A) phage K possessing a contractile tail, and B) phage K in a contracted state showing the core of the tail has been exposed and the head of the phage is now devoid of DNA.

1.4.4 Lifecycle of bacteriophage

Phage can be divided into three types; lytic, temperate or lysogenic⁴⁰¹⁻⁴⁰⁴. Lytic phages hijack the bacterial host cellular machinery to produce progeny phage, destroy the cell to re-enter the surrounding environment, and proceed to invade new bacterial hosts (figure 1.10A)⁴⁰¹⁻⁴⁰³. Lysogenic phages integrate their DNA into their host genome, and remain dormant benignly replicating with the bacteria (figure 1.10C)⁴⁰¹⁻⁴⁰³. When phage DNA is situated within the bacterial genome they are referred to as prophage. Temperate phage (figure 1.10B) are a combination of these two phage types and are able to enter the lytic cycle or integrate their genome into the host⁴⁰¹⁻⁴⁰³. Bacteriophage therapy employs primarily lytic phage to achieve maximal bacterial elimination^{389, 393, 405}. As such, lytic phage will be discussed in further detail below.

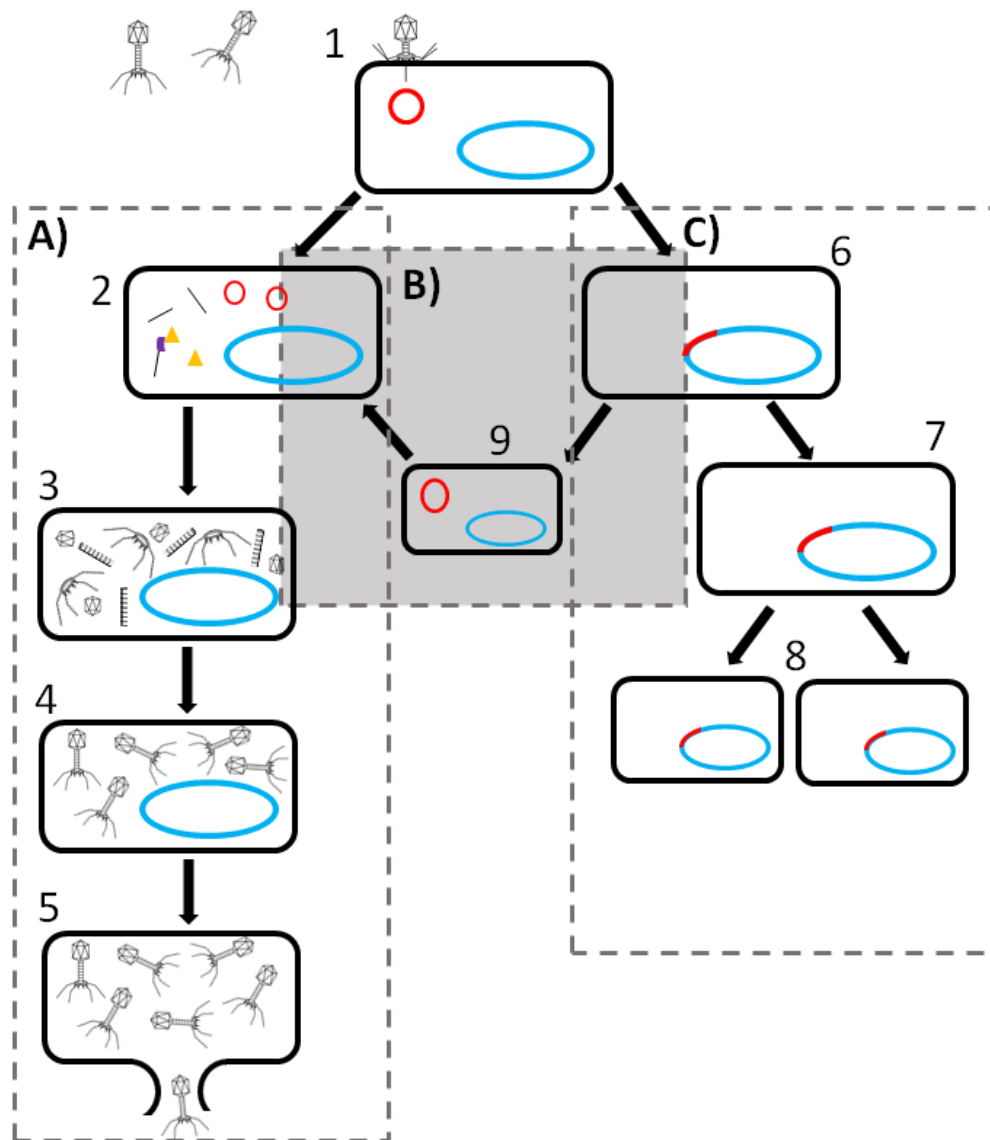


Figure 1.10: Bacteriophage lifecycle. Figure adapted from Lodish et. al 2000. Bacteriophage are divided into three different types. (1) Each of the three cycles begins with the bacteriophage coming into contact with the bacterial host and injecting its genomic material into the bacterial cell. Panel A) Describes the lytic phage cycle. (2) Following DNA injection, the DNA forms a circular plasmid like structure and by manipulating the host machinery phage specific proteins and DNA are produced. (3) These proteins begin to form whole phage elements (eg. Tail structure) and (4) eventually begin to form whole phage particles. (5) Once a critical number of phage have formed, proteins produced by the phage DNA cause holes to form in the cell wall and membrane and progeny phage are released into the environment. Panel C) describes the lysogenic phage cycle. (6) Following DNA injection the phage DNA integrates into the bacterial genome where it is described as a prophage. This prophage remains benign (7/8) replicating within the bacterial genome as the bacterial population grows. Panel B) describes the temperate phage cycle. (9) Following incorporation into the host genome, under certain environmental conditions prophage can be stimulated to exit the genome and form circular, plasmid like DNA structures stimulating switch to a lytic cycle.

1.4.4.1 Lytic Phage Lifecycle

As mentioned above, lytic phage are the most desirable for therapeutic applications. Thus far the most studied *Myoviridae* lytic phage is the *Escherichia coli* phage T4. This phage will be employed as an example.

The first step of lytic phage infection is adsorption. This involves attachment to a receptor or protein located externally on the surface of the bacterial target^{402, 404}. Bacteriophage T4 tail fibres bind to the surface receptor outer membrane porin C, and at this stage the binding is reversible⁴⁰⁶. At least three fibres must bind to the receptor for successful infection⁴⁰⁷. A further six shorter fibres bind to the lipopolysaccharides in the cell wall at which time phage-host binding becomes irreversible^{408, 409}.

The second stage is penetration. This is where the genetic material of the phage is injected into the host bacteria^{402, 404}. In regards to the T4 phage, this involves contraction of the sheath and protrusion of the tail core into the periplasm⁴⁰⁶. Lysozyme enzymes act to degrade the cell membrane region thus allowing passage of the core through to the cytoplasm into which the DNA is injected⁴¹⁰.

Following DNA injection, replication and translation occurs. The host cellular machinery is manipulated in an effort to begin producing phage specific proteins^{402, 404}. In T4 this includes DNA replication machinery to replicate phage DNA⁴¹¹ as well as 40 different structural proteins⁴¹².

Maturation is the next stage where structural elements start to form and the phage genome is encapsulated in the phage's head structure^{402, 404}. DNA encapsulation in T4 is mediated by a terminase enzyme which acts to pump the DNA into the phage capsid head⁴¹³.

The final stage is phage release from the host cell. This is mediated by pore-forming enzymes^{402, 404}. In the case of T4, lysozymes which act to hydrolyse peptidoglycan contribute to the lysis of host cells and liberation of the phage progeny⁴¹⁴. It is the action of bacterial cell lysis and destruction that make lytic phage desirable for therapeutic application.

1.4.5 Bacteriophage Therapy

The ability of phage to destroy bacterial cells makes them a strong anti-bacterial candidate. Despite being ignored for many years in Western medicine, a recent surge in research has re-ignited interest in bacteriophage as a therapeutic⁴¹⁵. This section shall review phage therapy over the last 100-years.

1.4.5.1 Early History of Phage Therapy

Assessing the efficacy of phage therapy against bacterial infection began in livestock. An example is provided by early work by d'Herelle who studied the prevention of *Salmonella gallinarum* infection in chickens³⁹³. He found that chickens inoculated with phage that were subsequently exposed to *S. gallinarum* were protected from infection³⁹³. Work progressed quickly to clinical application with the first recognised therapeutic use of bacteriophage in 1919^{389, 393}. In 1919 phage were employed to treat dysentery and results of the study were positive, with all four patients recovering swiftly^{389, 393}. Further work was published in 1921 which demonstrated effective clearance of *S. aureus* wound infections within 24-48 hours of phage application³⁸⁹.

Owing to these and other successes, application of phage therapy began to spread.

However, problems quickly surfaced. One of these was development of commercial phage

products without an appropriate knowledge of phage biology. This led to production of phage products that were shown to be ineffective against bacterial infection due to improper production techniques³⁹³. Furthermore, a lack of purity, and contamination of phage based medications with bacterial endotoxins also meant that some phage preparations were noted to be clinically unsafe³⁸⁹. Finally, poor scientific method including lack of placebo control groups contributed to a poor review of the therapy⁴¹⁶. Such problems, along with the success of then new antibiotics, extinguished interest in phage therapy in the western world⁴⁰¹. Despite this, research efforts continued in a number of eastern European countries^{389, 401}.

1.4.5.2 Early phage therapy in Eastern Europe

Over the last 60 years, a large volume of work in regards to phage therapy has been promulgated from Eastern Europe. This includes a review by Slopek et. al. 1987 detailing extensive clinical application of phage demonstrating that of 298 patients treated, 95.3% experienced successful infection elimination⁴¹⁷. Many of the published works originating from Poland and the former Soviet Union are not available as English texts. Fortunately, two studies have been produced reviewing this body of work^{389, 418}. A number of Polish investigations have noted efficacy of phage therapy. Stroj et. al. 1999 effectively treated cerebrospinal meningitis in newborn babies following a failure of antibiotic treatment³⁸⁹. Successful phage mediated elimination of bacteria has been also noted when treating sub-phrenic abscesses, lung and pleural infections, urinary tract infections, and postsurgical wounds⁴¹⁸. Studies conducted in the former Soviet Union are also positive. One large trial assessing phage treatment of paediatric *Shigella* dysentery (n = 30,000) indicated the rates of diarrhoea were 3.8 fold lower in the phage treated group compared to placebo^{389, 418}. Infection elimination using phage was also noted in lung, eye, urinary tract

and surgical wound infections³⁸⁹. Unfortunately, many of these studies were again of poor scientific rigour and many are not placebo controlled.

1.4.5.3 Resurgence of Phage Therapy

Research by Smith and Huggins in the 1980's was responsible for rekindling the western interest in phage therapy³⁸⁹. Their findings that phage was equivalent in efficacy to antibiotics in treating *E. coli* infection in mice highlighted the potential of phage⁴¹⁹. A significant volume of *in vitro* research was subsequently conducted following the success of this research which identified phage as a promising antimicrobial⁴²⁰⁻⁴²⁷. Phage efficacy against bacteria often differs from *in vivo* or more complex environments^{364, 365}. Therefore, we will focus on *in vivo* investigation of phage therapy.

In vivo assessment of phage therapy has shown successful prevention^{422-424, 427-432} and treatment^{421, 425, 426, 433-439} of bacterial infections. Some studies however have found phage to be ineffective *in vivo*⁴⁴⁰⁻⁴⁴³. These studies showing reduced efficacy however do find a phage mediated delay in time to mortality^{440, 442}, and non-significant reduction in infection load⁴⁴¹ suggesting some beneficial effect of the phage therapy. Complexities in phage biology, phage-host interactions and human immune system-phage interactions make development of phage therapy complicated. Extensive *in vitro* and *in vivo* research is essential prior to clinical application to ensure therapy efficacy.

The complexity of phage is perhaps the reason behind the relatively small number of clinical trials. Research has thus far primarily investigated the safety of phage application. Recent studies have found no side effects upon oral phage application⁴⁴⁴⁻⁴⁴⁶ and topical phage application to external wounds⁴⁴⁷, leg ulcers⁴⁴⁸ and the ear⁴⁴⁹. Efficacy of phage therapy has been assessed in recent literature in only one study. This study assessed the

efficacy of phage treating chronic otitis associated with *P. aeruginosa* infection. Phage treatment improved the clinical condition of patients and also reduced levels of infection⁴⁴⁹. Whilst these clinical trials show promising results, increased clinical work is required before phage can become accepted as a mainstream antimicrobial option. Research in this thesis will evaluate phage therapy in the context of CRS in an effort to develop an effective antimicrobial option when antibiotics fail.

1.4.6 Applications for bacteriophage in CRS

As mentioned previously, bacterial infection may exacerbate the CRS condition. Conventional antibiotic intervention can be ineffective. Thus, development of a novel antimicrobial will be of great benefit in the treatment of CRS. Biofilm formations are also noted to contribute to the pathogenesis of CRS. Furthermore, recent study suggests maintaining beneficial nasal cavity commensals is also of great importance. Therefore, bacteriophage may be a strong candidate therapy due to their efficacy against biofilms and their ability to target an individual species.

1.4.6.1 Bacteriophage versus biofilms

As mentioned biofilm formation in CRS is a particular issue. It is therefore essential that any treatment is capable of destroying biofilm bound bacteria for application in CRS bacterial infections. Importantly phage are noted to not only diffuse through the biofilm matrix⁴⁵⁰, but can also infect and subsequently replicate within bacteria in biofilm form⁴⁵¹. Research into the effect of treating biofilm with phage is an area of increasing interest⁴⁵¹⁻⁴⁶².

Significant reductions in biofilms of a number of bacterial species including *Campylobacter jejuni*⁴⁵⁶, *P. aeruginosa*⁴⁵⁷, *Escherichia coli*^{451, 452, 463}, *Aggregatibacter*

*actinomycescomitans*⁴⁶⁴, *S. epidermidis*⁴⁵⁸ and importantly *S. aureus*^{372, 437} have been noted *in vitro*. Looking more specifically at studies where biofilms have been cultivated *in vivo*, there has been limited research. Successful elimination has been noted against *P. aeruginosa* implant related infections⁴⁴¹. Combination therapy of debridement and phage has shown successful reduction of wound based *S. aureus* biofilms⁴⁴³. Lastly, a combination of antibiotics and phage elicited significant biofilm reductions in implant related *S. aureus* infection, whilst phage alone generated a non-significant reduction⁴⁴¹. This highlights that bacteriophage have potential to be applied clinically against preformed biofilms.

Bacteriophage are also noted to prevent biofilm formation of *E. coli*, *Proteus mirabilis*⁴⁵⁴, *P. aeruginosa*⁴⁵³ and *S. epidermidis*⁴⁵⁹ biofilm formation on catheters. This ability to prevent biofilms has an important application in CRS. If the bacteriophage have the capacity to remain in the sinus region they would be able to prevent and fend off further infection from *S. aureus*. Of note, recent research has discovered that certain bacteriophage adhere to mucous⁴⁶⁵. Therefore, potentially if a phage is introduced into the nose it may remain even after eliminating the pathogenic bacteria to prevent further infection.

The ability to prevent and treat biofilms is important when treating bacterial infections in CRS. Therefore, with a careful selection of phage with anti-biofilm activity, phage could be applied successfully to treat mucosally-anchored biofilms in CRS.

1.4.6.2 Specificity of phage

An individual's nasal cavity is not a sterile environment and the nasal nares of healthy subjects are populated by a multitude of microorganisms^{466, 467}. This complex

microorganism microcosm is defined as the microbiome. It has been suggested that an unbalanced microbiome, particularly a reduced level of bacterial diversity, may contribute negatively to CRS^{103, 104}. Antibiotics are common therapies against CRS but have been noted to reduce bacterial diversity in the nose⁴⁶⁸. Therapy specifically targeting pathogens may be beneficial for treating nasal infections, helping to maintain the commensal communities. Phage host range is often limited to one bacterial species³⁷³, and phage are able to target a pathogen whilst maintaining commensal flora⁴⁶⁹. This makes them a prime candidate for treatment of nasal infections. Additionally, commensal organisms are noted to compete with pathogens and prevent colonisation⁴⁷⁰⁻⁴⁷². Hence maintaining such commensals and preserving beneficial bacterial interference would also be beneficial in CRS.

1.5 Summary of Literature Review

CRS is a complex condition with a pathophysiology that is difficult to completely ascertain. A host of factors contribute to this condition, some of which interact not only with CRS but also with other factors. CRS is complicated by the fact that not all contributing factors must be present for an individual to experience the condition. This in turn means developing a 'one treatment fits all' style of medical management is extremely difficult. However, one strategy is to break these factors down into individual targets and delineate and develop treatment for each entity. Embarking on this notion, this thesis focussed on the contributing factor of *S. aureus* infection.

S. aureus infection has been shown to exacerbate CRS. As discussed, this bacteria possesses a myriad of virulence constituents capable of conferring host damaging and immune evading characteristics. To understand which virulence factors are important in the CRS context, understanding the prevalent strains and epidemiology of *S. aureus* in CRS is important. This has not been investigated in depth in CRS and is a potential area of research. Different strains will often possess or express different virulence factors. Determining which strains are particularly prevalent and damaging in CRS would be of great benefit. This knowledge could lead to identification of individual *S. aureus* virulence factors that could be targeted to improve a patient's quality of life.

Furthermore, biofilms of *S. aureus* are noted to exacerbate CRS and are found to encourage recurrent infection and disease recalcitrance. Biofilms are very tough structures and are resistant to numerous antimicrobial strategies. There is a call for development of a safe and effective treatment targeting these mucosally anchored bacterial biofilms in CRS

patients' noses. Thus far, no treatment has been accepted as an effective anti-biofilm agent in CRS.

Bacteriophage have been shown to be not only effective against bacterial infections, but are also regarded as anti-biofilm agents. Phage have been used extensively and successfully in Eastern Europe to treat bacterial infections resistant to antibiotics. Interest in phage research has intensified in the last few years, and is emerging as a promising therapeutic. Application of phage in the context of CRS bacterial infection has yet to be assessed. Therefore, assessing the potential of phage as an agent against *S. aureus* infection and biofilm is an attractive area of research.

1.6 Project Aims

A number of research aims have been devised in accordance with the gaps in knowledge and required research discussed in the foregoing section. The three main aims of the study are described subsequently.

1. Expand and enhance the knowledge and understanding of *S. aureus* epidemiology in CRS with a focus on furthering our understanding of the clonal relationships between repeated *S. aureus* nasal infections.
2. Determine the *in vitro* efficacy of a bacteriophage against biofilms of *ex vivo* *S. aureus* isolates harvested from CRS patients. Furthermore assess the development of *S. aureus* bacteriophage insensitive mutants.
3. Determine the *in vivo* safety and efficacy of phages against sinonasal, mucosally anchored *S. aureus* biofilms. Furthermore to determine whether phage therapy can synergise with other anti-biofilm agents to enhance the anti-biofilm capacities of the treatments.

2 Cousins, siblings or copies: the genomics of recurrent

Staphylococcus aureus infections in chronic rhinosinusitis

2.1 Statement of authorship

Statement of Authorship

Title of Paper	Cousins, siblings or copies: the genomics of recurrent <i>Staphylococcus aureus</i> infections in chronic rhinosinusitis
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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Amanda J Drilling	
Contribution to the Paper	Collected and stored bacterial isolates from CRS patients and harvested bacteria from patient tissue, analysed data and collated patient information, prepared manuscript.	
Signature	Date	08/10/2014

Name of Co-Author	Geoffrey Coombs	
Contribution to the Paper	Directed molecular typing, assisted in the analysis of results, assisted in the preparation of the manuscript.	
Signature	Date	09/09/2014

Name of Co-Author	Hui-leen Tan	
Contribution to the Paper	Performed molecular characterization of <i>S. aureus</i> isolates.	
Signature	Date	09/09/2014

Name of Co-Author	Julie C Pearson	
Contribution to the Paper	Analysed molecular typing results.	
Signature	Date	09/09/2014

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Name of Principal Author (Candidate)	Amanda J Drilling		
Contribution to the Paper			
Signature		Date	

Name of Co-Author	Samuel Boase		
Contribution to the Paper	Aided in experimental design, preparation of human ethics applicationn and manuscript editing.		
Signature		Date	30/08/2014

Name of Co-Author	Alkis Psaltis		
Contribution to the Paper	Aided in data analysis and collection and manuscript editing		
Signature		Date	08/09/2014

Name of Co-Author	Peter Speck		
Contribution to the Paper	Aided in manuscript editing		
Signature		Date	08/09/2014

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Name of Principal Author (Candidate)	Amanda J Drilling		
Contribution to the Paper			
Signature		Date	

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Aided in data analysis and manuscript editing.		
Signature		Date	07/09/2014

Name of Co-Author	Peter-John Wormald		
Contribution to the Paper	Aided in experimental design, data collection and analysis and manuscript editing.		
Signature		Date	08/09/2014

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Cousins, siblings, or copies: the genomics of recurrent *Staphylococcus aureus* infections in chronic rhinosinusitis

Amanda Drilling, BBtech (Hons)¹, Geoffrey W. Coombs, PhD^{2,3}, Hui-leen Tan, BSc (Hons)³, Julie C. Pearson, BSc³, Samuel Boase, BMBS (Hons), PhD¹, Alkis Psaltis, MBBS (Hons), PhD, FRACS¹, Peter Speck, PhD⁴, Sarah Vreugde, MD, PhD¹ and Peter-John Wormald, MD, FRACS¹

Background: *Staphylococcus aureus* infection is known to play a role in recalcitrant chronic rhinosinusitis (CRS). However, it is unknown if recurrent *S. aureus* infections are caused by the same strain or are due to independent acquisitions of different strains.

Methods: Samples were collected from patients with CRS from July 2011 to August 2012. *S. aureus* was isolated from mucosal swabs and tissue specimens from patients who underwent surgery during the study period, or from swabs of areas of purulence taken in the postoperative period under endoscopic guidance. Pulsed-field gel electrophoresis was used to characterize *S. aureus* isolates.

Results: Thirty-four patients were included in the study; 79% showed persistence of the same *S. aureus* strain in their paranasal sinuses ($p = 0.001$; $H_1 \neq 50\%$). Furthermore, a significantly high frequency of patients with known biofilm status were positive for *S. aureus* biofilm ($p = 0.002$; $H_1 \neq 50\%$). When patients were stratified according to dis-

ease evolution postsurgery, certain strains appeared to be more commonly associated with symptom persistence.

Conclusion: The same *S. aureus* strain appears to persist in the paranasal sinuses of CRS patients despite multiple courses of culture-directed antibiotics. This suggests that conventional antimicrobial therapies in patients with CRS may not eliminate the organism. This may be partly explained by the formation of biofilms in the paranasal sinus region. © 2014 ARS-AAOA, LLC.

Key Words:

Staphylococcus aureus; chronic rhinosinusitis; persistent infection; biofilms; intracellular; pulsed-field gel electrophoresis

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¹Department of Surgery–Otolaryngology Head and Neck Surgery, University of Adelaide, Adelaide, South Australia, Australia; ²Australian Collaborating Centre for Enterococcus and Staphylococcus Species (ACCESS) Typing and Research, School of Biomedical Sciences, Curtin University, Perth, Western Australia, Australia; ³Department of Microbiology and Infectious Diseases, PathWest Laboratory Medicine–WA, Royal Perth Hospital, Perth, Western Australia, Australia; ⁴School of Biological Sciences, Flinders University, Bedford Park, South Australia, Australia

Correspondence to: Peter-John Wormald, MD, FRACS, Department of Surgery–Otolaryngology Head and Neck Surgery, University of Adelaide, Adelaide, SA, Australia; e-mail: peterj.wormald@adelaide.edu.au

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Staphylococcus aureus plays a significant role in recalcitrant chronic rhinosinusitis (CRS).^{1–4} Microbiological culture-dependent and culture-independent techniques have identified *S. aureus* in 15% to 36% and 50% to 61% of CRS patients, respectively.^{5–9} When isolated during sinus surgery, *S. aureus* has been shown to be associated with a more severe patient symptomatic profile and an increased prevalence of postoperative infections.^{1,4}

Recently published research from our department has shown *S. aureus* may persist in patients with CRS despite culture-directed oral and topical antibiotic treatment¹⁰ and may contribute to refractory sinusitis.¹ It is not known, however, if recurrent *S. aureus* infections are caused by the same strain, and whether the source of the bacteria are from local biofilm^{1,3} or intracellular niches,^{11,12} or from external contamination.^{13,14} It is also unknown what factors influence strain persistence and whether patient demographic or clinical factors or previous antibiotic treatment may be involved.

Cousins, siblings or copies: the genomics of recurrent *Staphylococcus aureus* infections in chronic rhinosinusitis

Amanda Drilling¹ BBtech (Hons), Geoffrey W Coombs^{2,3} PhD, Hui-leen Tan³ BSc (Hons), Julie C Pearson³ BSc, Sam Boase¹ BMBS (Hons) PhD, Alkis Psaltis MBBS(Hons) PhD FRACS¹ Peter Speck⁴ PhD, Sarah Vreugde¹ MD PhD and Peter-John Wormald¹# MD FRACS.

¹ Department of Surgery-Otolaryngology Head and Neck Surgery, University of Adelaide, Adelaide, SA Australia

²Australian Collaborating Centre for *Enterococcus* and *Staphylococcus Species* ([ACCESS](#)) Typing and Research, School of Biomedical Sciences, Curtin University, Western Australia, Australia

³Department of Microbiology and Infectious Diseases, PathWest Laboratory Medicine – WA, Royal Perth Hospital, Western Australia, Australia

⁴Flinders University, Bedford Park, South Australia, Australia.

Short title: Genomics of *S. aureus* infections in CRS

Conflict of Interest: None relevant to this study

Key Words: *Staphylococcus aureus*, chronic rhinosinusitis, persistent infection, biofilms, intracellular, pulsed-field gel electrophoresis.

2.3.2 Abstract:

Background: *Staphylococcus aureus* infection is known to play a role in recalcitrant chronic rhinosinusitis (CRS). However it is unknown if recurrent *S. aureus* infections are caused by the same strain or are due to independent acquisitions of different strains.

Methods: Samples were collected from patients with CRS from July 2011 to August 2012. *S. aureus* was isolated from mucosal swabs and tissue specimens from patients who underwent surgery during the study period, or from swabs of areas of purulence taken in the post-operative period under-endoscopic guidance. Pulsed-field gel electrophoresis was used to characterise *S. aureus* isolates.

Results: Thirty-four patients were included in the study. 79% showed persistence of the same *S. aureus* strain in their paranasal sinuses ($p = 0.001$ $H_1 \neq 50\%$). Furthermore a significantly high frequency of patients with known biofilm status were positive for *S. aureus* biofilm ($p = 0.002$ $H_1 \neq 50\%$). When patients were stratified according to disease evolution post-surgery, certain strains appeared to be more commonly associated with symptom persistence.

Conclusion: The same *S. aureus* strain appears to persist in the paranasal sinuses of CRS patients despite multiple courses of culture-directed antibiotics. This suggests that conventional antimicrobial therapies in patients with CRS may not eliminate the organism. This may be partly explained by the formation of biofilms in the paranasal sinus region.

2.3.4 Introduction

S. aureus plays a significant role in recalcitrant chronic rhinosinusitis (CRS)^{33, 211, 216, 349}. Microbiological culture-dependent and –independent techniques have identified *S. aureus* in 15 to 36% and 50 to 61% of CRS patients respectively^{100, 103, 473-475}. When isolated during sinus surgery, *S. aureus* has been shown to be associated with a more severe patient symptomatic profile and an increased prevalence of post-operative infections^{33, 211}.

Recently published research from our department has shown *S. aureus* may persist in patients with CRS despite culture-directed oral and topical antibiotic treatment¹⁸⁰ and may contribute to refractory sinusitis²¹¹. It is not known however, if recurrent *S. aureus* infections are caused by the same strain, and whether the source of the bacteria are from local biofilm^{211, 349} or intracellular niches^{217, 261}, or from external contamination^{476, 477}. It is also unknown what factors influence strain persistence and whether patient demographic, clinical factors or previous antibiotic treatment may be involved.

To further our knowledge of *S. aureus* infection in patients with CRS, the current study investigated *S. aureus* strain relatedness in recurrent *S. aureus* CRS infections. In addition, biofilm forms of *S. aureus* were typed and compared to post-operative infections, to determine if biofilm was acting as a primary nidus for re-infection.

2.3.6 Materials and Methods:

Human ethics and patient inclusion criteria

The study was performed between July 2011 and August 2012 and was approved by the human ethics committee at the Queen Elizabeth Hospital. Inclusion criteria included patients with a diagnosis of CRS recalcitrant to appropriate medical therapy from whom *S. aureus* was cultured on more than one occasion during the study period. Patients were recruited from the tertiary clinic of the senior author (PJW). Immunocompromised patients were excluded.

Clinical Information

Retrospective review of the patients' case notes was performed. The reviewer was blinded to the patient's microbiological results. Patient demographics and clinical information including the presence of symptoms at time of post-operative review and whether the patient felt better or worse since the time of surgery was recorded.

Specimen collection

Mucosal swabs and tissue from patients who underwent surgery during the study period were collected. Culture swabs were also taken in the post-operative period under-endoscopic guidance from areas of purulence. Caution was exerted to avoid vestibular contamination by careful retraction of the alar cartilage as well as the use of guarded culture swabs (Figure 2.1). Specimens were referred to Adelaide Pathology Partners (APP, Mile End, South Australia) and processed for bacteriological culture.

Isolation and storage of *S. aureus*

Bacteria were isolated on Columbian blood agar plates, colistin nalidixic acid plates or cystine lactose electrolyte deficient plates. Colonies with *S. aureus* morphology were identified as *S. aureus* using latex agglutination testing. Isolates were subcultured into bovine cerebrospinal fluid broth (CSF, Thermo Fisher Scientific), incubated aerobically overnight and stored in 50% glycerol at -80°C. Antibiotic susceptibility profiles were determined using disc diffusion on Mueller-Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) recommendations⁴⁷⁸. A panel of nine antimicrobials was tested and included penicillin, oxacillin, erythromycin, clindamycin, trimethoprim, tetracycline, augmentin, cephalexin and mupirocin.

Isolation of *S. aureus* cultures from biofilm present on patient tissue

Tissue specimens were analysed for presence of biofilm forms of *S. aureus* using previously described methodologies^{261, 479}. Briefly, the tissue samples were initially washed three times in MilliQ water and then submerged in 0.0003% cetylpyridinium chloride (CPC; Sigma-Aldrich,) for five minutes removing excess planktonic *S. aureus* cells. The samples were washed a further three times in MilliQ water to remove the CPC and then sonicated (Soniclean) in phosphate buffered saline (PBS) for 10 minutes to dislodge the biofilm. The sonicate was cultured onto *S. aureus* ID agar plates (bioMérieux) and incubated at 37°C for 24-48hrs. *S. aureus* were subcultured overnight in CSF broth and stored in 50% glycerol at -80°C.

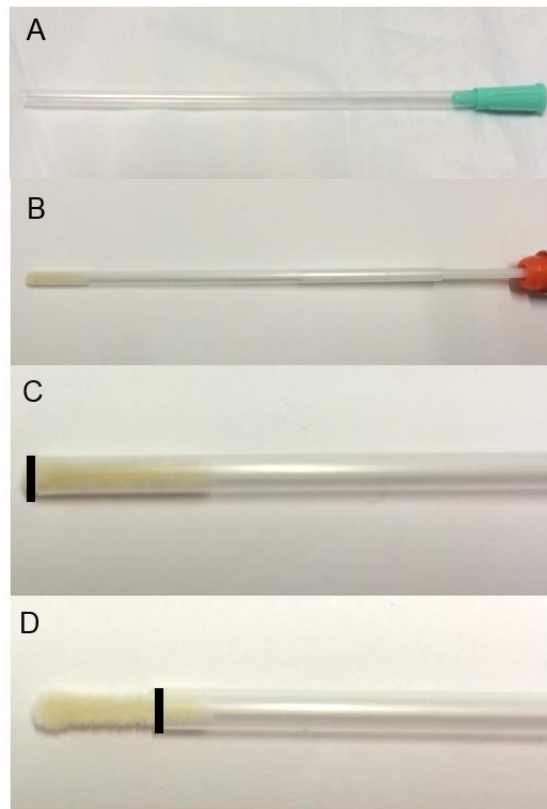


Figure 2.1: Guarded culture swabs. A) Mixing cannula used to protect the flocked swab from contamination. B) Flocked swab used for bacterial sampling. C) The end attachment of the mixing cannula is removed and the swab is inserted inside. When the swab enters the nose it is completely protected by the cannula (black line represents end of cannula). When the swab is in the appropriate nasal position it is pushed out of the protected sheath (D) and the sample is taken. When retreating out of the nose the swab can be retracted back into the cannula to be protected from contamination when the swab is removed.

Pulse-field Gel Electrophoresis (PFGE)

PFGE was performed on all isolates using a contour-clamped homogeneous electric field DRIII System (Bio-Rad Laboratories) as previously described⁴⁸⁰. Genomic DNA was prepared and digested with the *sma1* endonuclease restriction enzyme (Roche Diagnostics). PFGE was carried out in 0.5 Tris-borate-EDTA - 1% agarose gels at 14°C.

The pulse times were 1 – 40s over 20 hours. Chromosomal patterns were examined visually, scanned with a Quantity One device (Bio-Rad Laboratories) and digitally analysed using FPQuest (Applied Maths). *S. aureus* strain NCTC 8325 was used as a reference strain. The Dice coefficient and the unweighted pair group method with arithmetic mean were used with settings for tolerance and optimization of 1.25 % and 0.5 %, respectively. Isolates with $\geq 80\%$ similarity or less than six band difference were considered the same strain⁴⁸¹. The *S. aureus* pulsotype was analysed against the patients' symptomatic profile at the time of the appointment

Statistics

An asymptotic Wald test was used to compare the frequency of patients having a persistence of the same *S. aureus* strain to a null hypothesis of 50% using the software program SAS 9.3 (SAS Institute Inc., Cary, NC, USA). A null hypothesis of 50% was selected when assessing whether patients had a persistence of the same strain. This was selected assuming equal probability of having either the same or a different strain cultured from the nose of a patient over time. Similarly a null hypothesis of 50% was selected for biofilm presence. This is due to previous studies identifying a presence of 50% of CRS patients found to have *S. aureus* biofilm present^{211, 215}. Fisher's Exact Tests were used to compare the frequencies of patients with *S. aureus* strains that remained the same versus a *S. aureus* strain that changed in relation to demographics (eg. male versus female) and biofilm and intracellular *S. aureus* status (positive vs. negative). Fisher's exact tests were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

2.3.8 Results

Patient Characteristics

Thirty four patients were enrolled into the study over a 13 month period. The average age of the patients was 50yrs (STD \pm 18.2, range 18 to 82) with a female preponderance of 59%. Table 2.1 summarises the demographic and clinical information of the cohort. The majority of patients (59%) had undergone prior sinus surgery with the average number of previous surgeries being 2.85 (STD \pm 4.26, range 1-20).

Table 2.1: Patient Demographics

Patient demographic	Number of patients (%)
Nasal polyposis	18 (53)
Asthmatic	19 (57)
Allergic	20 (59)
Aspirin sensitivity	7 (21)
Undergone >1 sinus surgical procedure	20 (59)
Current smoker	0

Culture Results

Twenty five patients cultured *S. aureus* on two occasions, six patients on three occasions and three patients on four occasions. The time between positive cultures varied with an average of 98 days (STD: \pm 69, range 12-280 (Table 2.2). In 13 patients *S. aureus* was isolated from the biofilm attached to patient mucosa mucosal as well as sinonasal swabs.

Table 2.2: Time between isolation of *Staphylococcus aureus* cultures.

Patient	Biofilm isolated* (Y/N)	Time between cultures (days)		
		A-B	B-C	C-D
1	N	68		
2	N	47		
3	N	12		
4	N	105		
5	N	124		
6	N	61		
7	N	26	154	
8	N	119		
9	Y	0	47	280
10	Y	0	16	49
11	N	217		
12	N	245		
13	N	166		
14	N	201		
15	Y	0		
16	Y	89	63	91
17	Y	0	98	91
18	Y	0	72	215
19	N	203		
20	Y	0	62	
21	Y	31	156	
22	Y	0	67	
23	N	168		
24	N	91		
25	N	49		
26	N	40		
27	N	98		
28	N	63		
29	Y	0	14	
30	Y	0	54	
31	N	23		
32	Y	0		
33	Y	71		
34	N	63		

* = Y denotes a patient who had biofilm isolated from tissue harvested at the time of surgery, N = no tissue available for isolation of *S. aureus*.

PFGE Strains

For 27 of the 34 (79%) patients, the *S. aureus* isolated from each patient had the same PFGE pulsotype indicating the same *S. aureus* strain persisted within the patient over time ($p = 0.001$, $H_1 \neq 50\%$). The remaining seven (21%) patients showed a variation in *S. aureus* pulsotype, indicating a different strain had been acquired over time (time between cultures 160 ± 85 days). The distribution and relationship of the 16 strains identified in his study are depicted using a dendrogram (Figure 2.2). Table 2.3 summarises the relatedness of the *S. aureus* isolated from each patient who over time was infected with a different *S. aureus* strain.

Nine patients had an intraoperative swab and mucosal biofilm sample from which *S. aureus* was cultured. For all nine patients the biofilm isolate was the same as the *S. aureus* isolated from the intraoperative swab. Patient demographics, topical steroid application and oral/ topical antibiotic prescribing did not appear to influence the strains isolated (data not shown). 21 of the 34 (61.8%) patients included in the study had tissue specimens available for biofilm analysis. Eighteen of the 21 (85%) were found to be positive for *S. aureus* biofilms ($p = 0.001$, $H_1 \neq 50\%$).

Table 2.3: Percent relatedness of patient isolates with different PFGE pulsotypes.

Patient	Isolate	Pulsotype	Relatedness (percent relatedness)
4	A	K	59
	B	N	
9	A, B, C	H	54
	D	F	
12	A	C	59
	B	A	
16	A	N	A vs. B 79
	B	A	A vs. C/D 59
	C, D	L	B vs. C/D 59
19	A	A	54
	B	H	
21	A, B	M	67
	C	O	
33	A	P	66
	B	B	

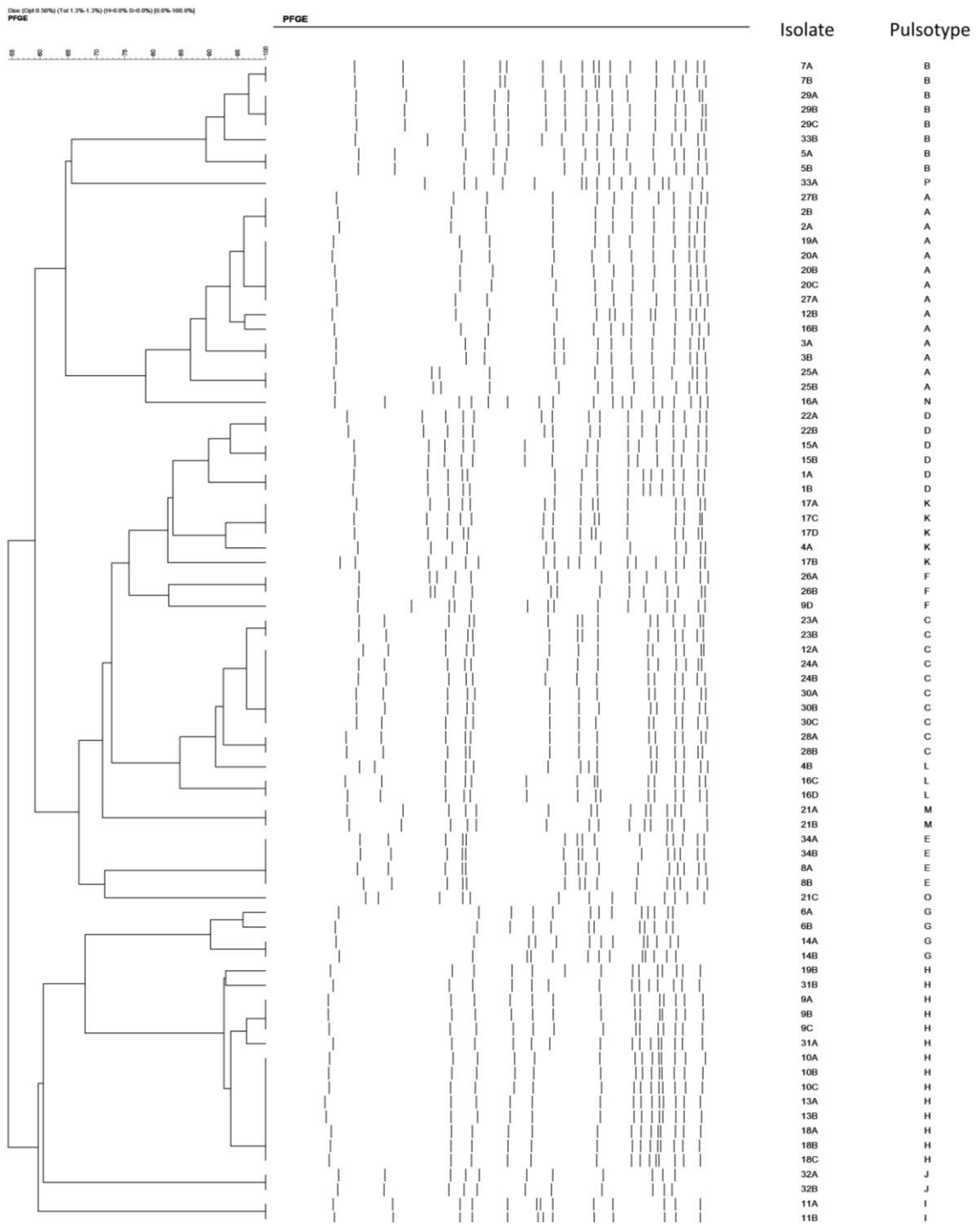


Figure 2.2: Dendrogram representing the relationships of *S. aureus* isolates harvested from patients experiencing recurrent *S. aureus* infection. Letters are used to distinguish between different pusotypes. Numbers denote each patient with the subsequent letter identifying the time of culture (eg. A = first culture, B = second culture ect.).

Changes in antibiotic susceptibility profiles between isolates

In seven patients (patient 4, 5, 6, 16, 18, 21 and 30) the *S. aureus* isolated in subsequent specimens had a different antimicrobial susceptibility profile to the original isolate (Table 2.4). Patients 5, 6, 18 and 30 maintained the same *S. aureus* PFGE pulsotype between cultures whilst patient 4, 16 and 21 had different *S. aureus* PFGE pulsotypes.

Patient symptomatology versus *S. aureus* pulsotype

Patient self-reported symptomatology and the associated *S. aureus* PFGE pulsotype were compared (Table 2.5). Pulsotypes B, D and H were associated with poorer clinical outcome. Pulsotypes E and L were more often associated with asymptomatic condition. However, some PFGE pulsotypes were evenly distributed between symptomatic and asymptomatic associations (Pulsotypes A and C).

Table 2.4: Antimicrobial susceptibility profile changes.

		Antibiotic Susceptible (R/S)								
Patient	Isolate	PEN	OXA	ERY	CLIND	TRI	TETRA	AUG	CEPH	MUP
4	A	S	S	S	S	S	S	S	S	S
	B	R	S	S	S	S	S	S	S	S
5	A	R	S	S	S	S	S	S	S	S
	B	R	S	R	S	S	S	S	S	S
6	A	R	R	S	S	S	S	R	R	S
	B	R	R	R	R	S	S	R	R	S
16	A	R	S	S	S	S	S	S	S	S
	B	R	S	S	S	S	S	S	S	S
	C	R	R	R	R	R	R	R	R	S
	D	R	R	S	S	R	S	R	R	S
18	A	S	S	R	R	S	S	S	S	S
	B	R	S	S	S	S	S	S	S	S
	C	R	S	S	S	S	S	S	S	S
21	A	R	S	S	S	S	S	S	S	S
	B	R	S	S	S	S	S	S	S	S
	C	S	S	R	R	S	S	S	S	S
30	A	R	S	S	S	S	S	S	S	S
	B	S	S	S	S	S	S	S	S	S
	C	S	S	S	S	S	S	S	S	S

Note: Grey areas indicate alterations in antibiotic resistance. R = resistant; S = sensitive; PEN = penicillin; OXA = oxacillin; ERY = erythromycin; CLIND = clindamycin; TRI = trimethoprim; TETRA = tetracycline; AUG = augmentin; CEPH = cephalexin; MUP = mupirocin.

Table 2.5: Correlation of pulsotype and patients clinical condition at the time of culture.

Pulsotype	Specimen number	Specimens	Ratio of S:As* patients
A	13	2A, 2B, 3A, 3B, 12B, 16B, 19A, 20A, 20B, 20C, 25A, 25B, 27A, 27B.	8:6
B	8	5A, 5B, 7A, 7B, 29A, 29B, 29C, 33B.	8:0
C	9	12A, 23A, 23B, 24A, 24B, 28A, 28B, 30A, 30B, 30C.	5:5
D	6	1A, 1B, 15A, 15B, 22A, 22B.	6:0
E	4	8A, 8B, 34A, 34B.	1:3
F	2	9D, 26A, 26B.	2:1
G	4	6A, 6B, 14A, 14B.	2:2
H	14	9A, 9B, 9C, 10A, 10B, 10C, 13A, 13B, 18A, 18B, 18C, 19B, 31A, 31B.	13:1
I	2	11A, 11B.	1:1
J	2	32A, 32B.	2:0
K	4	4A, 17A, 17B 17C, 17D.	4:1
L	2	4B, 16C, 16D.	0:3
M	2	21A, 21B.	2:0
N	1	16A.	1:0
O	1	21C.	1:0
P	1	33A	1:0

*Note: S = symptomatic, As = Asymptomatic

2.3.9 Discussion

This study demonstrates the majority of CRS patients with ongoing infection post-surgery have persistence of the same *S. aureus* strain in their sinuses, despite use of culture-directed antimicrobials. Strain persistence may be due to the production of *S. aureus* biofilms, which are less susceptible to antimicrobial therapy compared to planktonic forms of the bacteria⁴⁸². Intracellular *S. aureus* presence may also contribute to the persistence of a particular *S. aureus* strain^{212, 217}.

Thirteen patients had *S. aureus* detected peri-operatively in biofilms harvested from mucosal specimens. Eleven of the 13 patients cultured *S. aureus* on subsequent microbiological evaluation, with nine and six patients harbouring the same strain post-operatively at <6 months and >6 months respectively. Furthermore, *S. aureus* isolated from peri-operative swabs of nine patients were found to be the same strain as the biofilm isolate. These findings support the popular belief that bacteria in biofilm form may provide a nidus for active planktonic reinfection of sinus cavities^{483, 484}. To strengthen such conclusions, it would be of benefit to investigate further the strain pattern of *S. aureus* isolated from CRS patients over a longer period of time. Additionally, isolation of *S. aureus* from intracellular origin would allow us to compare long term post-operative *S. aureus* infections to this potential nidus.

On examining the influence of oral and topical antibiotics on the *S. aureus* strain, we did not find a correlation between the type and frequency of antibiotic use and the persistence of a *S. aureus* strain. However, given the small study size and the obvious difficulty with assessing patient compliance and prescriptions prescribed by other health care professionals (e.g primary care and emergency department physicians) this finding needs to be interpreted with caution. Antibiotics use did appear to influence the development of

antimicrobial resistance in this study. In seven patients the *S. aureus* isolated over the course of antimicrobial therapy had an altered antibiotic susceptibility profile. In four cases the *S. aureus* PFGE pulsotype did not change, indicating the strains had acquired antibiotic resistant determinants. In the remaining three cases a change in PFGE pulsotype was identified, suggesting the patients had acquired resistant strains from an external source or had preferentially selected a resistant strain during treatment.

When correlating a patient's condition, in terms of the patient's symptoms and the *S. aureus* strain cultured it appears that some *S. aureus* strains are more associated with a symptomatic state than others. This association could be explained by the production of different virulence factors by certain strains. However, the inflammatory response and subsequent symptom development is complex as evidenced by some strains isolated from symptomatic and asymptomatic patients. Patient associated factors, including local immune function, and individual susceptibility to *S. aureus* infection would also influence symptomatic development and progression. In addition one cannot discount the influence of *S. aureus* sinonasal bacterial load which was not quantified in this study. This finding also supports previous research that has shown *S. aureus* can colonize the nasal cavities of individuals without inciting disease¹⁰³ and that the mere presence of this bacteria is not a prognostic indicator of disease evolution in CRS patients.

Our study was not without limitations, including the patient sample size and the small number of known patients found to be negative for *S. aureus* biofilm. Furthermore, determination of patient symptomatology was undertaken retrospectively, and was determined by patients self-reporting as symptomatic or asymptomatic at the time of culture. Use of a more rigorous method in future studies including the SNOT-22 questionnaire⁴⁸⁵ or VAS score⁴, as well as objective endoscopic scoring techniques⁴ would

be necessary to strengthen findings. Another limitation of the study was that only CRS patients were recruited in the study, and patients were only swabbed when clinically indicated. The study strategy of swabbing patients only when clinically indicated also likely caused an overrepresentation of symptomatic patients. A further limitation is that the method employed relies on the ability of the bacteria to grow. Intracellular and biofilm bacteria often have a reduced metabolism making isolation by traditional microbiological techniques difficult. Using culture-independent methods such as whole genome sequencing may overcome this problem. Ability to compare an entire genome sequence compared to PFGE pattern comparison would allow for a more complex understanding of the *S. aureus* isolates and the difference between them. Therefore when conducting future more rigorous studies, such a culture-independent technique would be preferable.

2.3.10 Conclusion

In summary, the majority of CRS patients experiencing persistent or recurrent *S. aureus* infection had the same *S. aureus* strain persisting in their sinuses despite culture-directed antibiotics. Furthermore we were able to show *S. aureus* biofilm is frequently present when *S. aureus* is persistently cultured from the sinonasal region. This is suggestive of biofilm providing a niche for future reinfection. Our study also suggests certain *S. aureus* strains may be more detrimental in CRS than others, and some may even act as innocuous commensals. Further research on a larger patient cohort including control subjects is required to strengthen the findings of this pilot investigation.

2.3.11 Acknowledgments

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3 Bacteriophage reduces biofilm of *Staphylococcus aureus* ex vivo isolates from chronic rhinosinusitis patients

3.1 Statement of Authorship

Statement of Authorship

Title of Paper	Bacteriophage reduces biofilm of <i>Staphylococcus aureus</i> ex vivo isolates from chronic rhinosinusitis patients
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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Amanda J Drilling		
Contribution to the Paper	Involved in experimental design, performed all biofilm work and bacteriophage susceptibility work, interpreted the data and wrote the manuscript.		
Signature		Date	29/08/2014

Name of Co-Author	Sandra Morales		
Contribution to the Paper	Involved in experimental design, bacteriophage production, bacteriophage insensitive mutant experiments, data interpretation and manuscript production.		
Signature		Date	9/4/2014

Name of Co-Author	Camille Jardeleza		
Contribution to the Paper	Aided in biofilm experiment design and data interpretation.		
Signature		Date	10/09/2014

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Aided in experimental design, data interpretation and manuscript preparation.		
Signature		Date	29/08/2014

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Amanda J Drilling		
Contribution to the Paper			
Signature		Date	

Name of Co-Author	Peter Speck		
Contribution to the Paper	Aided in experimental design and manuscript editing.		
Signature		Date	08/09/2014

Name of Co-Author	Peter-John Wormald		
Contribution to the Paper	Aided in experimental design, data analysis and manuscript editing.		
Signature		Date	08/09/2014

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

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4 Safety and efficacy of topical bacteriophage and EDTA treatment of *Staphylococcus aureus* infection in a sheep model of sinusitis

4.1 Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Amanda J Drilling		
Contribution to the Paper	Involved in experimental design and wrote animal ethics application. Involved in all animal operations and experiments, tissue preparation and analysis, data interpretation and statistics, prepared the manuscript.		
Signature		Date	26/08/2014

Name of Co-Author	Sandra Morales		
Contribution to the Paper	Aided in experimental design, bacteriophage preparation, animal tissue preparation and analysis, preparation of manuscript.		
Signature		Date	9/4/2014

Name of Co-Author	Sam Boase		
Contribution to the Paper	Aided in experimental design, animal operations, tissue harvest and manuscript preparation.		
Signature		Date	30/08/2014

Name of Co-Author	Josh Jervis-Bardy		
Contribution to the Paper	Aided in animal operations and tissue harvest.		
Signature		Date	27/08/2014

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Amanda J Drilling		
Contribution to the Paper			
Signature		Date	

Name of Co-Author	Craig James		
Contribution to the Paper	Aided in the analysis and grading of tissue histology.		
Signature		Date	01/09/2014

Name of Co-Author	Camille Jardeleza		
Contribution to the Paper	Aided in experimental design, animal experiments and tissue harvest.		
Signature		Date	10/09/2014

Name of Co-Author	Neil Tan		
Contribution to the Paper	Aided in tissue harvest and preparation.		
Signature		Date	08/09/2014

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Author Contributions

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Name of Principal Author (Candidate)	Amanda J Drilling		
Contribution to the Paper			
Signature		Date	

Name of Co-Author	Edward Cleland		
Contribution to the Paper	Aided in organ harvest.		
Signature		Date	01/09/2014

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Aided in experimental design, data analysis and manuscript editing.		
Signature		Date	01/09/2014

Name of Co-Author	Peter Speck		
Contribution to the Paper	Aided in experimental design and manuscript editing.		
Signature		Date	08/09/2014

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Name of Principal Author (Candidate)	Amanda J Drilling		
Contribution to the Paper			
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Name of Co-Author	Peter-John Wormald		
Contribution to the Paper	Aided in experimental design, experiment implementation, data analysis and manuscript editing.		
Signature		Date	08/09/2014

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Safety and efficacy of topical bacteriophage and ethylenediaminetetraacetic acid treatment of *Staphylococcus aureus* infection in a sheep model of sinusitis

Amanda Drilling, BBtech (Hons)¹, Sandra Morales, MSc², Samuel Boase, PhD¹, Joshua Jervis-Bardy, PhD¹, Craig James, FRCPA, MD³, Camille Jardeleza, MD¹, Neil Cheng-Wen Tan, MBBS, MRCS¹, Edward Cleland, MD¹, Peter Speck, PhD⁴, Sarah Vreugde, MD¹ and Peter-John Wormald, MD¹

Background: Treatment of sinonasal bacterial biofilms continues to be a challenge in modern rhinology. This study's objective was to assess the safety and efficacy of topically applied Cocktail of *S. aureus* specific phage (CTSA) alone and in combination with ethylenediaminetetraacetic acid (EDTA) for treatment of *Staphylococcus aureus* biofilms in vivo.

Methods: Using a sheep model of sinusitis, frontal sinuses (n = 6 per treatment) were flushed once daily with a CTSA (2 × 10⁶ plaque forming units [PFU]/mL), with or without EDTA (0.075 mg/mL), and compared to a control flush containing saline and heat-inactivated CTSA. Safety was assessed using histology and scanning electron microscopy (SEM) after treatment for 3 days. Efficacy was assessed by quantifying the generation of *S. aureus* biofilms in the frontal sinuses after 5 days of treatment. Biofilm mass was compared between treatment groups and controls using LIVE/DEAD BacLight staining and confocal scanning laser microscopy to visualize the tissue sections. COMSTAT2 software was used to compute the biofilm mass present on tissue sections.

Results: Tissue morphology was conserved, with no significant signs of inflammation, when comparing control and

test treatments. Furthermore, SEM analysis indicated test treatments were not toxic or damaging to mucosal cilia. COMSTAT2 quantification of biofilm showed a significant reduction in biofilm levels when comparing the control with CTSA (p = 0.0043), EDTA (p = 0.0095), and CTSA-EDTA (p = 0.0022) treatments.

Conclusion: Results indicate that CTSA and EDTA are safe and efficacious for short-term topical application against *S. aureus* infection in a sheep sinusitis model, and have the potential to be translated to a clinical setting. © 2014 ARS-AAOA, LLC.

Key Words: biofilm; bacteriophage; EDTA; ovine; antimicrobial therapy

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Chronic rhinosinusitis (CRS) is a debilitating condition characterized by inflammation of the nose and

¹Department of Surgery-Otolaryngology Head and Neck Surgery, University of Adelaide, Adelaide, SA, Australia; ²Special Phage Services Pty Ltd, Brookvale, New South Wales, Australia; ³Adelaide Pathology Partners, Adelaide, South Australia, Australia; ⁴School of Biological Sciences, Flinders University, Bedford Park, South Australia, Australia

Correspondence to: Peter-John Wormald, MD, Department of Otorhinolaryngology Head and Neck Surgery, The Queen Elizabeth Hospital, 28 Woodville Rd, Woodville South, South Australia 5011, Australia; e-mail: peterj.wormald@adelaide.edu.au

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paranasal sinuses.¹ Bacterial infection is thought to play a role in the pathogenesis of CRS, justifying the recurrent use of local and/or systemic antibiotics and reflected by the fact that sinusitis is the 5th leading diagnosis for which antibiotics are prescribed.² *Staphylococcus aureus* infection is thought to exacerbate the CRS condition³ and enhance recalcitrance, particularly when in biofilm form.⁴ When encased in a biofilm, bacteria can be up to 1000-fold more resistant to antibiotic treatments^{3,5} compared to free-floating forms, and consequently antibiotic therapy

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4.3 Article

Safety and efficacy of topical bacteriophage and EDTA treatment of *Staphylococcus aureus* infection in a sheep model of sinusitis

Amanda Drilling#, BBtech (Hons), Sandra Morales^, MSc, Samuel Boase#, PhD, Joshua Jervis-Bardy#, PhD, Craig James*, FRCPA, MD, Camille Jardeleza#, MD, Neil Cheng-Wen Tan# MBBS MRCS, Edward Cleland#, MD, Peter Speck*, PhD, Sarah Vreugde#, MD, Peter-John Wormald#, MD

#Department of Surgery-Otolaryngology Head and Neck Surgery, The Queen Elizabeth Hospital, Woodville South, South Australia, Australia and the University of Adelaide, Adelaide, South Australia, Australia

*Adelaide Pathology Partners, Adelaide, South Australia, Australia.

α School of Biological Sciences, Flinders University, Bedford Park, South Australia, Australia.

^ Special Phage Services Pty Ltd, New South Wales, Australia

Correspondence to Professor Peter-John Wormald, Department of Otorhinolaryngology Head and Neck Surgery, The Queen Elizabeth Hospital, 28 Woodville Rd, Woodville South, South Australia 5011, Australia. E-mail: peterj.wormald@adelaide.edu.au

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4.3.1 Abstract

Introduction: Treatment of sinonasal bacterial biofilms continues to be a challenge in modern rhinology. This study's objective was to assess the safety and efficacy of topically applied Cocktail of *S. aureus* specific phage (CTSA) alone and in combination with ethylenediaminetetraacetic acid (EDTA) for treatment of *Staphylococcus aureus* biofilms *in vivo*.

Methods: Using a sheep model of sinusitis, frontal sinuses (n=6 per treatment) were flushed once daily with CTSA (2×10^6 pfu/mL), with or without EDTA (0.075 mg/mL), and compared to a control flush containing saline and heat-inactivated bacteriophage. Safety was assessed using histology and scanning electron microscopy (SEM) following treatment for 3 days. Efficacy was assessed by quantifying the generation of *S. aureus* biofilms in the frontal sinuses following 5 days of treatment. Biofilm mass was compared between treatment groups and controls using LIVE/DEAD BacLight staining and confocal scanning laser microscopy to visualize the tissue sections. The software COMSTAT2 allowed computation of the biofilm mass present on tissue sections.

Results: Tissue morphology was conserved, with no significant signs of inflammation, when comparing control and test treatments. Furthermore, SEM analysis indicated test treatments were not toxic or damaging to mucosal cilia. COMSTAT2 quantification of biofilm showed a significant reduction in biofilm levels when comparing the control with CTSA ($p = 0.0043$), EDTA ($p = 0.0095$) and CTSA-EDTA ($p = 0.0022$) treatments.

Conclusions: Results indicate CTSA and EDTA to be safe and efficacious for short term topical application against *S. aureus* infection in a sheep sinusitis model, and have potential to be translated to a clinical setting.

4.3.2 Introduction

Chronic rhinosinusitis (CRS) is a debilitating condition characterised by inflammation of the nose and paranasal sinuses⁵²⁶. Bacterial infection is thought to play a role in the pathogenesis of CRS, justifying the recurrent use of local and/or systemic antibiotics reflected by the fact that sinusitis is the 5th leading diagnosis for which antibiotics are prescribed¹⁷⁴. *Staphylococcus aureus* infection is thought to exacerbate the CRS condition²⁶⁶ and enhance recalcitrance, particularly when in biofilm form²¹¹. When encased in a biofilm, bacteria can be up to 1000-fold more resistant to antibiotic treatments^{266, 527} compared to free-floating forms and consequently antibiotic therapy against infection can become challenging and is often ineffective. It is therefore important, particularly in CRS patients harbouring *S. aureus* biofilms, to find novel antimicrobial agents to treat bacterial infections recalcitrant to antibiotics.

A potential anti-biofilm therapy is to employ bacteriophages (phages), bacterial viruses that infect and lyse bacterial cells. Importantly, phages have the ability to diffuse through the biofilm matrix⁴⁵⁰ facilitating phage access to biofilm bound cells, which are subsequently infected and destroyed by the phage^{450, 451, 455}. Phages have been shown in numerous studies to be effective against biofilms^{451, 453-456, 458-460}, including biofilms of *S. aureus*³⁷². Previous study has shown a Cocktail of *S. aureus* specific phage (CTSA) is effective against biofilms of *S. aureus* clinical isolates obtained from CRS patients *in vitro*⁵²⁸. Clinical administration of phage has also been shown to be safe when applied orally^{444, 445}, as well as topically to the ear⁴⁴⁹, external wounds/venous stasis⁴⁴⁷ and leg ulcers⁴¹⁸.

Another potential therapeutic targeting biofilms is ethylenediaminetetraacetic acid (EDTA), a metal chelator, shown not to induce adverse effects when used intravenously

infused at a concentration of 6mg/mL EDTA⁵²⁹. Research has indicated EDTA to be effective at reducing bacterial biofilm levels^{351, 530-532}. Such reduction is thought to be instigated by EDTA's metal chelation properties, as high levels of iron⁵³³ and magnesium⁵³⁴ are thought to be essential for biofilm formation.

Whilst both agents have been found to be effective *in vitro* it is important to assess their efficacy *in vivo* as biofilm formation in a complex niche can often occur differently compared to a biofilm grown *in vitro*^{364, 365}. Furthermore, the safety and efficacy of phage preparations must be well established prior to their use in clinical settings. Hence, this study topically applied CT-SA with and without EDTA to the frontal sinuses of sheep to assess the safety and *in vivo* capability of these treatments to reduce *S. aureus* infection and biofilm formation.

4.3.3 Materials and Methods

Bacterial and treatment stocks

S. aureus strain ATCC 25923 was used to inoculate and generate infection in frontal sinuses of sheep. A *S. aureus*-specific bacteriophage cocktail (CTSA) was provided by Special Phage Services Pty Ltd (BrookVale Sydney, Australia), at a concentration of 2×10^8 PFU/mL, suspended in SM buffer⁴⁹⁹ and stored at 4°C. Heat-inactivated CT-SA (inactivated at 121°C for 15min) was from the same source. EDTA stocks were stored at room temperature at a concentration 7.5mg/mL (ASL Pharmacy, Camarillo, CA).

Sheep treatment groups

Approval for this project was granted both by the University of Adelaide and the Institute of Medical and Veterinary Science Animal Ethics Committees. A total of 27 Merino cross wethers (1yr of age) were used, 12 employed in a treatment safety arm and 15 in a treatment efficacy arm. Sheep were divided into 4 treatment groups; (1) no treatment control (NT, 2×10^6 PFU/mL of heat inactivated phage in 0.9% saline), (2) EDTA (0.075mg/mL EDTA in 0.9% saline), (3) CT-SA (2×10^6 PFU/mL of active CT-SA phage in 0.9% saline), and (4) CT-SA and EDTA in combination (2×10^6 PFU/mL of active phage and 0.075mg/mL EDTA in 0.9% saline). For both the safety and efficacy arms, three sheep were included in each treatment arm. Trephinations to both the right and left sinuses were performed on each sheep and each sinus was treated as an independent sample. Therefore each treated group contained an $n = 6$ sinuses. Three sheep in the efficacy arm were euthanized early due to poor condition. Two were euthanized during biofilm development stage prior to treatment application. The third was observed to have very poor condition prior to treatment application, including blood in the urine thought to be unrelated to the project. This sheep was treated for a period of 2 days with EDTA, however as its condition did not improve, it was subsequently euthanized.

Safety arm treatment protocol

To achieve access to the frontal sinuses, mini-trephinations were performed as described in Ha et. al. 2007. Briefly, sheep were anaesthetised by administering phenobarbitone (19 mg/kg) to the external jugular vein (induction). Anaesthesia was maintained with 1.5-2% inhalational halothane. Prior to trephination, nasal cavities were decongested using cophenylcaine forte nasal spray (ENT Technologies, Victoria, Australia). Trephines were placed 1 cm lateral to the sagittal plane between the bony orbits. Flourescein (1%, Phebra, Lane Cove) instilled into the frontal sinus was visualised inside the nasal passage using an endoscope to ensure correct placement of the trephine. Sheep were allowed to recover for one day.

Each of the four treatment groups (NT, EDTA, CTSA and CTSA-EDTA) consisted of 3 sheep, treated once a day for 3 days with 100mL of the associated treatment into both the left and right frontal sinuses. Treatment period was based on previous treatment safety studies^{522, 535} and was approved by the animal ethics committee to be sufficient time to assess safety. Sheep were then euthanized by administering pentobarbitone (<100mg/kg) to the external jugular vein and the sinuses extracted for analysis of the tissue.

Efficacy arm *S. aureus* inoculation and treatment protocol

In the efficacy arm, sheep had their frontal sinus ostium blocked, trephines placed into the frontal sinuses and sinus inoculated with *S. aureus* as described previously⁵³⁶. After 7 days the gauze was removed using endoscopic guidance, and the sheep were treated. The natural history of the model indicates *S. aureus* infection is not resolved within 2 weeks of gauze removal. Each treatment group (NT, EDTA, CTSA and CTSA-EDTA) consisted of 3 sheep, was treated once a day for 5 days with 100mL of the associated treatment flushed

through the trephines into both the left and right frontal sinuses. Treatment time was based on previous studies assessing efficacy of therapies against biofilm^{367, 522}. Sheep were then euthanized as above and extracted sinus tissue was histologically analysed and assessed for biofilm presence.

Safety analysis of treatments

Treatment safety was assessed by visualising tissue using light and scanning electron microscopy (SEM). To visualise using SEM, a previously defined method was employed⁵³⁶. To visualise using light microscopy, a 1 x 1 cm section of tissue was placed in 10% formalin (Sigma-Aldrich, Castle Hill) and sent to an external institute (Adelaide Pathology, Adelaide) for processing. Tissue sections were stained with hematoxylin and eosin⁵³⁷, and viewed using an Eclipse 90i light microscope (Nikon Instruments Inc, NY, USA). Three random images were taken of each sinus and from each image, two sections of the epithelial layer (section length of 0.1mm) and two sections of sub-epithelial layer were selected (area 0.1mm²). Within each epithelial section the number of inflammatory cells and goblet cells were counted, and within each sub-epithelial section the number of inflammatory cells were counted, identified in collaboration with a pathologist.

Inflammatory cells counts were divided into two categories; acute (neutrophils) and chronic inflammatory cells (lymphocytes, plasma cells, eosinophils).

BacLight staining and analysis of biofilm present on sheep tissue

To image biofilm present on sinus mucosa a previously defined protocol was employed⁵²². Briefly tissue sections were stained using a LIVE/DEAD BacLight stain (Life Technologies, Mulgrave, Victoria, Australia). Tissue was imaged using a Leica TCS SP5 spectral confocal scanning laser microscope (Leica Microsystems, Wetzlar, Germany). Two 1 x 1 cm sections of tissue were stained and two images (Image properties: Line

average 4, 512 x 512 px, z-stack 100 steps, volume $4.2 \times 10^7 \mu\text{m}^3$) were taken on each pieces of tissue in the area of highest biofilm presence. The software COMSTAT2 was employed to measure the level of biofilm present on the tissue as previously described⁵²². Biofilm presence on the left and right frontal sinus mucosa was analysed for all sheep in the efficacy arm. Biofilm presence was also assessed for 3 NT treated sheep from the safety arm as an un-infected biofilm negative control denoted UI.

Quantification of phage in sheep sinuses, organs and faeces

For all faecal and organ samples, phage were titrated in triplicate using a published methodology⁵⁰². Faecal samples were harvested from NT and EDTA treated sheep the day prior to treatment and the day of euthanasia. For CTSA and CTSA-EDTA treated sheep, faecal matter was harvested the day prior to treatment and every morning (prior to phage treatment) during topical therapy. To assess sheep faecal matter for phage concentration, 1g of faeces was suspended in 10mL of SM buffer, centrifuged at 3214 xg for 10 minutes (Eppendorf 5810 centrifuge, North Ryde, NSW, Australia), filtered (0.22 μm) and titrated for phage concentration.

Liver, spleen, kidney and brain were harvested from four CTSA-treated sheep; 2 from the safety arm and 2 from the efficacy arm. For the CTSA treated safety sheep, 1g of each organ was ground in 10mL of SM buffer using a Qiagen TissueRuptor (Qiagen, Germany). Samples were then centrifuged at 3214 xg, (spleen: 20 mins, liver, brain, kidney: 1hr), filtered using a 0.22 μm filter and titrated for phage. For the CTSA-treated efficacy sheep 2g of each organ was ground in 20 mL SM buffer using a Qiagen TissueRuptor. Samples were then centrifuged at 3214 xg, (spleen: 20 mins, liver, brain, kidney: 45mins), filtered using a 0.22 μm filter and titrated for phage.

For two of the efficacy sheep treated with CTSA-EDTA, fresh sinus tissue was assessed for phage concentration. Again 2g of each sinus was ground in 20mL SM buffer using a Qiagen TissueRuptor. Samples were then centrifuged at 3214 xg for 30 minutes, filtered using a 0.22µm filter and titrated for phage.

Phage enrichment:

A select number of filtered samples were enriched for phage (table 1). Briefly *S. aureus* ATCC 25923 was grown overnight at 37°C with shaking, and diluted 1:100 with PBS. Samples (volumes shown in table 1) were incubated overnight with 100µl of nutrient broth and 10µl of the diluted overnight *S. aureus* culture. Enriched samples were filtered using a 0.22 µm filter and titrated for phage.

Statistics:

To compare the levels of inflammatory cell infiltration, number of goblet cells, and mucosal biofilm levels between the various treatments, a Kruskal-Wallis one-way analysis of variance was employed with a Wilcoxon posthoc test using the software SAS Version 9.3 (SAS Institute Inc., Cary, NC, USA).

4.3.4 Results

Safety analysis of topical application of EDTA, CT-SA and CTSA-EDTA

Analysis of H&E stained sinus mucosal sections

To assess the safety of the test treatments (EDTA, CT-SA and CTSA-EDTA), H&E stained mucosal tissue was grossly assessed for tissue damage and inflammation (Figure 4.1) in collaboration with a pathologist. Almost all sinus samples (excluding two sinus sections) obtained from NT, EDTA, CT-SA and CTSA-EDTA treated sheep in the safety arm showed varying degrees of inflammation both chronic (lymphocytes and plasma cells) and acute (neutrophils) in nature in the epithelial and sub-epithelial sections. No squamous metaplasia of epithelium was identified in any of the samples.

One of the sinuses treated with CTSA showed some damage. Approximately 70% of the tissue section was observed to be hyperplastic respiratory epithelium with extensive loss of cilia, reactive atypia and associated intraepithelial neutrophils. The subepithelial layer in this tissue section was noted to be oedematous with high levels of red blood cells present suggesting haemorrhage. As all other CTSA treated sections did not show such an appearance the damage observed may have been due to sampling of an area adjacent to the site of trephination.

H&E stained sections of one of the sinuses treated with EDTA had a similar hyperplastic appearance; however the damage was not as extensive as the aforementioned sinus with approximately 40% of the tissue section showing damage. Again the sub-epithelial layer of this tissue section was quite oedematous. As all other EDTA treated tissue sections showed no such damage it is thought the tissue may have been sampled close to the site of trephination, or may have been a localised sinus infection.

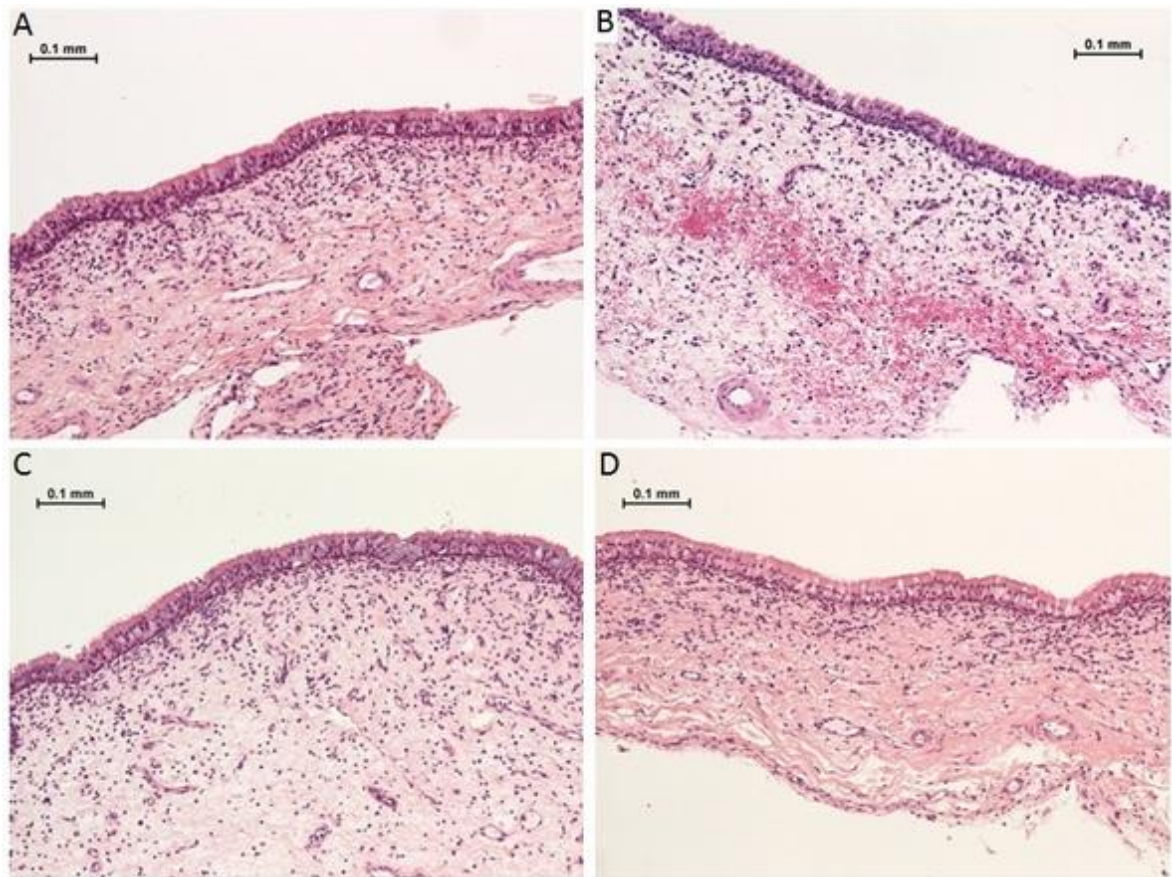


Figure 4.1 Representative images of the histology of the sinus mucosa harvested from sheep in the safety arm. Histology images were taken at 10X magnification. Similar mucosal architecture is noted in the (A) NT, (B) CTSA, (C) EDTA and (D) CTSA-EDTA treated sheep showing a pseudo-stratified columnar epithelial layer intersected with goblet cells. The images highlight the test treatments appear safe to apply topically to sinus mucosa.

Enumeration of acute and chronic inflammatory cell infiltrates of H&E stained tissue

Due to the variation in acute and chronic inflammation observed between different sinuses treated with the same solution, acute and chronic cells present in the epithelial as well as sub-epithelial regions were enumerated. No difference in the number of goblet cells or acute cellular infiltrates was observed between the treatment groups. There was also no difference noted in the number of chronic inflammatory cells in the epithelial layers.

There was however a significantly lower level of chronic inflammatory cells seen in the

sub-epithelial layer in the EDTA group compared to the NT ($p = 0.03$) and CTSA-EDTA ($p = 0.03$) group (Figure 4.2).

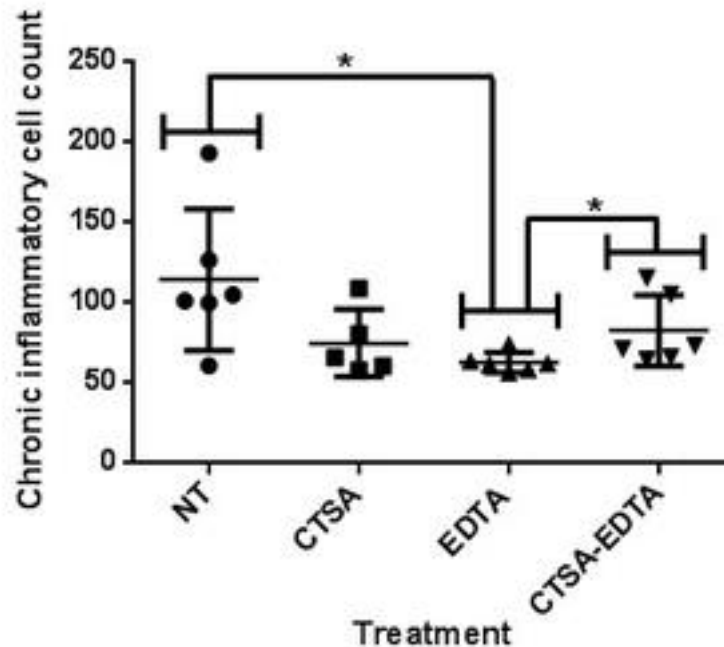


Figure 4.2: Comparison of the chronic inflammatory cell infiltrate in the sub-epithelial layer of sheep in the safety arm. Chronic inflammatory cells present in 0.1mm^2 sections in images taken at 20X magnification of the sub-epithelial layer were counted and compared between treatments. Significantly lower inflammatory cell counts were observed in the EDTA treated sheep compared to the NT and CTSA-EDTA treated sheep. * = $p < 0.05$.

Assessment of cilia using scanning electron micrographs

SEM was employed to assess the presence and integrity of the cilia present on the sinonasal mucosal surface. Cilia were observed to be present on all mucosal samples harvested from all treatment groups (Figure 4.3). The majority of mucosal specimens were observed to have sheets of cilia intersected with occasional small patches of cilia denudation (excluding one NT-treated sheep mucosal sample observed to be devoid of cilia thought to be due to errors in sample processing).

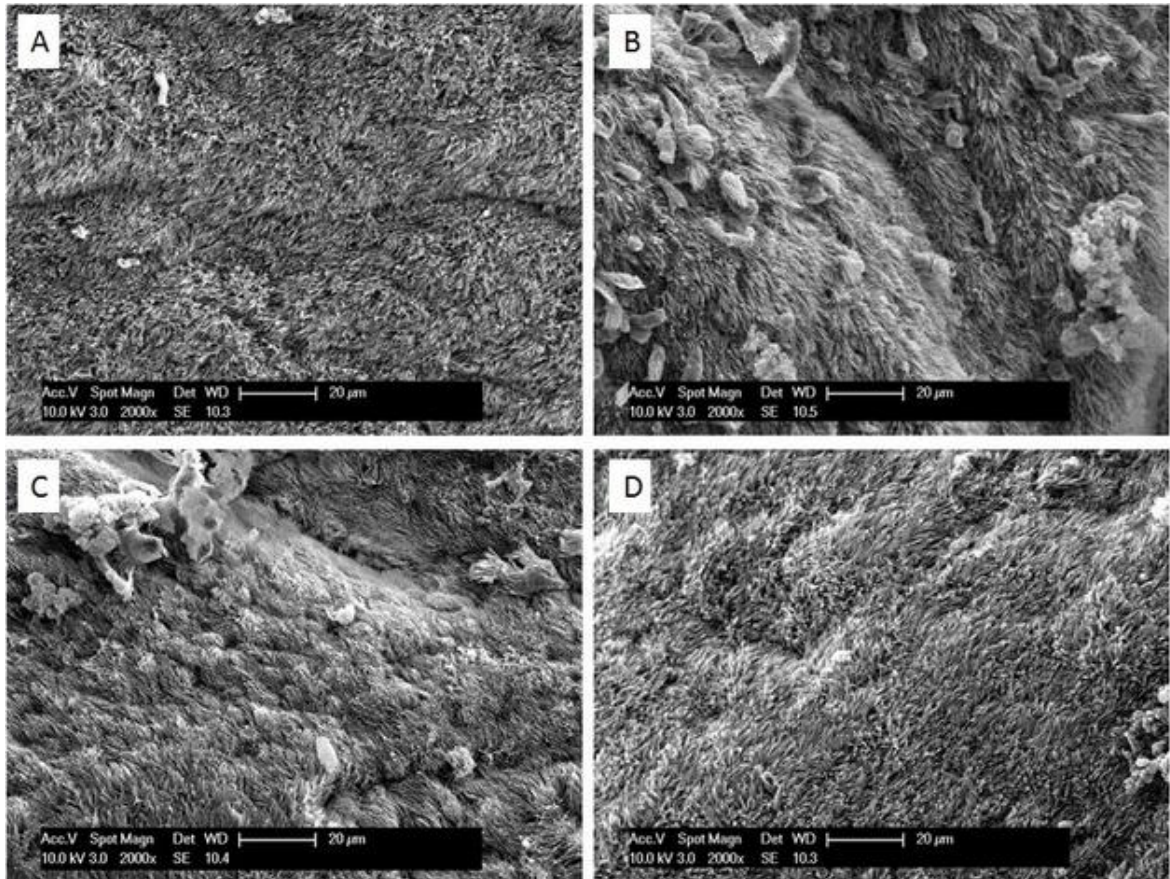


Figure 4.3: Representative images of scanning electron micrographs of sinus mucosa

harvested from sheep in the safety arm. Micrographs allowed comparison of the presence and morphology of cilia present on the sheep sinus mucosa of (A) NT, (B) CTSA, (C) EDTA and (D) CTSA-EDTA. Similar morphology and presence was generally seen when comparing the treatments, indicating the test treatments did not severely impact cilia morphology and did not cause cilia denudation.

Histological analysis of sinus mucosa harvested from sheep in efficacy arm

Histology of the mucosa harvested from sheep in the efficacy arm was compared between treatments for levels of goblet cells and acute and chronic inflammatory cells (Figure 4.4). There was no significant difference between the levels of goblet cells, acute inflammatory cells in the epithelial layer or chronic inflammatory cells in either the epithelial or sub-epithelial layers. There was however, a significant decrease in the levels of sub-epithelial

acute inflammatory cells in the EDTA ($p = 0.03$) and CTSA-EDTA ($p = 0.01$) when compared to the NT group (Figure 4.5).

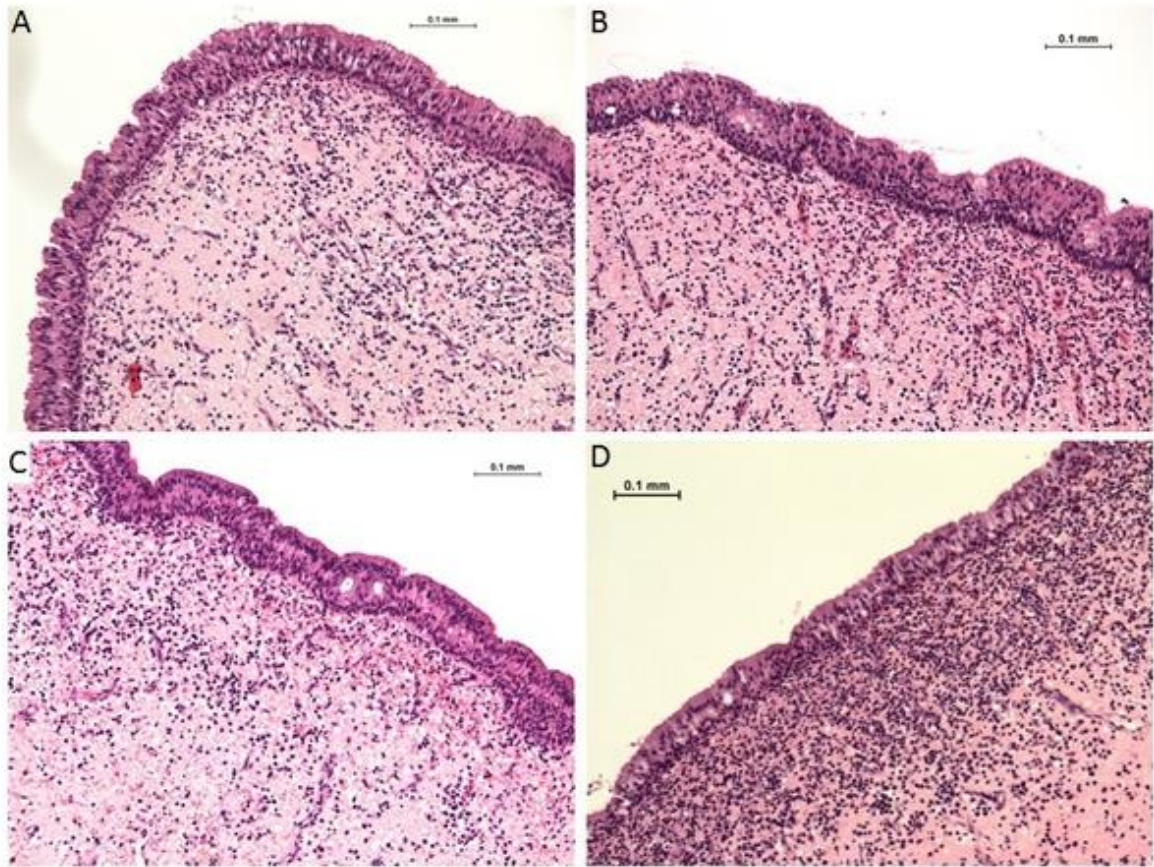


Figure 4.4: Representative images of the histology of the sinus mucosa harvested from sheep in the efficacy arm. Histology images were taken at 10X magnification. Histology of the sinus mucosa of the treatments (A) NT, (B) CTSA, (C) EDTA and (D) CTSA-EDTA treated sheep showed again similar architecture. There appeared to not be much difference in the levels of inflammation or goblet cells when compared the test treatments to the NT control.

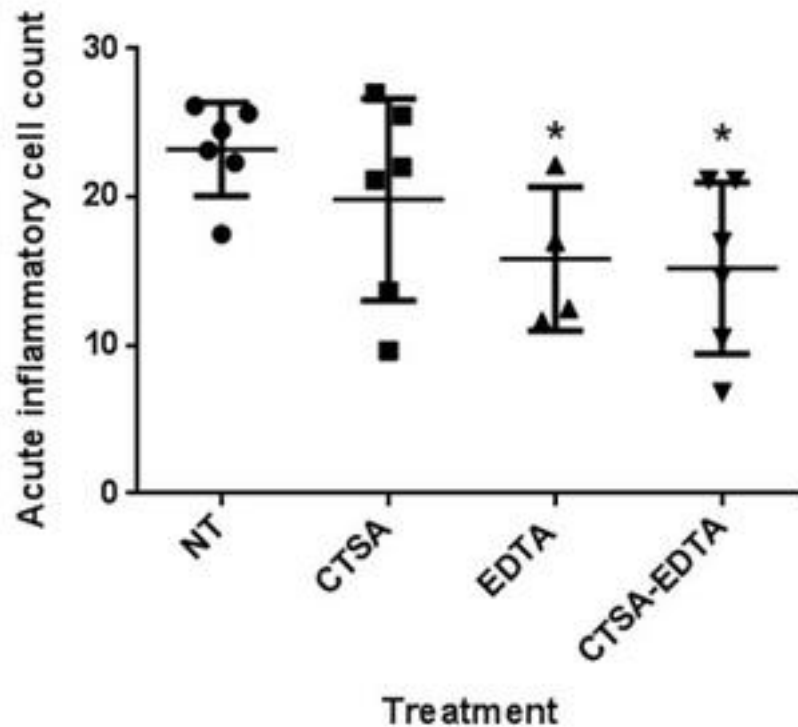


Figure 4.5: Comparison of the acute inflammatory cell infiltrate in the sub-epithelial layer of sheep in the efficacy arm. Cell infiltrates were counted from 0.1mm² tissue sections imaged at 20X magnification. Similar levels of acute inflammatory cells were observed in the sub-epithelial layer of the CTSA treated sheep compared to NT treatment. Significantly lower levels were observed in the EDTA and CTSA-EDTA treated sheep sub-epithelial layer compared to the NT treated sheep. * = p < 0.05.

Biofilm biomass reduction induced by CTSA, EDTA and CTSA-EDTA treatment

Mucosal biofilms were imaged using Live/Dead BacLight stain and CSLM and biomass levels computed using the software COMSTAT2 (Figure 4.6). Statistical analysis indicated that the single EDTA ($0.149 \pm 0.0484 \mu\text{m}^3/\mu\text{m}^2$) and CTSA treatment ($0.102 \pm 0.0197 \mu\text{m}^3/\mu\text{m}^2$) significantly reduced biofilm mass levels in comparison to the NT control ($0.608 \pm 0.174 \mu\text{m}^3/\mu\text{m}^2$). Similarly CTSA-EDTA treatment ($0.138 \pm 0.0405 \mu\text{m}^3/\mu\text{m}^2$) also significantly reduced biofilm mass levels when compared to NT control

levels. No significant differences were noted between the different treatment types and CTSA and EDTA did not act synergistically to remove biofilm. The Live/Dead BacLight stain caused background staining of sheep mucosal DNA, hence uninfected sheep mucosa was stained and used as a no biofilm control. CTSA, EDTA and CTSA-EDTA treatments all had significantly higher levels of biofilm biomass compared with the no biofilm control ($0.0466 \pm 0.0183 \mu\text{m}^3/\mu\text{m}^2$), so whilst biofilm biomass is reduced, it is not eradicated completely.

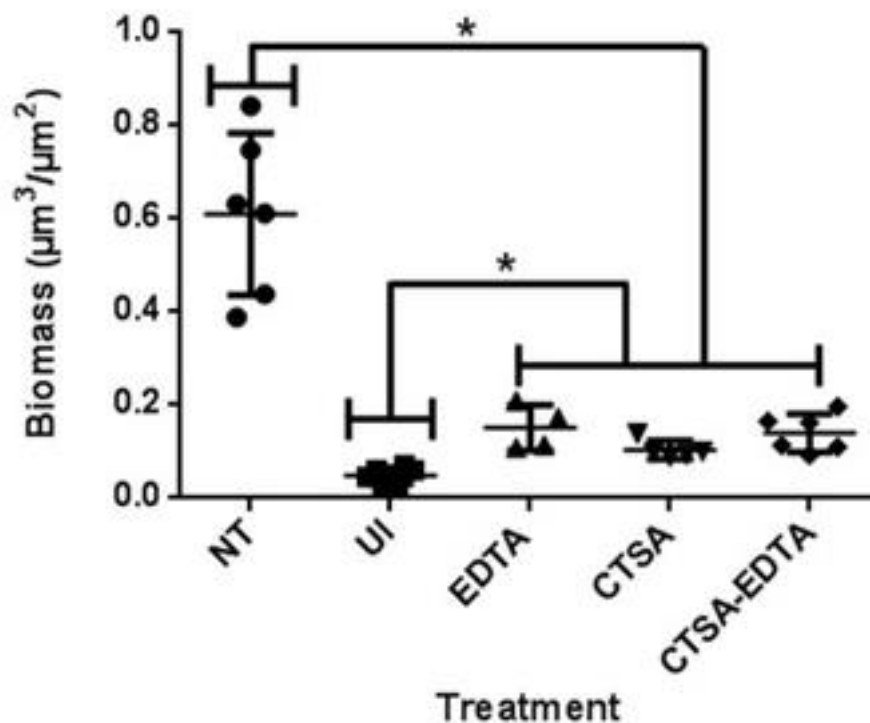


Figure 4.6: COMSTAT2 computation of biofilm mass levels present on sheep sinus mucosa. Significantly higher levels of biofilm mass were observed in the NT treated sheep compared to the test treatments CTSA, EDTA and CTSA-EDTA. Significantly higher levels of biofilm mass were observed in the CTSA, EDTA and CTSA-EDTA compared to the uninfected (UI) sheep. Results highlight CTSA, EDTA and CTSA-EDTA are able to significantly reduce levels of biofilm mass, however levels were still significantly higher than the background control indicating biofilms were not completely removed from the sinuses.

Titration of phage in faecal samples

Faecal matter for all NT and EDTA treatment sheep were free of phage both pre and post treatment (phage count < 100pfu/g of faeces). Faecal matter of the CTSA and CTSA-EDTA treated sheep were assessed prior to treatment, during treatment and post-treatment. In the safety sheep no phage were detected in any of the faecal samples (phage count < 100pfu/g of faeces). Due to no phage being identified in the safety sheep faecal matter, larger volumes were titrated for the efficacy sheep to allow increased detection of phage. Again however no phage were identified in the faecal samples (phage count < 2pfu/g faeces). However, phage enrichments of 2 of the CTSA treated sheep spleen samples and excess faecal samples showed phage were present only in the faecal matter of sheep 12 on days 3 and 4 (Table 4.1).

Table 4.1: Presence or absence of phage plaques following phage enrichment of spleen and faecal samples harvested from two CTSA treated sheep

Sheep number/treatment	Sample	Volume (mL)	Plaques identified (Y/N)
10/CTSA	Spleen	0.9	N
	Faecal D2	0.9	N
	Faecal D3	0.7	N
	Faecal D4	0.9	N
	Faecal D5	0.9	N
12/CTSA	Spleen	0.9	N
	Faecal D2	0.6	N
	Faecal D3	0.7	Y
	Faecal D4	0.9	Y
	Faecal D5	0.9	N

Note: Samples were enriched in duplicate, no results showed variation between the replicates. CTSA = Cocktail of S. aureus specific phage; D = day.

Phage titration in kidney, spleen, liver and brain samples

To determine if CTSA phage crossed the nasal mucosal barrier and dispersed throughout the body following topical sinonasal application, kidney, spleen, liver and brain samples were harvested and phage titre determined. Four CTSA treated sheep (two from the safety arm and two from the efficacy arm) were analysed for the presence of phage in the brain, liver, kidneys and spleen. No phage (<1 pfu/mL) were detected in any of the samples.

Phage titration of sinus mucosa harvested from CTSA-EDTA treated sheep

Owing to the observation that CTSA treated sheep showed no active phage present in kidney, liver, spleen and brain samples, the sinuses of two CTSA-EDTA treated sheep were assessed for phage titre immediately following harvest. Three of the four sinuses were observed to have low levels of phage isolated from sinus tissue (average 3.6 ± 3.4 PFU/0.1g sinus mucosa), the low number likely due to the majority of phage being present in the mucus rather than inside the tissue.

4.3.5 Discussion

Safety and efficacy of topical application of CT-SA alone or in combination with the metal chelator EDTA was assessed in this study using an *in vivo* sheep model of sinusitis. All three test treatments were observed to be both safe for short term application to sinus mucosa and efficacious against *S. aureus* mucosal biofilms. Synergy was not observed when combining the EDTA and CT-SA therapies (CTSA-EDTA), and owing to the high levels of efficacy noted in both the EDTA and CTSA groups, CTSA-EDTA combination therapies do not appear to bring added value. With similar safety and efficacy profiles, both treatments have the ability to be translated clinically.

Focusing on treatment safety this study noted phage to be safe for short term topical application to the sinuses, in accordance with a previous study where phage was found to be safe both in animal models^{420-427, 538} and human clinical settings^{389, 418, 444, 445, 447, 449}. In a clinical setting, safety of phage has been noted consistently over the last 50 years of phage therapy in Eastern Europe³⁸⁹, however many of these studies have the limitation of being uncontrolled. Recently, a number of controlled clinical trials have highlighted the safety of oral^{444, 445} applications, as well as topical application to the ear⁴⁴⁹, external wounds/venous stasis⁴⁴⁷ and leg ulcers⁴¹⁸. One potential side effect of phage treatment is the Jarisch–Herxheimer reaction, or an inflammatory response to the endotoxins or lipoteichoic acids released upon bacterial lysis as seen in antibiotic therapy⁵¹⁸. Such a reaction has yet to be described in the context of phage therapy⁵¹⁸ and in fact previous work has noted in some cases the debris produced from phage mediated bacterial lysis is not pro-inflammatory⁵³⁹.

Results of our study indicated EDTA to be safe for short term topical application to the sinus mucosa, with the maintenance of both the sinus mucosal architecture and cilia morphology. However previous studies assessing intravenous application of EDTA for treatment of heavy metal poisoning has been associated with some serious side effects, including renal damage⁵⁴⁰ and interference on trace mineral metabolism⁵⁴⁰. Such side effects must be considered, however, it is unknown whether topical nasal application of EDTA would allow absorption of EDTA into the blood stream. A previous study has assessed intravenous application of EDTA at a dosage of 3g/wk with no adverse side effects attributed to EDTA therapy⁵²⁹. This is comparable to the dose used nasally in our study (0.25g/wk). Previous studies have shown that EDTA does not bio-accumulate⁵⁴¹, so it is unlikely that there would be renal or mineral metabolism side effects. Poor gastrointestinal absorption of EDTA⁵⁴² also indicates that if a patient was to accidentally swallow the solution during flushing, only low levels are likely to pass into the blood stream⁵⁴².

An observation in our study was that sinus mucosa of sheep in the safety arm, treated with EDTA alone, showed a significant reduction in chronic inflammatory cell numbers in the sub-epithelial layer when compared to both the NT and CTSA-EDTA treated tissue.

Whilst interesting, further research is warranted to confirm these findings and to understand the molecular mechanism that causes this phenomenon. A potential mechanism could be that by capturing essential metal trace elements, a localised zinc deficiency could exist within the sinonasal mucosal surfaces, causing a decrease in the recruitment of T-cells and inhibiting the maintenance of T cytolytic cells, as reported⁵⁴³. Interestingly such a difference was not noted between the CTSA-EDTA group and the NT control, and the EDTA treated sheep showed a significantly lower level of chronic cell infiltrate in the sub-

epithelium compared to the CTSA-EDTA group. Such a finding may be attributed to subtle differences in inflammatory cell infiltration induced by the presence of phage⁴¹⁸.

All three test treatments were noted to be efficacious against established *S. aureus* biofilms. As well, reductions in acute inflammatory cell infiltrate in the sub-epithelial layer further supports the concept that treatments are reducing the infection load in the sinuses, mediating a reduction in the host immune response to pathogens on the surface of the tissue.

Efficacy of both phage and EDTA therapies in the context of treating bacterial infections have been studied thus far mainly in the laboratory and animal models. Phage have previously been noted to be effective *in vitro* against *S. aureus* infection^{400, 544-546} and biofilm^{372, 528, 547} and *in vivo* against *S. aureus* planktonic infection^{422, 425, 548-550}. However, two studies assessing phage therapy against *S. aureus* biofilms contrast to our work, in that biofilm load was not reduced in the phage treated animals^{551, 552}. As different phage were used in these studies, the phage cocktail used may have had enhanced anti-biofilm capacity. Furthermore, we investigated treatment of mucosal biofilms whilst one study assessed treatment of an intramedullary catheter in mice⁵⁷. As biofilms show differential gene expression when grown on abiotic compared to biotic surfaces⁵⁵³ this may also contribute to the contrasting results.

No studies have yet assessed EDTA in an *in vivo* model of biofilm eradication, however results in this study reflect similar *in vitro* observations indicating EDTA to be a potent inhibitor of biofilms^{351, 554-556} as well as being effective against preformed biofilms⁵³⁰⁻⁵³². The mechanism of EDTA's effect on biofilm is likely due to its metal chelation action. Divalent ion concentrations are known to impact biofilm formation. High calcium levels

have been shown to inhibit biofilm growth⁵⁵⁷, whilst high levels of iron⁵³³ and magnesium⁵³⁴ are thought to be essential for biofilm formation, so metal chelation may also play a part in the anti-biofilm effect of EDTA.

Previous study of *in vivo* topical phage application^{431, 436, 551, 558} did not investigate phage distribution in the body. However, phage distribution following intravenous phage injection has shown phage to predominantly settle in the liver, spleen and kidneys, whilst also being identified to a lower degree in the brain and skeletal system⁵⁵⁹, leading us to investigate the liver, spleen, kidney and brain for phage presence. The absence of phages in these organs suggests that the phages may not have been able to cross the sinus mucosal barrier into the bloodstream in detectable amounts. However, it has been observed that immune response to the foreign phage can inactivate and remove phage quite quickly⁵⁶⁰. Therefore the failure to identify phage in the brain, kidney, liver and spleen samples may have been due to immune mediated inactivation prior to organ harvest.

Phage persistence at the site of injection is another parameter that is often investigated in phage therapy *in vivo* research^{425, 426, 431, 561}. Therefore to investigate phage persistence in the sinus region, we assessed sinus samples taken at the time of euthanasia (24hrs post administration) where we identified low levels of active phage still present in the sinuses. This indicates phages are persisting for at least 24 hours in sinuses infected with *S. aureus*. Previous study has found varying degrees of phage persistence within the body, ranging from 48hrs⁴³¹ to 25 days⁴²⁵ post inoculation, suggesting future analysis of phage viability both within *S. aureus* infected and uninfected sinuses would be of great benefit to expand our knowledge of CT-SA persistence in sinuses.

In this study both CTSA and EDTA treatments have been identified to be effective anti-*S. aureus* biofilm agents when used alone, but this study also showed a lack of synergy in

combination indicating little value in using both treatments together. EDTA has been shown to enhance the action of the antimicrobial activities of other compounds against *S. aureus* infection⁵⁶²⁻⁵⁶⁴ and biofilm^{530, 565}. This enhancement may be due to the action of EDTA to increase the permeability of bacterial cells^{566, 567} and allowing an enhanced entrance of these antimicrobials into the bacterial cells. As the first step in phage infection is attachment and adsorption to specific bacterial membrane components⁵⁶⁸, it is likely that this increase in permeability would not enhance phage action.

When comparing the two treatments, the specificity of CT-SA for the bacterial species *S. aureus*⁵²⁸, is an advantage due to the treatments minimal impact on commensal flora of the nose⁵²¹. Therefore in a situation where the primary pathogen is known to be *S. aureus*, CT-SA would be a more desirable therapy. In comparison, a situation where for example both *P. aeruginosa* and *S. aureus* are causing sinonasal infections, EDTA would be a more efficient therapy due to the broad spectrum nature of EDTA³⁵². Another desirable property of phages are their ability to self-proliferate in the presence of bacteria⁵⁶⁹. Therefore it is possible that even after cessation of topical CT-SA application the phage could continue to eliminate *S. aureus* biofilm⁴⁴⁹.

Results of this study found the treatments EDTA and CT-SA to be safe for short term topical sinonasal application. Furthermore, treatment was able to reduce levels of biofilm however it was not completely eradicated, hence a longer period of treatment would be required for complete removal. Therefore to progress to clinical trials assessment of safety and anti-biofilm ability for a longer period of treatment in the sheep would be required, and is currently being investigated.

4.3.6 Conclusion

Shown to be both safe for short term application and capable to significantly reducing mucosal biofilm load, the metal chelator EDTA and the bacteriophage cocktail CT-SA have the potential to be translated into a clinical treatment of *S. aureus* infection and mucosal biofilms. Whilst phages have the advantage of specifically targeting a single pathogen, EDTA has the advantage of offering a broad spectrum alternative when multiple pathogens are involved. Future research will involve assessing the safety and efficacy of topical application of CT-SA or EDTA for a longer period to investigate if biofilm load can be eradicated by these treatments.

4.3.7 Acknowledgments

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5 Thesis summation

Staphylococcus aureus infection and biofilms have been noted to exacerbate CRS^{211, 213, 349}. No treatments are available that are capable of treating recalcitrant *S. aureus* biofilms from the sinonasal region. Therefore, one of the main goals of this research was to develop a novel therapeutic agent capable of eliminating mucosally anchored biofilms. Work in this thesis initially began investigating the role of *S. aureus* in recalcitrant CRS. Does the same strain of *S. aureus* persist in a patients' nose or is re-infection catalysed by a new strain?

The outcome of this study showed that the majority of recurring *S. aureus* infections in recalcitrant CRS was caused by a reoccurrence of the same *S. aureus* strain. This is a new finding and indicates that culture directed systemic antibiotics (which all of these patients had) was unsuccessful in eliminating the infection. Rather the bacterial load was reduced, the patient felt better and the bacteria now under threat adopted a more resistant phenotype – the biofilm phenotype^{527, 570, 571}. Previous research in this department has highlighted the incidence of *S. aureus* biofilms in the poor prognostic patients²¹¹. Whilst this has been previously hypothesised, direct assessment of this at a strain level has not been previously undertaken.

Furthermore, study outcomes indicated some CRS patients that were prescribed long-term antibiotics were observed to develop antibiotic resistant *S. aureus* isolates. As antibiotics are now the mainstay of the treatment of recalcitrant CRS there is significant need to develop therapies that can be effective with a short term application, as well as developing strategies that help to reduce the incidence of bacterial resistance. To overcome this problem of resistance it is also important to test bacterial susceptibility profiles prior to

treatment. To test the susceptibility of biofilm in an outpatient setting is difficult as invasive methods such as tissue collection are not feasible. This study therefore also compared the molecular types of *S. aureus* isolated from biofilm and mucopus, harvested at the time of surgery. In all cases (9/9) both biofilm and mucopus isolates were indicated to be the same strain. This highlights that to assess biofilm antimicrobial susceptibilities it is appropriate to take a non-invasive swab of mucopus to determine the resistance profile of the biofilm bacteria.

Results of the epidemiological analysis of *S. aureus* in CRS suggested that it would be beneficial to develop a novel antimicrobial therapy aside from antibiotics. To determine a suitable antimicrobial therapy recent studies of the CRS microbiome were reviewed. Microbiome investigations of the nasal region suggest that a healthy nose is colonised by a highly diverse and large community of bacteria^{103-105, 572, 573}. These studies have identified that patients with CRS have a reduced bacterial diversity⁵⁷² and often an overgrowth of pathogens such as *S. aureus*^{103, 104}. Furthermore, commensals including *Staphylococcus epidermidis* have been shown to impede nasal colonisation of *S. aureus*. Such findings suggest that use of a treatment that can specifically target a pathogen whilst maintaining commensal bacteria would be beneficially. Review of the literature would suggest bacteriophage is a therapy may therefore be suitable for treatment against *S. aureus* sinonasal infection and biofilm.

Viruses that infect bacteria are ubiquitous. These viruses are known as bacteriophage or phage, a name which essentially means 'bacterial eater'. Phage have a long history of successful treatment of bacterial infections in a number of eastern European countries³⁸⁹. One important characteristic phages possess is efficacy against bacterial biofilms. A number of studies have found success when applying phage against biofilms *in vitro*^{372, 437}

as well as *in vivo*⁴⁴¹. With this knowledge, this work then set out to assess the potential of phage therapy against *S. aureus* infection and biofilms in a CRS context.

The first step was to acquire or isolate phage to test their ability to destroy *S. aureus* bacteria. Special Phage Services Pty. Ltd. possessed a cocktail of *S. aureus* specific phage (CTSA). This phage had already shown preliminary efficacy against biofilms of *S. aureus* isolated from cystic fibrosis patients. The phage product designated CTSA had also been extensively characterised. This product had been shown to be sterile and not cytotoxic to human cells *in vitro*. This therefore presented an excellent candidate phage cocktail to test for CRS applications.

To confirm that CTSA was effective against *S. aureus* biofilms of CRS origin *in vitro* studies were performed. This work found the cocktail was effective against *S. aureus* biofilms and planktonic cells obtained from the paranasal sinuses of CRS patients. CTSA was also found to infect a broad range of *S. aureus* cultures isolated from CRS patients. Furthermore, CTSA as a cocktail of phages was able to reduce the generation of bacterial resistance to the phage treatment in comparison to a singular phage preparation. These results taken together highlight strong potential of CTSA for application against biofilms. These positive *in vitro* results allowed progression to assessing phage application *in vivo*.

A series of animal studies on phage efficacy were conducted using a sheep model of frontal sinusitis. As previous animal research in our department on the frontal sinusitis model had indicated high efficacy of EDTA against biofilms (unpublished data) investigation into whether phage could synergise with EDTA was also undertaken. Work indicated both CTSA and EDTA to be safe for short-term topical sinonasal administration. Furthermore both EDTA and CTSA when applied alone significantly reduced the level of

S. aureus sinonasal biofilms following five days of treatment. When used in combination the biofilm reduction was maintained, however the treatments did not work synergistically. *In vivo* work also endeavoured to assess whether phage could cross the mucosal barrier in the sinuses, pass into the blood stream and accumulate in various organs. Organ samples (spleen, kidney, liver, brain) were found to be devoid of phage 24 hours after final treatment application in both the safety and efficacy CTSA treated sheep. Investigation of the longevity of phage in the sinus region identified phage to still be present in the sinuses 24 hours after final treatment application when *S. aureus* was present. Results of this work indicated that both EDTA and CTSA could be advanced to a clinical trial setting. However some additional safety testing is required to ensure the safety of long-term application.

There are a number of future directions that can extend from this research. An important extension of this work would be to further confirm the safety of topical sinonasal application for longer periods of treatment. *In vivo* work presented in this thesis highlighted phage reduced *S. aureus* mucosal biofilms, but five days of treatment was not long enough to eradicate the biofilm structures. It follows that clinical application will likely need to be applied for a longer period of time and the safety and efficacy of such a period of treatment is essential.

Further to this we did not observe synergy between the EDTA and phage therapies. *In vivo* success against *S. aureus* biofilms has been noted when combining the antibiotic teicoplanin with phage⁴⁴¹. There is also potential to evaluate combination of phage and other antimicrobials including antibiotics. Combination therapies would help to reduce bacterial resistance generation to both phage and antibiotics by exerting multiple selection pressures. We could also begin employing the information gained from nasal microbiome studies. Information is beginning to suggest beneficial nasal bacteria exist in the nose⁴⁶⁶,

and combining probiotics with phage could help boost protection against and treatment of nasal pathogens.

Another important future direction is strengthening our knowledge of whether phage can cross the sinonasal mucosal barrier to access the blood stream. A limitation of the *in vivo* work described here was the 24 hour time lapse between final treatment application and organ harvest. Further experiments need to be carried out to collect samples at multiple time points. As organ harvest involves animal euthanasia, this work will be extended by taking blood samples from sheep which will allow sampling of a number of time points. It would also be of interest to study the organ deposition of phage following intravenous application.

Further study in animal models would allow investigation of the metabolism of phage in the body and enrich our knowledge of treatment safety. It would also be of benefit to know whether phage are able to remain in the sinus region to inhibit future infections. Therefore investigation of the longevity of phage in the sinus region would also be of interest. As previously discussed, phage have been found to adhere to mucous in some cases⁴⁶⁵. It may be possible to select a panel of phage that have this characteristic in an effort to confer protection from future sinonasal *S. aureus* infections.

The logical progression of the bacteriophage work, pending confirmation of the long-term safety in animal studies, is to conduct clinical trials. Clinical trials will assess the ability of phage at eliminating *S. aureus* from the sinonasal mucosa. Additionally, research into whether phage are able to confer long term protection against further *S. aureus* nasal infections will be important. One interesting parameter for clinical study is to observe the effect that phage therapy has on the nasal microbial microbiome. It would be expected that

phage would have minimal impact on commensal communities due to their infection specificity. Therefore whether this is the case, and whether this is of benefit to CRS patients would be important information. It is hoped that development of an effective anti-biofilm therapy would not only eliminate biofilm structures, but also improve patient symptomatology and quality of life.

Research in this thesis has primarily focused on the pathogen *S. aureus*. Whilst this is an important bacteria to develop therapies against it is not the only pathogen of interest in CRS. Other harmful bacteria include species such as *Pseudomonas aeruginosa*.

Increasing our knowledge of epidemiology and developing targeted treatment strategies against these bacteria is also important. Phage are already available that are specifically targeted against these pathogens. A benefit of phage is that for the majority of bacterial species, effective targeted phage can be isolated from the environment. Therefore phage technology is not only limited to the treatment of *S. aureus* but can also be expanded to target other bacterial species. Potential is there to target any pathogenic bacteria in the nose of CRS patients, whilst maintaining commensal and beneficial flora. Future research will therefore expand this technology to target other CRS specific nasal pathogens.

CRS is not the only condition where *S. aureus* is noted to be detrimental to wound healing or patient health. *S. aureus* is noted to be a significant problem in cystic fibrosis causing lung infections and burn wound infections. Topical therapies are preferable in these infections as they are classically in a biofilm form and systemic antibiotic therapy is often ineffective. Development of a phage cocktail effective topically against *S. aureus* infection therefore has potential to be translated to treatment of alternative conditions in which it is of benefit to eliminate *S. aureus* infection. Further to this a number of studies have indicated that eradication of *S. aureus* from the nares of patients helps to reduce post-

surgical infections and is of particular importance in joint surgery. Therefore if this bacteriophage cocktail is effective at eliminating *S. aureus* infection it could be employed to cleanse a patient of *S. aureus* infection prior to surgical procedures.

6 Conclusion

CRS is a complex and inimical condition that affects an individual's quality of life and impairs their daily functioning. Enhancing understanding and improving management techniques is paramount to efficiently manage or even cure this illness. Work in this thesis has both expanded our knowledge of *S. aureus* epidemiology in the context of CRS and identified a potential anti-biofilm agent. With further *in vivo* development of bacteriophage therapy, there is potential for this treatment to be applied clinically against detrimental *S. aureus* infections and biofilms in CRS patients. Additionally, preliminary work investigating *S. aureus* role in CRS has developed a number of avenues of research. Building our knowledge of the pathobiont *S. aureus* is essential to allow an understanding of how and why *S. aureus* switches to a pathogenic state. A stronger understanding of this complex bacteria will likely help us to control and treat this bacteria more effectively.

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