INTRACELLULAR *STAPHYLOCOCCUS AUREUS* IN CHRONIC RHINOSINUSITIS

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Degree of Doctor of Philosophy

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Dedicated to my wonderful wife, Harriet

and our precious children, Thomas and Arabella.

DECLARATION

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ABBREVIATIONS

APC	Antigen presenting-cells
ARS	Acute rhinosinusitis
ABRS	Acute bacterial rhinosinusitis
AEC	Airway epithelial cells
AERD	Aspirin-exacerbated respiratory disease
ATCC	American Type Culture Collection
CDS	Codon sequences
ClFA	Clumping factor A
ClFB	Clumping factor B
CF	Cystic fibrosis
CRS	Chronic rhinosinusitis
CSLM	Confocal scanning laser microscopy
CSR	Cell surface receptors
CT	Computed tomography
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EB	Elementary body
ECF	Extracellular fluid
ECM	Extracellular matrix
EM	Electron microscope
EPS	Extracellular polymeric substances
ESS	Endoscopic sinus surgery
F(ab′)	Fragment antigen-binding region of antibody
FACS	Fluorescence-activated cell sorting
FC	Flow cytometry
Fc	Fragment crystallisable
FcR	Fc receptor
FCS	Fetal calf serum
FISH	Fluorescence in situ hybridisation
FESS	Functional endoscopic sinus surgery
FnBPA	Fibronectin-binding protein A
FnBPB	Fibronectin-binding protein B
HGT	Horizontal gene transfer
HNP	Human neutrophil peptides
HRQoL	Health-related quality of life
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
INCS	Intranasal corticosteroids
IQR	Interquartile range
LDH	Lactate dehydrogenase
LLO	Listeriolysin O
MEM	Minimum essential medium
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MMP	Matrix metalloproteinases
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
NOD	Nucleotide Oligomerization Domain
OCT	Optimal cutting medium
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PFGE	Pulsed field gel electrophoresis
PI	Propidium iodide
PMN	Polymorphonuclear leukocytes
PNA	Peptide nucleic acid
PRR	Pattern recognition receptors
PV	Panton-Valentin
QS	Quorum Sensing
RAST	Radioallergosorbent test
RB	Reticulate body
RCT	Randomised controlled trial
RNA	Ribonucleic acid
rRNA	Ribosomal RNA

SaPI	Staphylococcal pathogenicity islands
Sbi	Staphylococcal binder of immunoglobulin
SCV	Small colony variant
SE	Staphylococcal enterotoxins
SEA	Staphylococcal enterotoxin A
SE-L	Staphylococcal enterotoxin-like toxins
SFB	Serum free protein block
SPA	Staphylococcal protein A
TB	Tuberculosis
TBS-T	Tris-buffered saline and 0.05% tween-20 buffer
TCR	T-cell receptor
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of MMP-1
TLR	Toll-like receptor
TMB	(3,3′,5,5′-Tetramethylbenzidine
TSS	Toxic shock syndrome
UIFM	Upright immunofluorescence microscopy

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ABSTRACT

Chronic Rhinosinusitis (CRS) is a heterogeneous disease characterised by recurrent and persistent episodes of nasal obstruction, discharge and facial pain or pressure. Patients suffering from CRS experience considerable morbidity and have impaired qualities of life. The gold standard treatment of cases that fail medical therapy is endoscopic sinus surgery (ESS). Despite the proven efficacy of ESS, the modern sinus surgeon will see a subset of patients who persistently fail any attempt to improve their disease profile. Recent research into CRS had identified bacterial biofilms, in particular those mediated by *Staphylococcus aureus* to hold a potential role in the aetiopathogenesis of this disease. Patients with biofilms suffer from more severe preoperative symptoms and have worse postoperative outcomes. As a consequence, numerous anti-biofilm therapies have been developed including biofilm dispersal agents and biocidal agents. Despite showing early promise *in vitro*, the use of these therapeutic agents *in vivo* has not translated to a conclusive clinical benefit. Recent studies have identified that *S. aureus* can invade non-professional phagocytic cell types such as epithelium with the ability to survive and replicate intracellularly. This led to the hypothesis that by exploiting the intracellular environment, bacteria may evade host immunity, topical antimicrobial therapy and establish a niche for survival with potential reservoirs for chronic or relapsing *Staphylococcal* infections. Therefore, this PhD thesis set out to investigate whether intracellular *S. aureus* plays a disease modifying role in CRS.

Chapter 1 critically reviews the context of the work included in this thesis pertaining to CRS, *S. aureus*, biofilms and intracellular infections.

Chapter 2 validates a novel imaging technique using confocal scanning laser microscopy (CSLM) coupled with dual staining of fluorescence in situ hybridisation (FISH) probes and nucleic acid counterstains (propidium iodide, [PI]), to identify the presence of intracellular *S. aureus* in whole mucosal specimens, with a direct comparison to previously reported techniques of immunohistochemistry (IHC). The study reported the benefits and drawbacks of each technique, and identified specific roles for their use when examining tissue specimens. The major advantage of CSLM-FISH/PI was that simultaneous biofilm analysis was possible in the same piece of tissue.

Chapter 3 investigated the unexpected phenomenon of false-positive antibody binding in *S. aureus* infected tissue specimens when performing IHC in paraffin embedded tissue sections. This was hypothesised to be caused by protein A expression in the bacterial cell wall that continued to bind IgG-class antibodies with high affinity. A methodology was developed and validated to overcome this issue, with significant implications when performing future IHC experiments.

Chapter 4 utilised the previously reported CSLM-FISH/PI protocols for intracellular *S. aureus* detection in a cohort of CRS and control patients. For the first time the association between biofilms and intracellular infection was reported, suggesting that the biofilm may offer a conditioned environment to allow invasion of *S. aureus* to deeper tissue layers.

Chapter 5 followed a wider cohort of patients in their postoperative course in order to ascertain whether a relationship between intracellular infection and disease recalcitrance could be identified. The results found that intracellular *S. aureus* infection at the time of surgery was significantly associated with failure of medical and surgical therapy in the

postoperative patients. This reinforced the theory that the intracellular location provides bacteria with a protective niche where they can avoid host elimination and topical antimicrobial therapy.

Chapter 6 investigated whether the concept of bacterial phenotype switching following intracellular infection in airway epithelial cells occurs as a mechanism of allowing these organisms to decrease their virulence and evade innate immunity. It was found that *S. aureus* reduces production of its superantigenic enterotoxins as a consequence of internalisation; however, this reduction in virulence was reversible after lysing the host cells and a single sub-culture step. Additionally, for the first time we demonstrated that intramucosal organisms harvested from sinonasal biopsies demonstrate altered phenotypic growth patterns and lack of coagulase activity consistent with small colony variants (SCV). This represented another potential explanation for why bacteria are so capable of internalising and persisting in epithelial tissues.

The findings of this thesis have provided novel insights alluding to a role of intracellular *S. aureus* in CRS. The versatility of *S. aureus* in altering its phenotypic characteristics to take advantage of the local environment makes it troublesome to fully eradicate and significant associations can be made between intracellular infection and recalcitrant disease. Future research should be directed towards identifying novel treatment strategies that can effectively target intracellular organisms.

CHAPTER 1

INTRODUCTION

1.1 CHRONIC RHINOSINUSITIS

1.1.1 DEFINITION

Rhinosinusitis is the term used to describe a group of disorders characterised by symptomatic mucosal inflammation within the nasal cavity and paranasal sinuses. Initially termed sinusitis, it is now recognised that the disease process affects the mucosa that is continuous within the nasal cavity and paranasal sinuses. In 1996, the Task Force on Rhinosinusitis[1] recognised that for high quality research to occur into the disease, a consistent definition must be established. Thus, the first consensus opinion was reported and broadly sub-classified adult rhinosinusitis based on duration and onset of symptoms;

1) Acute rhinosinusitis (< 4 weeks duration)
2) Subacute (4-12 weeks duration)
3) Chronic (> 12 weeks duration)

Indicative symptoms of rhinosinusitis were also divided into major and minor symptoms (Table 1.1). The diagnosis of acute rhinosinusitis (ARS) required two major factors or one major and two minor factors. It is often virally mediated with symptoms occurring suddenly and resolving spontaneously within its time-course, however a prolonged infection with duration of more than 5-10 days is suggested to be caused by bacterial elements and is thus termed acute bacterial rhinosinusitis (ABRS).

Table 1.1: Major and minor symptoms of rhinosinusitis

Major Symptoms	Minor Symptoms
Facial pain/pressure	Headache
Facial congestion/fullness	Fever (all nonacute)
Nasal obstruction/blockage	Halitosis
Hyposmia/anosmia	Fatigue
Purulence in nasal cavity on examination	Dental pain
Fever (acute rhinosinusitis only)	Cough
Nasal discharge/purulence/discoloured postnasal drainage	Ear pain/pressure/fullness

In this set of guidelines, CRS held a similar diagnostic criterion as acute, with the extended duration being the main factor in distinguishing the diseases. However, as the aetiopathogenic understandings evolved with time, the purely temporal distinction was felt inadequate. Therefore, a group of 30 physicians from North America representing 5 specialties including otolaryngologists, paediatricians and respiratory physicians set out to further refine guidelines for evidence-based rhinosinusitis definitions.[2] A group of European physicians also set out similar guidelines in the form of the European position paper on rhinosinusitis and nasal polyps[3] (Table 1.2) which although updated recently,[4] held identical diagnostic criteria. In addition to the diagnostic definition of CRS, a common clinical sub-division exists based on the presence (CRSwNP) or absence (CRSsNP) of nasal polyps.

Table 1.2: Comparison of American and European guidelines for CRS diagnosis.

	American Consensus	EPOS
Duration	>12 weeks	> 12 weeks
Subjective symptoms	≥ two of the following symptoms; • Anterior and/or posterior mucopurulent drainage • Nasal obstruction • Facial pain-pressure-fullness • Decreased sense of smell	≥ two symptoms, one of which should be • nasal blockage, obstruction, congestion • nasal discharge (anterior or posterior) ± facial pain/pressure ± reduction or loss of smell
Objective evidence	Nasal endoscopy to demonstrate; • presence or absence of polyps • signs of mucosal inflammation *and* Radiologic investigation • CT scan	Nasal endoscopy to demonstrate: • Nasal polyps, and/or • Mucopurulent discharge , and/or • Oedema/mucosal obstruction primarily in middle meatus

1.1.2 EPIDEMIOLOGY

The prevalence of rhinosinusitis has been rising steadily in epidemiologic surveys that have been carried out over the past 30 years.[5-7] Although limitations in self reported assessments of rhinosinusitis may lead to over-reporting, the prevalence in US population studies has been suggested to be between 12.5-16%.[5,8,9] This suggests that chronic rhinosinusitis is the second most prevalent chronic condition, and more common than arthritis, hypertension and cardiac disease.[10] A multi-centre European study reported as The Global Allergy and Asthma Network of Excellence (GA2LEN) study[11] found that the prevalence or CRS was 10.9% (range 6.9-27.1) using the EPOS diagnostic criteria.

The socio-economic consequences of this disease are vast, with direct medical costs in the US estimated at between $4.3 and $8.6 billion US.[12-14] This does not include indirect costs accounted by factors such as decreased productivity (presenteeism) whilst at work, and absenteeism due to disease-related sickness or recovery post surgery so the true amount is likely to be significantly higher. It has been reported that 85% of patients with rhinosinusitis are of a working age (18-65 years)[15] and calculations have estimated CRS patients miss an average of 4.8-5.7 days of work per year,[12] giving a total of 12.5 million lost work-days per year. Presenteeism is less easily quantified due to differences in individual productivity; however, the impact on indirect costs is likely to be immense. There is also a significant burden on the individual, with studies demonstrating that patients with CRS undergo an additional 3.5 outpatient visits and require 5.5 prescriptions more when compared to non-CRS patients,[14] as well as suffering excess financial costs of $773 +/- 300 per year.

Health-related quality of life (HRQoL) is another aspect that is negatively impacted by CRS with patients having worse QoL scores when compared to healthy, non-CRS subjects.[16] Physician administered questionnaires to CRS patients demonstrate significantly worse mood, vitality and sexual functioning.[17,18] These patients have worse scores for bodily pain and social functioning than patients with angina, back pain, congestive cardiac failure and chronic respiratory disease.[19]

1.1.3 AETIOPATHOGENESIS

Despite significant research into the aetiopathogenesis of CRS in the past 30 years, no single 'Rosetta stone' has been recognised as a unifying cause of the disease. It is now considered to be a heterogeneous, multi-factorial collection of disease processes that ultimately lead to the characteristic inflammation found within the sinonasal cavities.[4] Numerous causative associations have been made, broadly divided into intrinsic (related to the host) and extrinsic (related to the surrounding environment) factors. Intrinsic factors include disorders of the epithelium or cilia,[20,21] allergy,[22,23] asthma,[24] aspirin sensitivity,[25] host immune system dysfunction[26] and genetic factors[27,28]. Extrinsic factors are suggested to act as triggers that drive the inflammatory response within the sinonasal mucosa and include environmental toxins such as cigarette smoke[29] and the impact of micro-organisms, specifically viruses,[30] bacteria[31] and fungi.[32] In all likelihood, this categorisation is probably an oversimplification of what is a complex interplay between the host and surrounding environment. The focus of this thesis is to characterise the role that bacterial phenotypes, in particular *S. aureus*, may play on the pathogenesis of recalcitrant CRS.

1.1.4 MICROORGANISMS AND CRS

In an attempt to identify causation between microbes and disease, the German bacteriologist Robert Koch described four postulates related to the investigation of anthrax and tuberculosis. The widely understood description is as follows; (i) The microorganism is present and discoverable in every case of the disease, but should not be found in healthy organisms, (ii) it should be isolated from a diseased organism and is cultivated in a pure culture, (iii) inoculations from such culture must reproduce the disease in susceptible animals, and (iv) it must be re-obtained from such animals and again grown in pure culture. [33] At the time, many diseases conformed to this pattern and the success in applying the postulates led to their adoption as a doctrine for any potential microbial infection. Coupled with the emergence of antimicrobial therapy in the form of antibiotics; the ability to deal with, understand and treat infections led to significant advances in the diagnostic and therapeutic aspects of health-care. At the turn of the 20th century, the leading cause of mortality was from infectious diseases such as tuberculosis or diarrhoea, however, by the end of this century these conditions were replaced with mortality from chronic diseases such as cardiac disease, respiratory disease or cancer.[34] The rigid adherence to the dictum of the postulates has undoubtedly affected the evolution of the field of microbiology, which remains to this day skewed to focus on microbial culture techniques.

As ABRS so convincingly fulfilled the criteria of Koch's Postulates, with readily identifiable pathogens such as *Streptococcus pneumoniae* or *Haemophilus influenzae* responding well to antimicrobial therapy; a logical conclusion was that CRS merely represented a failure to eliminate the pathogenic source. Unfortunately, it soon became clear that this concept did not hold true and despite courses of culture-directed antibiotics, CRS patients remained symptomatic. In addition, traditional culture techniques

demonstrated a wide range of detection rates in CRS patients, with anywhere between 12% and 80% of patients demonstrating polymicrobial aerobic and anaerobic bacteria growth within the sinonasal cavity.[35] It was then recognised that the paranasal sinuses are not sterile areas and similar bacterial strains may be identified within healthy controls and CRS patients.[36] The fact that no single pathogen could be identified in CRS patients but not healthy controls, coupled with the lack of consistency of bacterial identification meant that Koch's first postulate not could be fulfilled. In combination, these reasons turned the focus of scientists away from the concept of CRS as an infectious disease.[37]

The applicability of Koch's Postulates to modern disease processes has come into question in recent years. With the understanding that certain bacteria are incapable of pure culture or can exist in a viable but non-cultureable state,[38] the basic tenets of bacterial causation as proposed by Koch are brought into doubt. Bacteria are now understood to exist in numerous phenotypes including biofilm, intracellular and small colony variant forms, with each potentially playing an individual role in disease aetiopathogenesis. The role of the different bacterial phenotypes as regards to CRS will be discussed further in subsequent chapters.

1.1.5 TREATMENT OF CRS

Despite the wide range of aetiopathogenic origins associated with CRS, the treatment follows a fairly standardised regime including both medical and surgical interventions. Medical therapy includes topical and systemic agents such as steroids or antibiotics. In patients whose symptoms are refractory to these treatments, surgical intervention may be warranted.

1.1.5.1 MEDICAL THERAPY

A substantial body of evidence supports the use of medical therapies in the treatment of CRS. Researchers and clinicians are in agreement about the efficacy of intranasal corticosteroids (INCS) delivered by small-volume, metered dose sprays such as fluticasone, mometasone and budesonide. Numerous randomised controlled trials (RCT) have documented level 1a evidence as to the benefit of INCS with regards to symptomatic improvement[39,40] and reduction in size of nasal polyps.[41,42] On the other hand, a group of other studies have also reported contradictory evidence that INCS did not offer any advantages to placebo.[43,44] Nevertheless, the general consensus amongst rhinologists is that the short and long term therapeutic benefits far outweigh the minor side effects of epistaxis and nasal irritation, and thus INCS are considered in the "first-line" of medical treatment. In recent years the use of high dose INCS delivered by different methods such as atomisers[45] or saline irrigation[46] have gained interest. Although efficacy studies have shown a benefit in prospective single-arm pilot studies,[45,47] only one study has compared it to standard small-volume, metered dose sprays reporting no significant difference between the two.[48] Clearly an area that requires further research is in performing a three-arm RCT comparing low dose INCS, high dose INCS and placebo.

Fewer studies have examined the use of systemic corticosteroids in CRSwNP, however level 1a evidence is available demonstrating their benefits in terms of symptomatology and reduction of polyp size[49-51] after short treatment periods of 2-6 weeks. Despite the evident short term improvements, a number of studies identified that these benefits were short-lived and did not translate to a significant long-term improvement in symptomatic outcomes compared to placebo or INCS alone.[51,52] The major limitation of long term systemic corticosteroid use is the risk of adrenal suppression and side effects such as

osteoporosis, immunosuppression, fluid and electrolyte imbalances, behavioural changes, skin thinning, and alterations to endocrine and metabolic functions[53]; thus, their long-term use is not advised and usually restricted to 2-3 courses of up to 2 weeks a year.

The use of oral antibiotics is widespread amongst otolaryngologists and primary care physicians. In recent surveys of the US[54,55] and UK,[56] it has been reported that over 90% of doctors will prescribe antibiotics for CRS. Despite their widespread use, there remains a paucity in high level trials reporting the efficacy with only 2 RCT's in CRSwNP and none in CRSsNP. Van Zele et al[52] reported that doxycycline offered benefits including reduction of polyp size and nasal symptoms compared to placebo in a placebo controlled RCT. Caution has to be applied when interpreting this study, however, due to the immunomodulatory effect of doxycycline which may have been a confounding factor. Other studies have shown that oral anti-staphylococcal agents are not effective in treating *S. aureus* positive CRS patients.[57]

No high level placebo controlled RCT's exist examining the impact of short term antibiotic usage in CRSsNP. The only studies available compared different antibiotics for acute infections exacerbations of CRS, and concluded that they were effective in treating this sub-group of patients.[58,59]

The role of long-term macrolide antibiotic therapy is another area that has come under scrutiny. Doxycycline is recognised to have the ability to downregulate the production of endopeptidases known as matrix metalloproteinases (MMP). These molecules are capable of degrading components of the extracellular matrix (ECM), and thus doxycycline acts as an inhibitor of tissue damage. In the treatment of CRSwNP the only evidence is in the form

of uncontrolled open trials[60-62] where it has been reported that patients improve both clinically and radiologically after at least 8-12 weeks treatment. It is somewhat surprising that no high level RCT's have been performed to compare long-term treatment to placebo in this subgroup of patients. Conflicting evidence has been reported for their use in CRSsNP patients; Wallwork et al[63] reported significant subjective and objective improvements following Roxithromycin and advocated its use, however, Videler et al[64] did not find these improvements in their RCT of 60 patients. Numerous other observational cohorts have suggested that macrolides do have a benefit in both symptom scores and objective findings of disease. The lack of reliable evidence highlights an additional area that would benefit from multi-centre high level trials in order to effectively guide our medical management.

A recent review critically examined the evidence for intranasal saline irrigation in the treatment of CRS and found five studies that examined the impact of saline irrigations prior to any surgery.[65] Of these, there were a wide range of study designs that compared; saline irrigation vs no treatment,[66] saline irrigation vs placebo in the form of reflexology,[67] hypertonic vs isotonic saline irrigation[68,69] and high volume irrigation vs saline spray.[70] Despite the variety of modalities examined, the general consensus was that saline irrigations may improve symptoms when used as an adjunct to INCS. Their effectiveness alone, however, could not match the effects of INCS and thus they are considered an adjunctive treatment.[71]

The role of anti-fungals has been a subject of much debate in recent years. After the fungal hypothesis was raised as a unifying aetiological cause of CRS, a great deal of interest emerged and as a consequence, numerous trials of anti-fungal medications. Unfortunately,

high level, multi-centre randomized studies demonstrated that both intranasal and systemic anti-fungals were not effective in treating CRS[72-75] and thus recommendations have been made against their use.[76,77]

A wide variety of other medical treatments have been suggested, including antileukotrienes, anti-IgE, proton-pump inhibitors, furosemimde, immunosuppressants, probiotics and mucolytics. On the whole, the efficacy of these agents has not been proven and thus their routine use not recommended.[4]

1.1.5.2 SURGERY

When medical treatment fails it is universally recognised that surgery in the form of Functional Endoscopic Sinus Surgery (FESS) offers potential improvements in symptoms, quality of life and the endoscopic appearance of the nasal cavity.[78,79] Due to the methodological and ethical limitations of structuring RCT's of invasive surgical therapies, there remains an understandable paucity of level 1 evidence with regards to specific surgical treatment modalities. Only two RCT's have directly compared FESS to medical therapy and although they did not demonstrate significant differences in overall symptomatology, the limitations of these studies must be considered in the study design and age. In 1997, Hartog[80] compared FESS to sinus irrigation in cases of isolated maxillary sinusitis reporting no difference in symptomatic outcomes, however the pathology in question is clearly different from the pan-sinus disease that is seen in medically recalcitrant patients. The second study by Ragab et al[81] in 2004 compared surgery to medical therapy and although they found no difference between outcomes in the groups, it was concluded that surgery should still be reserved for those cases refractory to medical therapy, a concept that remains in effect today.

Fairly unsurprisingly, the insistence of Cochrane Group systematic reviews to base advice on RCT studies alone has led to conclusions that FESS does not confer additional benefits to those obtained by medical treatment alone in CRS.[82] Despite the so-called lack of proof in this review, numerous level III cohort studies that demonstrate the efficacy of FESS[83,84] in providing symptomatic relief were not included. Therefore, to make an evidence-based suggestion discounting these studies leads to an obviously flawed conclusion. A recent multi-institution study has made probably the best effort thus far in attempting to directly compare medical versus surgical treatment. The group prospectively enrolled 180 patients who had failed maximal medical therapy into two study arms as per patient choice; on-going medical therapy or immediate FESS.[85] A third crossover group emerged as the study progressed, comprised of medically managed patients who subsequently elected to undergo FESS. The baseline characteristics of the cohorts were similar, however at 1 year follow-up it was identified that patients who underwent FESS, including those in the crossover group, showed improved QoL scores in almost all outcome measures. It was concluded that surgery offers several advantages over failed medical treatment for CRS.

Despite the proven efficacy of well-performed FESS, sinus surgeons are recognising a subgroup of patients with recalcitrant symptoms that persistently fail medical and surgical treatment.[86] Intensive research efforts are focusing on these patients[87,88] with mechanisms such as irreversible fibrosis or long-term bacterial colonisation potentially playing a part in their disease process. The recognition of different phenotypic bacterial forms that do not abide by the traditional mechanisms of disease causation has generated significant interest into their purported role in chronic diseases. The contribution of bacteria in their biofilm, intracellular and small colony variant (SCV) forms to CRS will be discussed further in this thesis.

1.2 *STAPHYLOCOCCUS AUREUS*

1.2.1 TAXONOMY AND BIOLOGY

Figure 1.1: Scanning electron micrograph of *S. aureus*. Image from Centre for disease control (CDC) Public Health Image Library.

S. aureus is a species from the *Staphylococcus* genus that is recognised as one of the leading causes of pathogenic infections in humans in both community and hospital environments. The earliest identification of *S. aureus* was in 1884 when Friedrich Julius Rosenbach distinguished it from *Staphyloccus albus*, now currently known as *Staphylococcus epidermidis*. Although capable of causing life threatening illness, *S. aureus* is also one of the commonest colonizing organisms found in the skin, hair follicles, anterior nares and oral cavity of humans.

This bacteria has a remarkable ability to persist in otherwise hostile environments, being capable of resisting factors such as dessication, low pH, nutrient starvation and high temperatures in its drive to survive.[89,90] Indeed it has been recognised that *S. aureus* organisms persist on fabric or dust particles months after contamination,[91] as well as adhering and surviving on desquamated skin fragments.[92]

1.2.2 BIOCHEMICAL AND GENETIC PROPERTIES

S. aureus exists as a non-motile, coccal structure roughly 0.8-1 μm in size that forms in grape-like clusters due to cellular division occurring in two planes. It is classed as a facultative anaerobic organism that is also capable of generating energy by aerobic respiration. When cultured on blood agar plates it forms large, yellow colonies, thus the etymological derivation of its name; from the Greek term *staphyle* (grape-like) and the Latin term *aureus* (golden).

To distinguish *S. aureus* from other species it is often tested on the basis of positive results to catalase, coagulase, mannitol fermentation and trehalose tests.[93] A disadvantage of traditional bacterial identification methodology is the prolonged time for definitive identification.[93] More recently, the advent of molecular detection tests has led to rapid methods of bacterial identification. Probes designed to target specific DNA or protein sequences can identify the "molecular fingerprint" left by an organism from culture, tissue or blood samples.[94,95]

The genetic outline of *S. aureus* is the most widely studied and sequenced bacteria with more complete genomes submitted to public databases than any other pathogen. The first complete genomes published were of N315 and Mu50 in 2001[96], and since then almost 75

genomes have been completed or reported in draft format.[97] The single chromosome of *S. aureus* is between 2.8-3.0 Mbp in size and encodes roughly 2700 codon sequences (CDS),[98] that are subcategorised according to their purpose. Functional groups include clusters of genes regulating structure, transport mechanisms, biosynthesis and virulence; however, there remain large numbers of CDS to which a function is unknown.

The pan-genome of a species is divided into the core genome, which is strictly conserved across all different strains[99] and a dispensable genome which comprises partially shared or strain specific genes.[100] In *S. aureus*, the core is understood to comprise 75% of all CDS with the remainder being variably distributed. This genomic diversity is understood to be a result of horizontal gene transfer (HGT), the process whereby genetic material from one bacterium is transferred to another in a unidirectional manner.[101] HGT can occur through three main processes; (i) transformation, where genetic material can be taken up from the environment; (ii) transduction, where DNA is moved from one bacterium to another by a virus or phage; and (iii) bacterial conjugation, where DNA is transferred directly by cell-to-cell contact.

In addition to HGT, it is recognised that staphylococcal pathogenicity islands (SaPI) play a role in the transfer of virulence factors between the species. These are highly mobile genetic sequences roughly 15-17kbp in size[102] that are highly conserved as a gene sequence. SaPI's typically encode for two or more superantigen genes and are responsible for superantigen mediated disease such as toxic shock syndrome.[103,104] They occupy specific chromosome sites and appear related to certain bacteriophages due to similarities in function and mechanism of DNA transfer.

The ease of transfer of virulence factors by HGT and SaPI's contribute to the heterogeneity that exists in terms of the combinations of resistance genes and virulence factors occurring within *S. aureus* strains. The ability to transfer and share these factors no doubt adds to the ability of the organism to adapt and evolve so as to overcome the immune system or antimicrobial therapies.

1.2.3 PATHOGENICITY MECHANISMS

In the *Staphylococcus* genus, there are at least 40 species, of which 16 are found in humans. This bacterium has a unique capability of acting as both a commensal colonizer and invasive pathogen,[105] affecting humans of diverse genetic backgrounds.[96] It is responsible for a wide range of diseases ranging from minor acute infections to severe, life threatening diseases. In addition to causing disease in humans, *S. aureus* can cause acute infections in animals,[106] and is capable of colonizing a wide range of species such as dogs, cats or rabbits.[107] The *Staphylococcal* virulence factors that play a part in bacterial pathogenesis are summarised in Table 1.3.

Table 1.3: *Staphylococcal* virulence factors involved in bacterial pathogenesis.

ACTIVITY	FACTOR	GENE
Anti-phagocytosis anti-opsonic	Protein A	*spa*
	Capsular polysaccharide type 1, 5 & 8	*cap1, 5 & 8*
Fibronectin binding	Fibronecting binding protein (FnBPA/B)	*fnbA fnbB*
Fibrinogen binding	Clumping factor A / B (ClFA/B)	*clfA clfB*
	Collagen binding (Collagen BP)	*cna*
Extracellular matrix binding	ECM binding protein	*ebh*
Extracellular adherence	Extracellular adherence protein	*eap*
Haemolytic toxins	Alpha	*hla*
	Beta	*hlb*
	Gamma	*hlg*
	Delta	*hld*
Leukolysin	PVL	*lukF lukS*
Extracellular enzymes	Staphylokinase	*sak*
	Catalase	*katA*
	Thioredoxin	*trxA*
Non-proteinaceous factors	Staphyloxanthin	*NWMN_0218*
Anti-lysozyme	O-acetyltransferase	*oatA*
Anti-inflammatory peptides	Chemotaxis inhibitory protein	*chp*
	Staphylococcal complement inhibitor	*scn*
Staphylococcal enterotoxins	Enterotoxin A-E	*sea-e*
	TSST-1	*tst*
Pro-inflammatory cell wall component	Lipoteichoic acid	*lta S*
	Wall teichoic acid	*wta*

1.2.3.1 HOST COLONISATION

In order to colonize a host and cause subsequent disease, the bacteria produce and regulate a wide range of pathogenic proteins in order to gain, and then maintain their environmental foothold. These proteins include cell surface proteins related to adhesion, factors that inhibit or subvert the innate immune system, or cytolytic toxins to allow further transgression into deeper tissue layers.

1.2.3.2 SURFACE PROTEINS

S. aureus produces more than 20 cell wall proteins capable of binding to a wide range of host cells and components of the ECM.[108] Termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), they serve a range of functions including the evasion of innate immunity as well as conferring the ability for bacteria to adhere to cell surfaces as an initiation of colonization or pathogenicity.

1.2.3.2.1 PROTEIN A

Staphylococcal Protein A (SPA) is a surface protein anchored to the peptidoglycan cell wall of *S. aureus*[109] that is known to bind to the constant fragment crystallisable (Fc) portion of circulating immunoglobulin G (IgG) antibodies *in vivo*. A consequence of this binding is that the IgG molecules are mis-oriented so that Fc receptors on polymorphonuclear leukocytes (PMNs) are unable to appropriately identify and phagocytose these organisms.[110] This may explain why protein A deficient strains of *S. aureus* are phagocytosed more efficiently by neutrophils *in vitro*[111] and show decreased virulence in animal models.[112] Protein A's anti-opsonic and anti-phagocytic capabilities are potent mechanisms to avoid destruction by the host innate immune system.

1.2.3.2.2 ADHESINS

As opposed to being anti-phagocytic, other MSCRAMMs, termed adhesins, are capable of binding to host proteins. Of these, the most widely characterised are fibronectin-binding protein A and B (FnBPA/B) and fibrinogen binding proteins called clumping factor A and B (ClFA/B). FnBPA has been identified as having a key role in the aetiopathogenesis of *S. aureus* mediated endocarditis[113] and ClFA is recognized to bind platelets and areas of tissue injury,[114] as well as being a virulence factor in *S. aureus* mediated arthritis.[115] ClFB is significantly associated with nasal colonization of *S. aureus* due to its ability to bind strongly to cytokeratin 10, a major component of keratinizing squamous epithelium found in the anterior nares.[116,117] Other molecules are also recognized to play roles in bacterial-cell adhesion dynamics including Staphylococcal binder of immunoglobulin (Sbi), coagulase, collagen binding-adhesin, ECM binding protein and extracellular adherence protein.[108]

1.2.3.3 SECRETED PROTEINS

Once the bacteria have secured an environmental niche, the next stage in its pathogenicity is to secrete exoproteins that directly interact with the local environment and host immune system. These may be cytolytic toxins that exhibit local pathogenic effects or factors that confer a protection from host elimination.

1.2.3.3.1 CYTOLYTIC TOXINS

S. aureus produces four cytolytic toxins termed alpha (α-), beta (β-), gamma (γ-) and delta (δ-) hemolysin, and a fifth called leukocydin.[118] Of these, the most widely studied is alpha-hemolysin, also known as alpha-toxin, which is expressed by the majority of *S. aureus* clinical isolates.[119] All the toxins exert their pathogenic effects by creating 1.1-2.5 nm non-

selective ion channels (pores) within cell membranes, thus allowing intracellular ions to leak out and cause apoptosis[120] or colloid osmotic cell lysis.[121] Alpha-toxin is the most potent of this group with well documented cytolytic, dermatotoxic and neurotoxic effects.[122] Beta-hemolysin is also known as sphingomyelinase C, and is toxic to cell types that express sphingomyelin. It has been demonstrated to play a role in lung injury[123] and can inhibit normal cilial activity in sino-nasal mucosal specimens.[124] Gamma-hemolysin is a bi-component toxin similar in structure to Panton-Valentin (PV) leukocydin. Both these toxins are constructed of two distinct proteins (S and F components) that, when bound together, exhibit their cytolytic capabilities. They have an important protective role for bacterial survival as they target host mammalian neutrophils and macrophages. Delta-hemolysin is another pore-forming toxin, capable of lysing erythrocytes and other mammalian cell types *in vitro,* as well as subcellular structures such as membrane-bound organelles, spheroplasts and protoplasts.[125]

1.2.3.3.2 PROTECTIVE PROTEINS

S. aureus secretes a number of proteins that exhibit protective properties, mainly to prevent degradation and elimination by host defences but also to increase its disease dissemination. Staphylokinase is a molecule that facilitates conversion of host plasminogen to plasmin and has multiple effects. Firstly, staphylokinase itself binds and inactivates components of the innate and adaptive immune system; human neutrophil peptides (HNPs) and α-defensins.[126] Secondly, plasmin is a potent enzyme that degrades extracellular matrix proteins such as fibrin, collagen and elastin.[127] By facilitating damage to the local environment, *S. aureus* can provide itself with further avenues of invasion and disease dissemination. Additional protective agents include antioxidants such as catalase,[128] thioredoxin[129] and staphyloxanthin[130]; the carotenoid pigment that gives *S. aureus* its

characteristic golden colour. These factors offer protection from the innate immune system by ameliorating the destructive effects of targeted reactive oxygen species.[131,132]

Another protein that has been described in *S. aureus'* impressive range of defence factors is O-acetyltransferase. This bacterial enzyme acts to confer resistance to lysozyme through acetylation of the peptidoglycan cell wall.[133] Therefore, even if the organism is phagocytosed, it is capable of protecting itself from destruction by the host innate immune system.

1.2.3.4 STAPHYLOCOCCAL ENTEROTOXINS

A different class of secreted proteins are known as staphylococcal enterotoxins (SE). They are defined separately from the hemolysin class of toxin as they exhibit their pathogenic effects by an immunomodulatory effect rather than a direct membrane lysis action. Since the first enterotoxin was described in the mid-20th century and designated Staphylococcal Enterotoxin A (SEA), over 10 further enterotoxins have been described,[134,135] each classified according to a standardised nomenclature (A, B, C, etc). To be classed as an enterotoxin, a number of criteria have to be met; (i) the ability to cause emesis and gastroenteritis in a primate model, (ii) superantigenic capability, (iii) intermediate resistance to heat and pepsin digestion, and (iv) tertiary structural similarity.[122] A further 10 toxins have been described that share the structural homology and superantigenic capabilities, however they do not exhibit emetic properties, and thus are referred to as staphylococcal enterotoxin-like toxins (SE-L) The most widely studied SE serotypes are A, B, C, D, E and toxic-shock syndrome (TSST)-1. Genes coding for SEs are located on mobile genetic elements including bacteriophages, pathogenicity islands, genomic island and plasmids.[104,136] The ease of transfer of these virulence factors between sub-species

using the above mechanisms has undoubtedly led to the diversity of *S. aureus* and as a consequence almost all human clinical isolates will contain one or more SE gene.

SE's exhibit their pathogenic effects through their capability of superantigenic T-cell stimulation. Superantigens are capable of bridging and bypassing the specific antibody-antigen complex between Major Histocompatibility Complex (MHC) Class II molecules in antigen presenting-cells (APC) and the T-cell receptor (TCR) on T-helper (Th) cells (Fig. 1.2),[137] thereby stimulating a massive polyclonal T-cell expansion and an overwhelming immunogenic response. The response stimulated is a predominantly inflammatory one, focusing on the stimulation of Th-1 cells leading to the production of cytokines such as IL-1, IL-2, IL-6, TNF-α and gamma-interferon. This undermines one of the underlying principles of the adaptive immune system; the specificity of its antigen presentation system. Therefore these toxins are often referred to as superantigens.

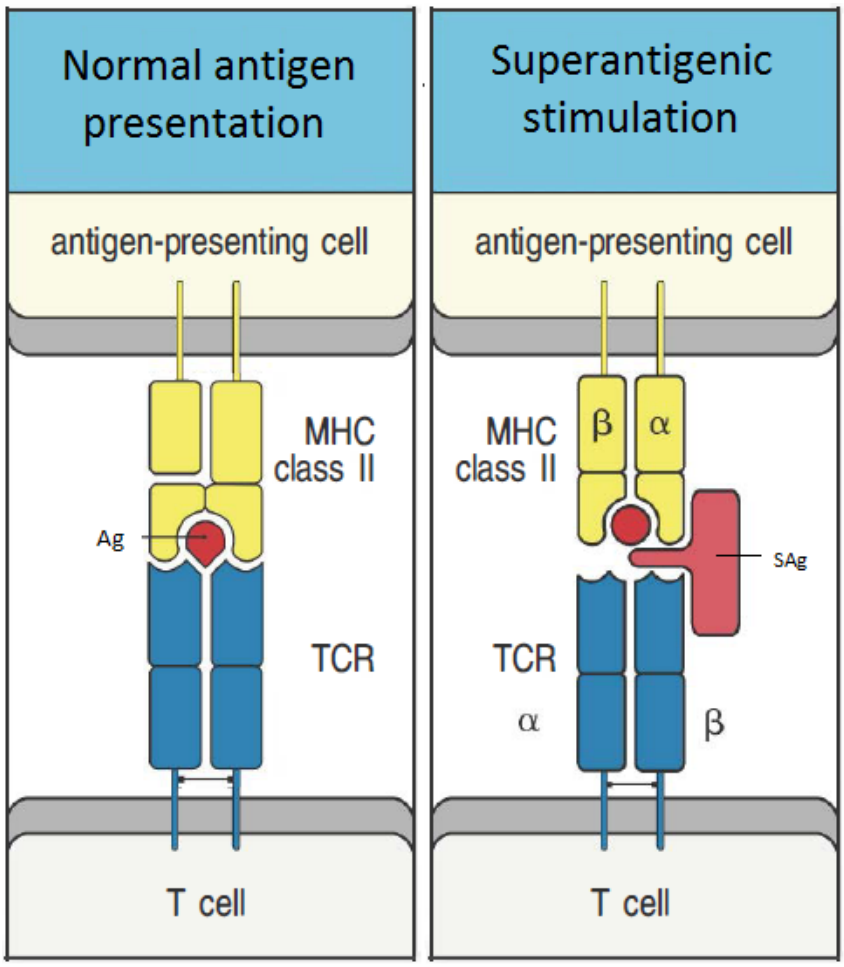

Figure 1.2: Comparison of antigen and superantigen binding to T-cell receptor (TCR); *left*: normal antigen presentation demonstrating the specificity required to bind all subunits of the MHC Class II and TCR; *right*: superantigen non-specific binding of MHC Class II α-subunit to TCR β-subunit. Yellow = MHC, Blue = TCR, Red = Ag or SAg. Copyright 2011 from *Janeway's Immunobiology,* Eighth Edition by Murphy et al. Adapted by permission of Garland Science/Taylor & Francis LLC.

1.2.4 *STAPHYLOCOCCUS AUREUS* INFECTION IN HUMANS

With the wide range of host-pathogen interactions defined, it is not surprising that *S. aureus* is such a versatile organism capable of causing significant morbidity to one individual and yet simultaneously colonising another with no deleterious effects. A wide range of illnesses can be attributed to invasive *Staphylococcal* infection (Table 1.4), and are summarised below.

Table 1.4: *S. aureus* related infectious processes in humans

Type of infection	
Skin related	Impetigo, boils, furuncles, folliculitis, abscesses
Deep-seated	Endocarditis, pneumonia, osteomyelitis, meningitis
Blood-borne	Bacteraemia, sepsis
Toxin-mediated	Food poisoning, Toxic Shock Syndrome

1.2.4.1 SKIN RELATED INFECTIONS

S. aureus is the leading cause of superficial infections such as impetigo, boils, furuncles and folliculitis. Typically a breach in the epidermis or epithelial mucosal barriers will allow *S. aureus* to gain a minor foothold where it begins to exhibit its pathogenicity. Localised tissue putrefaction may lead to the formation of abscesses, which are collections of bacteria and dead cells including ECM, neutrophils and macrophages.

1.2.4.2 DEEP-SEATED INFECTIONS

When the host fails to deal completely with a localised infection, the bacteria may disseminate by using the blood and lymphatic systems, or via loco-regional spread. Alternatively, *staphylococci* may invade an internal organ directly, for example via the upper respiratory tract or surgical wounds. Practically any organ can be affected, and *S. aureus* endocarditis, pneumonia and osteomyelitis have all been described.

1.2.4.2.1 ENDOCARDITIS

Infectious endocarditis results from fungal or bacterial infection of the endocardial surface of cardiac valves[138] with *S. aureus* being the leading bacterial pathogen, occurring in 31% of cases.[139] The majority of cases occur in native valves; however, external prosthesis such as mechanical and biomechanical valves can act as foci for bacterial attachment in up to

30% of cases. Up to 70% of cases are also related to intravenous drug use (iatrogenic or illicit). Once the bacterium attaches itself to the valve, platelet and thrombus aggregations form around the multiplying bacteria, known as vegetations. These can cause valvular rupture through direct local injury or detach themselves and cause metastatic infection in the lungs, kidneys or brain.

1.2.4.2.2 PNEUMONIA

S. aureus may gain entry to the lungs either through inhalation or blood-borne spread. In community acquired pneumonia it is considered a minor pathogen responsible for only 5.5% of cases, with *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* being the major organisms.[140] However, when we consider subgroups of patients who have already suffered another form of physiological insult, we can see that *S. aureus* plays a much more aggressive role. Up to 36.5% of hospital acquired pneumonias can be attributed to *S. aureus*[141] and alarmingly, 53% of these cases are antibiotic resistant strains.[142] This clearly highlights the ability for the organism to take advantage of a weakened physiological state in gaining a foothold to then cause significant further pathology.

Cystic fibrosis (CF) is a pulmonary condition caused by a defective gene that leads to abnormal chloride and sodium transport across the epithelium. Although it affects numerous organs through different means, a significant consequence is that cilia lining the airway do not function correctly, and thick, mucoid secretions accumulate within the lungs. Secondary bacterial infections are extremely common, with up to 68% of patients being persistently colonized with *S. aureus.*[143]

1.2.4.2.3 OSTEOMYELITIS

S. aureus is by far the most prevalent organism in osteomyelitis, being cultured in almost 80% of cases.[144,145] Osteomyelitis is an infectious process that is defined as bone infection secondary to trauma, surgery and joint prosthesis, vascular insufficiency or spread from haemotogenous origins.[146] Antibiotic resistance is a major problem[147] and foci of infection can remain within sequestered bone making this condition very difficult to treat.

1.2.4.3 BLOOD-BORNE INFECTION

If *S. aureus* is able to disseminate itself into the blood stream it can have devastating consequences. The frequency of *S. aureus* bacteraemia and sepsis is rising, with an estimated 26% of cases linked to this organism.[148] Mortality rates are high at between 20-25%, with factors such as antibiotic resistant strains, immunocompromised hosts and the need for invasive medical equipment such as central venous catheters all playing a part.[149] Sepsis and septic shock arise as a consequence of massive pro-inflammatory cytokine secretion with an overload of the immune system and multi-system organ dysfunction.

1.2.4.4 TOXIN-MEDIATED INFECTIONS

Amongst bacteria involved in food-poisoning related illness, *S. aureus* is the most commonly related organism.[150] Symptoms do not result from direct interactions between the *staphylococci* and host, rather contamination of food and drink by the superantigenic SEs. As these proteins are stable against heat, moderate cooking may not be sufficient to denature them, and their resistance to pH and pepsin can make them impervious to both acidic and enzymatic degradation in the upper gastrointestinal tract.[134] The incubation period is often short, between 1-6 hours, which is followed by a rapid onset of symptoms including nausea, vomiting and diarrhoea. However, illnesses are often short-lived and

self-terminating with minimal morbidity and mortality rates of only 0.03% in immunocompetent individuals.[151]

On the contrary, toxic shock syndrome (TSS) is another toxin mediated infection with potentially fatal consequences in around 5% of cases.[151] First described in menstruating females, it has since been associated with other diseases such as influenza, sinusitis, tracheitis, intravenous drug use, HIV infection, cellulitis, burn wounds, allergic contact dermatitis and postoperative wound infections.[152] After an initial infectious insult, typically from a hospital-acquired source, the patient's condition will rapidly deteriorate with fever, central nervous system dysfunction, renal complications, superficial desquamation and profound hypotension as a result of cytokine mediated systemic vasodilatation. The early recognition and effective treatment of this condition has reduced the mortality significantly.

1.3 BACTERIAL BIOFILMS

1.3.1 DEFINITION

The first historical description of the bacterial form that is now understood to be a biofilm was in the 17th century by the Dutch microbiologist Anton van Leeuwenhoek, who took scrapings of dental plaque and examined them using his rudimentary microscope. He was able to identify "animalcule", translatable from Latin as "lowly or tiny animals". However, he was unable to delve further into its potential impact thus the early description of the biofilm form became ignored. Subsequent microbiologists, later driven by the doctrine of Koch's postulates, shifted focus to the planktonic or free-floating form of bacteria believed to be the cause of acute disease. It was not until the 1970's when the concept of bacterial biofilms re-emerged when Costerton et al[153] described how organisms produce a complex mass of tangled polysaccharide molecules as a mechanism of attaching themselves to surfaces or to other cells. Initial research focused on the environmental impact of biofilms; however, its translation to medical research initiated a paradigm shift in the understanding of infectious disease processes. Over 17000 articles have been published on biofilms since Costertons initial article, with over half of these since 2008.

As a result of advances in the understanding of biofilms, its definition is constantly evolving. Initial attempts at defining biofilms focused on static bacterial populations encased in their extrapolymeric matrix.[154,155] However, as the understanding progressed it became recognised that biofilms were not a static homogenous bacterial population, but rather a collection of organisms exhibiting temporal heterogeneity with phenotypic diversity and altered physiological attributes. Therefore the recognised definition of a biofilm is "*a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a*

matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription".[156] This encompasses the essential aspects of biofilms that have direct relevance to their role in acute and chronic disease processes.

1.3.2 STRUCTURE, FORMATION AND LIFE-CYCLE

To conceptualise the biofilm ultrastructure is essential in understanding its abilities to behave pathologically and the mechanisms by which it provides protection in hostile environments. Microorganisms secrete a collection of extracellular polymeric substances (EPS) which provide the building blocks necessary for a biofilm to form. The EPS is responsible for creating the microenvironment affecting porosity, density, water content, charge, hydrophobicity, and mechanical stability.[157] Although initially referred to as an exo- or lipo-polysaccharide matrix,[155] the polysaccharide component is, in fact, but a small component with proteins, glycoproteins, glycolipids and extracellular DNA all combining to constitute the matrix.[158]

Biofilm formation is understood to be a regulated procedure which converts free-floating planktonic bacteria into their adherent form[159] as a response to specific environmental cues[160] that may vary according to bacterial species. The first step occurs when bacteria develop a reversible association to a surface in a process dependant on many factors including properties of the cell surface, the hydrodynamics of the aqueous medium and the bacteria themselves.[161,162] If the bacteria determine that an environmental advantage may be gained by remaining in place, a series of bacteria-bacteria communications known as Quorum Sensing (QS) begins.[163] Signalling molecules are secreted by the bacteria that, when reaching a specific threshold, trigger receptors on their neighbours to initiate a

cascade of transcriptional gene changes[164] leading to production of EPS, irreversible attachment and the formation of bacterial microcolonies. These gene changes can occur incredibly rapidly, even within 15 minutes of initial cell adhesion.[165]

The next stage of development is known as biofilm aggregation and maturation, in which the biofilm EPS organises itself to a complex three-dimensional structure with channels, pores and a redistribution of encased bacteria.[166] Proteomic studies have shown that the highest number of gene changes occurs in this stage of biofilm development[167,168] which are, in the most part, responsible for the altered phenotypic biofilm characteristics.

The final stage of the biofilm lifecycle is when detachment or dispersal occurs. Single organisms or groups of cells may separate from the main biomass[169] as a result of the biofilm reaching a critical mass or from alterations in the micro-environment. The proteomic characteristics of detached organisms are more analogous to that of planktonic bacteria rather than those within the maturing biofilm,[167,170] with a similar virulent phenotype and the capability of forming new, distant biofilms; thus the life cycle of the biofilm is complete.

1.3.3 FUNCTION AND PROPERTIES

The biofilm exists to provide an environmental advantage for the bacteria held within. This has been demonstrated both *ex vivo* and *in vivo*, with factors such as a resistance to disinfectants,[171,172] surfactants,[173] phagocytosis[174] and antiobiotic therapy[175-178] all combining to provide mechanisms by which it can remain a continuing nidus of living bacteria long after host factors and antimicrobials have eradicated planktonic organisms.[155] In addition to advantages from the secreted EPS, bacteria themselves undergo significant

phenotypic alteration with genes responsible for structure, metabolism, virulence and nutrient transport all changing when bacteria are enclosed in a biofilm.[179]

As previously discussed, the diversity of bacterial pathogenicity and antibiotic resistance genes is due, in part, to horizontal gene transfer. Biofilms are polymicrobial in nature, capable of hosting a broad spectrum of bacterial genera and species.[180] It is believed that these polymicrobial biofilms potentiate HGT and the formation of new strains with novel combinations of genes.[101]

1.3.4 IDENTIFICATION TECHNIQUES

The phenotypic changes in the metabolism of bacteria encased in biofilms have rendered traditional culture techniques inadequate for the detection and species identification of biofilms. Major technological advancements in microscopic imaging have undoubtedly played a part in the modern understanding of biofilms. Although van Leeuwenhoek used his crude light microscopes to visualise what he thought were bacterial biofilms, the true appreciation of the structure only came about after the development of the electron microscope (EM). For the first time, scientists were able to image on the sub-cellular level and identify the three dimensional structures bound to surfaces. Scanning electron microscopy (SEM) allows for a highly magnified topological analysis of both inert and biological surfaces (Fig. 1.3), whereas transmission electron microscopy (TEM) is used on ultra-thin tissue sections in order to image the sub-cellular ultrastructure. Despite this, limitations still remained in their use for imaging biofilms. Although SEM may visualise the characteristic shape of a bacterial genus, definitive species identification may be impossible.[181] Due to the incredibly thin sections imaged by TEM (as fine as 50 nm), a significant risk of sampling error exists, as well as providing difficulty in recognising the

true ultrastructure of the biofilm. Furthermore, these imaging modalities suffer from limitations of the processing technique which requires a number of dehydration steps in graded ethanol, acetone and xylene. As the biofilm EPS matrix is extremely hydrated, constituted of up to 95% water, significant processing artefacts and distortion may occur making true interpretation difficult.[156]

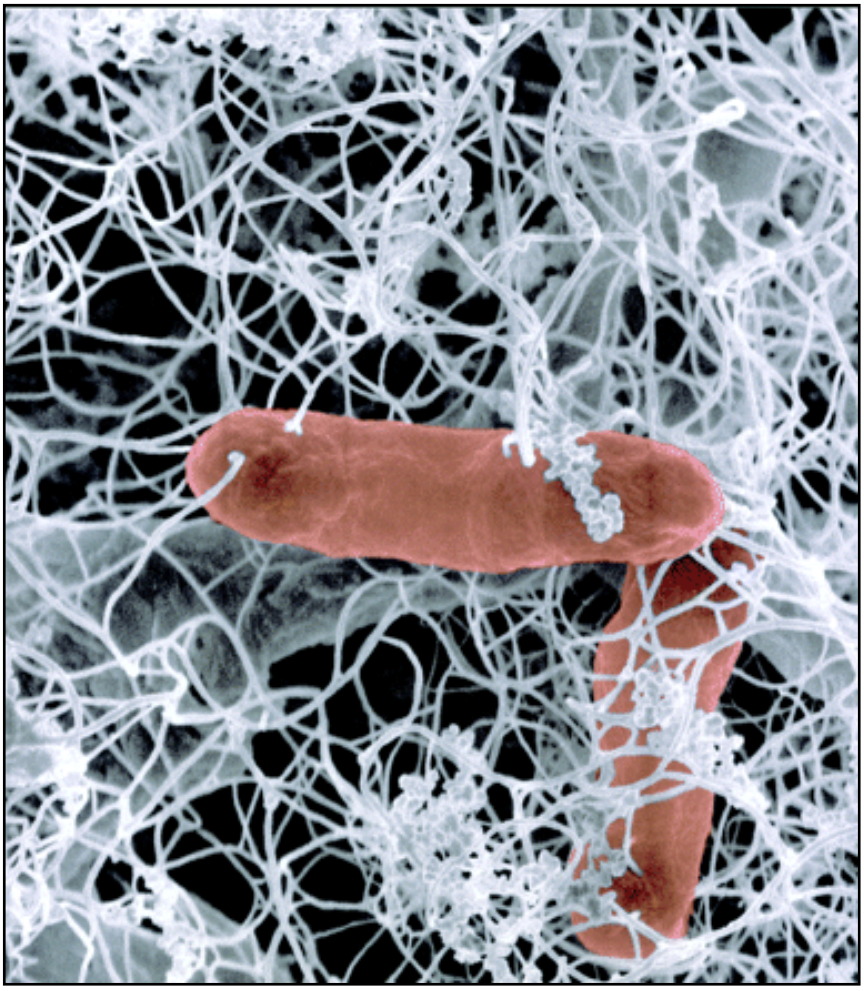

Figure 1.3: Scanning electron micrograph of a bacterial biofilm demonstrating the organism (pink) surrounded by extrapolymeric matrix. Reproduced with permission from "Applied and Environmental Microbiology", vol. 48(1).

The next advance in imaging was the introduction of the confocal scanning laser microscope (CSLM). CSLM allows for the capture of high resolution optical or fluorescent images from fully hydrated tissue samples, with the key capability of acquiring an ultra-fine plane of focus within the sample. By the use of a pinhole to remove out of focus light and being able to select the imaging depth, it is possible to create a three dimensional image reconstruction from multiple consecutive images. Using these imaging techniques, researchers were able to understand the complex biofilm ultrastructure.[182]

The second advancement that facilitated the use of CSLM in biofilm detection has been in the development of molecular fluorescence in situ hybridisation (FISH) probes. FISH probes are designed from peptide nucleic acid (PNA) sequences that are stable, resistant to enzymatic degradation and capable of crossing a cell membrane.[183,184] By creating a complementary nucleic acid probe that targets the highly conserved 16S subunit of bacterial ribosomal RNA (rRNA), it is possible to identify even a single bacterium.[185] Each sequence will be labelled with a specific fluorophore so that it can be identified with an epifluorescence microscope (Fig. 1.4). Thus, when coupled with the CSLM, FISH techniques allow for quantitative[186] and qualitative[187] imaging of biofilms on whole mucosal samples and inert surfaces.[188]

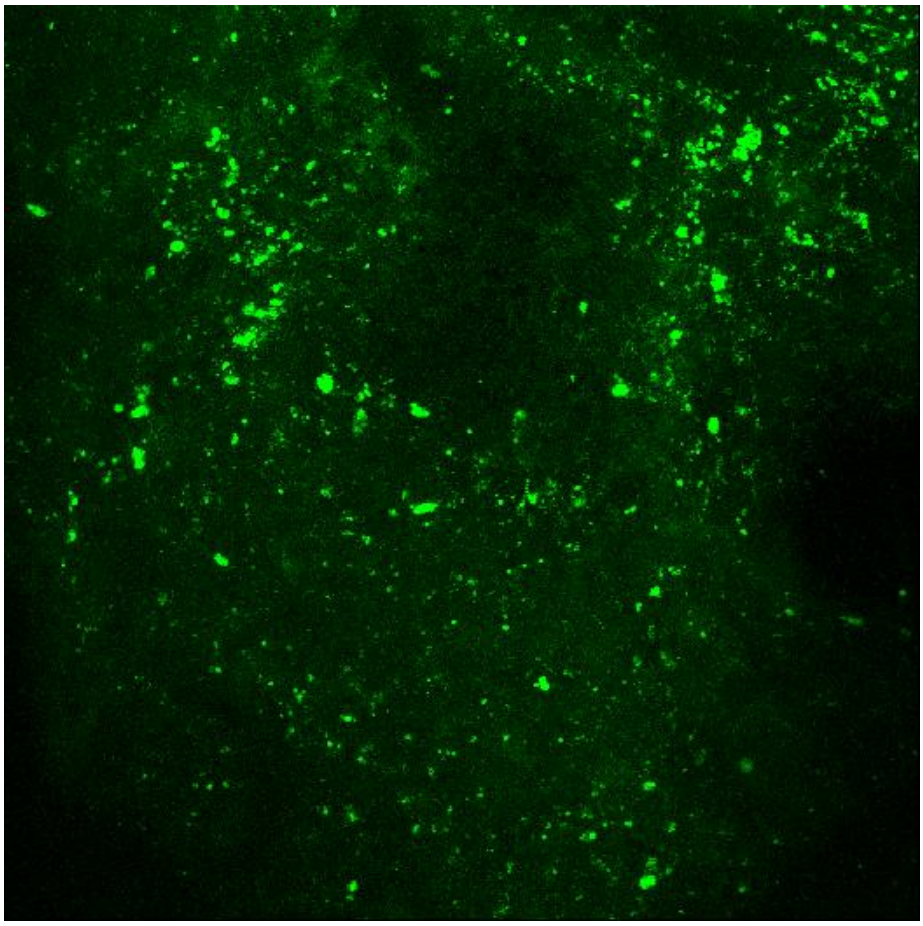

Figure 1.4: CSLM image of *S. aureus* biofilm labelled with FISH probe demonstrating brightly fluorescing organisms (green) surrounded by a "blush" of extrapolymeric matrix.

1.3.5 BIOFILMS IN DISEASE

The difficulty in attributing bacterial causation to biofilm-related disease stems from the reliance on Koch's Postulates, with a key limitation in being able to isolate pure cultures from affected individuals. As previously discussed, a fundamental part of biofilm pathogenicity is to decrease bacterial metabolism, which therefore causes inconsistencies in culture rates. With the advent of molecular identification techniques that unequivocally identify the presence and metabolic activity of bacteria in what are otherwise un-cultureable sites,[189,190] it was clear that a paradigm shift had to occur in the conceptualisation of the relationship between bacteria and disease. To this end, Parsek and

Singh[191] established a set of criteria to more clearly define biofilm-related pathogenesis; (i) pathogenic bacteria are adherent to a surface, (ii) direct examination of infected tissue shows bacteria living in cell clusters, or microcolonies, encased in an extracellular matrix, (iii) the infection is localised, although dissemination may occur as a secondary phenomenon, and (iv) the infection is difficult or impossible to eradicate with antibiotics despite the antibiotic sensitivity of planktonic bacteria.

Biofilms have therefore been attributed to infections of both biological tissue and artificially implanted devices including orthopaedic joints,[192] cardiac devices such as pacemakers,[193] urinary catheters[194] and even prosthetic ocular lenses.[195] Human tissues act as ideal, nutrient rich surfaces for bacterial attachment and it is increasingly recognised that biofilms contribute to pathogenicity in diseases that typically suffer from relapsing and remitting course, such as CF,[196] osteomyelitis[197] and endocarditis.[158]

1.3.6 BIOFILMS IN CRS

The reasons why researchers and clinicians no longer believed in a bacterial aetiopathogenic role in chronic diseases such as CRS have been discussed, and even now controversy remains. However, as knowledge of the pathogenesis of biofilms has evolved, so has the conceptual understanding of the role it may play in CRS. Since the first report associating biofilms to CRS in 2004 by Cryer et al,[198] over 150 scientific articles have documented the presence of biofilm and its interactions with host tissues.

Techniques including SEM, TEM and CSLM have identified a high prevalence of biofilms in CRS patients but not healthy controls.[32,187,199,200] Studies have reported the clinical relevance of biofilms in CRS, with biofilm positive patients having more severe disease

preoperatively (objectively and radiologically),[201] and in the postoperative period these patients required more outpatient visits as well as scoring significantly worse on quality of life and symptomatology scales.[202]

In addition to clinical evidence of the effect that biofilms may have on outcomes, molecular evidence of cellular alterations caused by biofilms has been reported. Numerous genetic regulatory changes have been identified in biofilm infected patients including changes within the innate immune system,[203,204] and an altered response of the adaptive immune system.[205] Although there is much work to be done, it does appear that biofilms can have a profound effect on the infected host.

As evidence for the role of biofilms in CRS has increased, coupled with the recognition that current therapeutics do not appear effective in their treatment, researchers have also focused on developing mechanisms for biofilm eradication. These treatments can broadly be divided into biofilm dispersal or biocidal agents. Biofilm dispersal may be either chemical or mechanical. The use of high-pressure, pulsed saline has been suggested as a method of physically disrupting the biofilm, and has been tested in conjunction with a chemical surfactant; citric acid/zwitterionic surfactant (CAZS).[206] Although effective at treating biofilms in an animal model, the chemical agent caused significant deciliation and therefore its use was discontinued. Whether the pressurised saline when used alone is suitably efficacious in removing the entire biomass of an infection is as yet, not tested. Other surfactant agents have been suggested including ones derived from baby shampoo,[207] and although evidence is mounting as to their safety profile,[208,209] no concrete proof exists for their efficacy. Clearly this is an area of anti-biofilm therapy that may benefit from further investigation.

A number of biocidal agents have been tested *in vitro* and demonstrated to effectively reduce the biomass of a biofilm. Mupirocin is an antibiotic agent with well known anti-*Staphylococcal* properties.[210,211] Its efficacy against *S. aureus* in its planktonic and biofilm forms *in vitro* is not questioned,[212] and although early studies demonstrated its effectiveness at decolonising the nose of *S. aureus* infected patients,[213,214] other clinical trials to date have failed to meet expectations. Recently, a trial investigated the efficacy of mupirocin rinses to treat *S. aureus* positive, recalcitrant CRS in a prospective double-blinded, placebo controlled trial.[215] In this study, 25 patients who had previously failed medical and surgical therapy were randomised into two blinded treatment arms. The first group received a 1 month, twice daily treatment course of 0.05% mupirocin diluted in saline and the control arm received saline flushes and oral antibiotics. In the mupirocin treatment arm, 11 patients were enrolled but only 6 reached the end point predominantly due to the prescription of oral antibiotics for other conditions by different physicians or withdrawal from the study. Of these 6 patients, 5 had eliminated their *S. aureus* infections in the short term. This was associated with an improvement in the appearance of the nasal cavity on endoscopy and a small benefit in patient-reported subjective measures of disease severity. As this trial was only a short term one, it is impossible to make conclusions on the long-term benefits. Another study investigated whether the anti-*S. aureus* effects of mupirocin treatment were long-lasting or not.[216] In this retrospective study of 61 *S. aureus* positive CRS patients treated with mupirocin, over 70% of patients suffered a long-term microbiological failure after cessation of treatment. These two studies highlight the fact that even after elimination of *S. aureus*, patients will become re-colonised by bacteria. Manuka honey and its active ingredient methylglyoxal, has been long used for its antimicrobial properties. It has well documented anti-bacterial effects,[217-219] however in the only clinical trial thus far, these effects did not translate to an improvement in patient

outcomes.[220] Limitations in that trial were significant, however, in that the study only recruited patients with allergic fungal rhinosinusitis with no attempts at biofilm detection and thus no conclusions can be made as to its efficacy in treating biofilm-related CRS.

Although research into the treatment of biofilms is in its infancy, the failure of anti-biofilm therapies to produce clinically relevant improvements in early trials raises certain questions. Firstly, whether the test agents themselves are fully optimised is unknown. While these treatments may eradicate a significant proportion of the biofilm biomass, it is certainly possible that small foci remain that may allow regrowth of bacteria on cessation of treatment. Secondly, it is unlikely that complete sinus mucosal coverage occurs when applying these topical agents to the post-operative sinuses, and therefore there may be areas of untreated mucosal biofilm. Whether novel anti-biofilm agents or topical delivery devices may improve upon these aspects is unknown. Thirdly, the question as to whether bacteria may utilise other protective mechanisms in order to evade or shield themselves from topical treatment has been raised. In recent years it has been identified that pathogens such as *S. aureus* can invade non-professional phagocytic cells as a potential mechanism of persistence in chronic disease. The intracellular location may provide bacteria with environmental advantages due to avoidance and resistance of topical and systemic antimicrobial therapies,[221] and induce phenotypic changes that confer protection from the host innate immune system.[222] Furthermore, these intracellular bacteria may act as reservoirs of infection to seed recurrent rhinosinusitis infections.[223] As discussed previously, topical anti-*S. aureus* medications may be effective in the short term, but relapse of disease occurs in a significant proportion of CRS patients. Whether intracellular invasion of sinonasal epithelium by *S. aureus* could play a role in recalcitrant CRS is

unknown, and therefore a source of research interest. The mechanisms and concepts of intracellular bacterial pathogenicity will be discussed in the following sub-chapter.

1.4 INTRACELLULAR BACTERIA

1.4.1 DEFINITION

Bacterial pathogens have been exploiting the intracellular environment since before Hooke discovered the cell in 1665. Mechanisms of bacterial internalisation depend on the organism in question and the type of host cell. Organisms may be facultative intracellular pathogens in which they are able to replicate either inside or outside cells, or obligate intracellular pathogens in which they can only replicate inside infected cells. The latter highlights one of the major limitations in applying Koch's Postulates to bacterial causation in certain intracellular infectious diseases where organisms are incapable of being grown in pure culture outside a host. An example of this is *Mycobacterium leprae (M. leprae)*, the organism responsible for leprosy. Historical specimens have suggested that leprosy has affected humans for 4000 years, however, it was only in 1873 that *M. Leprae* was identified and since then it has still never been grown in pure culture due to the lack of necessary genes for independent growth. Modifications in culture technique had to be applied and the organism has since been cultured in an animal model.

Intracellular pathogens are responsible for a wide range of pathological processes including respiratory, gastrointestinal and genitourinary infections. In these diseases the offending bacteria are usually engulfed by professional phagocytes such as macrophages, neutrophils, monocytes or dendritic cells before the host cellular machinery is subverted for the bacteria's own use to initiate its pathogenic effects. The ability to evade host immunity and establish protective niches, coupled with antibiotic resistance mechanisms are critical steps in the pathogenesis of intracellular infections.

1.4.2 MECHANISM OF INTERNALISATION

The plasma membrane is the barrier that separates the intracellular, cytoplasmic setting from the surrounding extracellular space. Rather than being a rigid structure, the mammalian cell membrane is a dynamic one whose function is also to coordinate the uptake and removal of small and large molecules.[224,225] Although ions and small molecules are transported through specialised channels or protein complexes, entry of larger macromolecules or cellular structures is broadly divided into two categories; "pinocytosis" or cell drinking and "phagocytosis" or cell eating.[224]

1.4.2.1 PINOCYTOSIS

Pinocytosis involves so called "cell ruffling", a process by which small extracellular fluids are actively taken into a cell. A number of methods have been described which involved non-specific binding or the close association of solutes to the cell membrane (adsorptive pinocytosis)[224] or specific receptor-mediated binding (receptor-mediated endocytosis).[226] Subsequently, the solute invaginates the cell membrane before the ruffles collapse to form a small endocytic vesicle enclosing the solute and a portion of extracellular fluid (ECF) (Fig. 1.5).

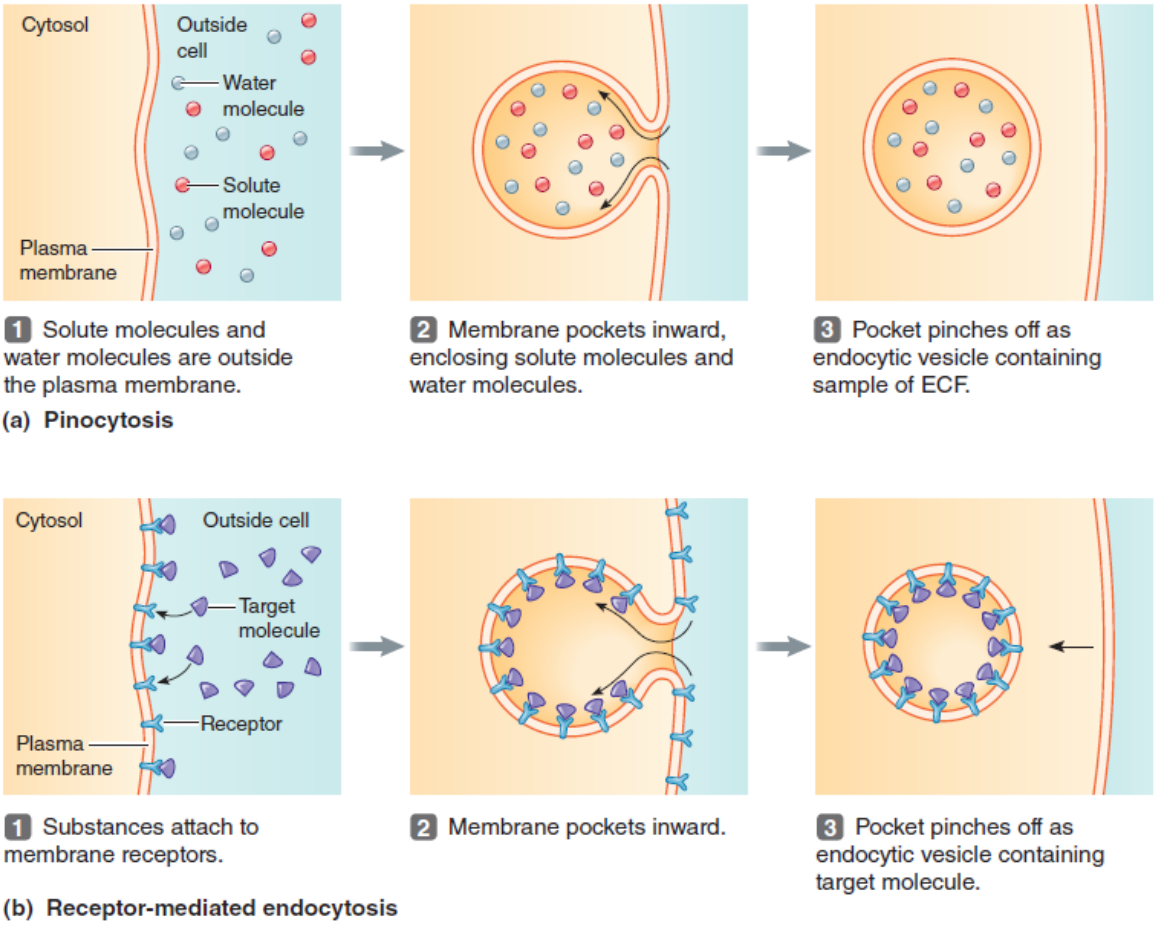

Figure 1.5: Pinocytosis and receptor-mediated endocytosis: *(a)* Representation of pinocytosis; the surface membrane dips inwards to form a pouch, then seals the surface, forming an intracellular endocytic vesicle that nonselectively internalises a portion of ECF. *(b)* Representation of receptor-mediated endocytosis. When a target molecule attaches to a specific surface receptor, the membrane pockets inwards and then pinches off to selectively internalise the molecules in an endocytic vesicle. From Sherwood. Human Physiology, 7E. © 2010 Brooks/Cole, a part of Cengage Learning, Inc. Reproduced by permission. www.cengage.com/permissions.

1.4.2.2 PROFESSIONAL PHAGOCYTOSIS

Professional phagocytes are fundamental components of the innate immune system, and by their very nature are specialised in their task to engulf and attempt to eliminate pathogens or dead-cell debris.[227] Their specificity relies on the ability for phagocytes to identify self

from non-self,[228] else inadvertent host cellular degradation might occur with disastrous consequences. Macrophages express pattern recognition receptors (PRR) to sense their local environment[228] as well as cell surface receptors (CSR) such as CD16, CD32, CD64 and CD23, responsible for pathogen recognition and phagocytosis.[229] Pathogens either bind directly to CSRs,[230] or are opsonised when circulating antibodies within the extracellular space bind the pathogen to the CSR,[231] triggering signalling cascades that rearrange the actin cytoskeleton.[227,232] Two mechanisms have been described; the trigger or zipper mechanism phagocytosis.[233]

The trigger mechanism has been likened to a form of macropinocytosis in which cellular binding initiates actin driven membrane ruffling coupled with invagination of the pathogen to form a macropinocytic pocket.[234] As these extend they may or may not fully enclose the bound organism depending on whether the terminal projections fuse, however, if this occurs the target pathogen is internalised within a relatively large spaced vacuole.

The zipper mechanism is the more commonly utilised technique of how phagocytes internalise bacteria with most of the mechanistic understanding coming from studies of the Fc receptor (FcR) pathway. CSR and bacterial ligand binding initiates the extension of cell membrane pseudopods to progressively envelop the target. In this case, circumferential ligand binding is necessary in maintaining the drive to engulf the bacteria[230] (Fig. 1.6). Studies have demonstrated that full circumferential expression of bacterial ligands are the critical part of zipper phagocytosis. Early in the 1970's, Griffin et al[235,236] demonstrated that lymphocytes diffusely coated with ligand were effectively phagocytosed by macrophages, however when only half the lymphocyte was coated, they remained bound to the macrophage but not internalised.

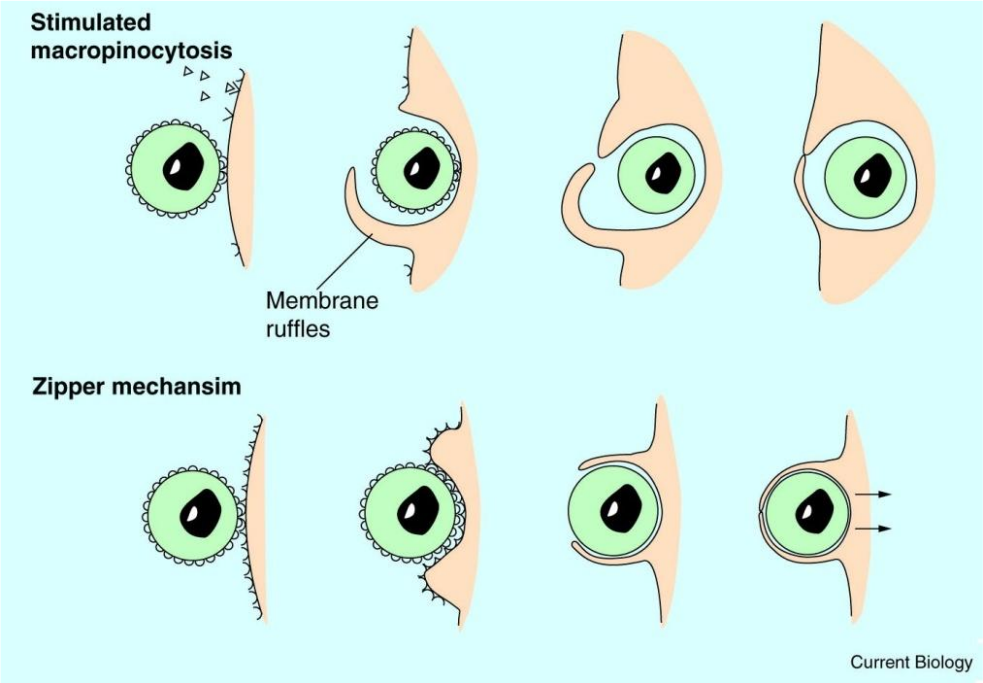

Figure 1.6: Macropinocytosis and zipper mechanisms of internalisation; *Top*: Diagram of stimulated macropinocytosis, or the trigger mechanism in which specific binding initiates actin driven membrane ruffling, invagination of the pathogen to form a macropinocytic pocket. Subsequent collapse or connection of membrane ruffles encloses the pathogen in a large vacuole. *Bottom*: Diagram of the zipper mechanism of phagocytosis that requires specific CSR and bacterial ligand binding coupled with pseudopod extension to engulf the pathogen in a tight vacuole. (Adapted from "Current Biology", vol. 11, Henson PM, Bratton DL and Fadok VA, Apoptotic cell removal,[237] with permission from Elsevier).

Following phagocytosis of a target pathogen into a professional phagocyte, a series of maturation steps occur with binding of late endosomes to form a mature phagosome and ultimately fusion with lysosomes to form phagolysosomes. Lysosomes release hydrolase enzymes and acidify the phagolysosome in order to attempt to deal with the unwanted intruder.

1.4.2.3 NON-PROFESSIONAL PHAGOCYTOSIS

Although phagocytosis was initially considered to be a function of professional phagocytic cells,[227] internalisation of bacteria by non-professional phagocytes[238] such as epithelial or endothelial cells has been recognised in recent years. Using *in vitro* cell culture models, bacterial pathogens have been recognised to internalise within numerous cell types including epithelium, endothelium, osteoblasts and fibroblasts (Table 1.5).

Table 1.5: Non-professional phagocyte cell types capable of internalising bacteria

Cell type	References
Epithelium	222,239-241
Endothelium	242-244
Osteoblast	144,221,245,246
Keratinocyte	247

In order to counteract the effectiveness of the innate immune system, pathogens have developed intrinsic mechanisms of host immune evasion. These may be factors that prevent phagocytosis or protect the organisms from host degradation and will be discussed in the following section with specific reference to common intracellular pathogens.

1.4.3 FUNCTION AND PROPERTIES

There are numerous advantages that the intracellular location may provide to an invading organism. It may offer a protective environment where organisms are shielded from the innate immune system or antimicrobial therapy. By internalising into non-professional phagocytes, *S. aureus* can hide its presence from professional phagocytes that exist in the extracellular spaces.[248] A number of reasons exist as to why intracellular organisms

demonstrate a relative resistance to any given antibiotic dose or extracellular concentration. Certain antibiotics, aminoglycosides for example, cross the membrane of eukaryotic cells very poorly or if endocytosed remain sequestered within intracellular vacuoles.[249] Efflux pumps may actively expel other antibiotics that can enter cells such as β-lactams or macrolides.[250] As a consequence, the intracytosolic concentration of antibiotics may be too low to effectively kill internalised organisms. In addition to the poor intracellular action of certain antibiotics, even those known to concentrate within the cell cytoplasm may be ineffective. Studies have demonstrated that *S. aureus* acquires a remarkable resistance to rifampicin in response to an osteoblastic intracellular environment *in vitro*, thought to be due to a significant thickening of its cell wall.[221]

The capability of *S. aureus* to alter itself from an innocuous coloniser to a dangerous pathogen has already been recognised, and evidence is mounting that the intracellular milieu provides environmental pressures to induce additional phenotypic alterations. Whole genome transcriptional changes have been described within *S. aureus* following internalisation into epithelial cells *in vitro,* including a profound downregulation of genes controlling metabolic functions and transport, suggesting that the bacteria enters a quiescent or dormant phase as a response to internalisation.[222] This is accompanied by a downregulation of virulence genes and believed to be why intracellular bacteria may persist in non-professional phagocytes for days or even weeks.[251]

1.4.4 IDENTIFICATION TECHNIQUES

When attempting to detect intracellular bacteria, a number of methodological limitations exist when considering traditional culture techniques. Although it is understood that swabs may dislodge and remove surface epithelial cells, any cultured bacteria are likely to be

from surface pathogens. It is possible to culture whole biopsy specimens; however, it would be difficult to isolate intracellular bacteria without contamination from those that may exist on the surface as well. Previously discussed alterations in bacterial metabolism may cause delays in growth, and contamination with normal wild-type surface bacteria may lead to the overgrowth of bacterial strains that may be residing intracellularly.

Imaging modalities that made biofilm identification possible can be used for intracellular detection in swab or biopsy specimens. TEM is an excellent tool, able to visualise the ultrastructure of cells and their contents. The processing steps that may disrupt surface biofilms will not affect intracellular bacteria and a significant volume of work has used this technique to study both the mechanisms of internalisation and the identification of intracellular bacteria *in vitro* and *in vivo*[240,243,252] (Fig. 1.7).

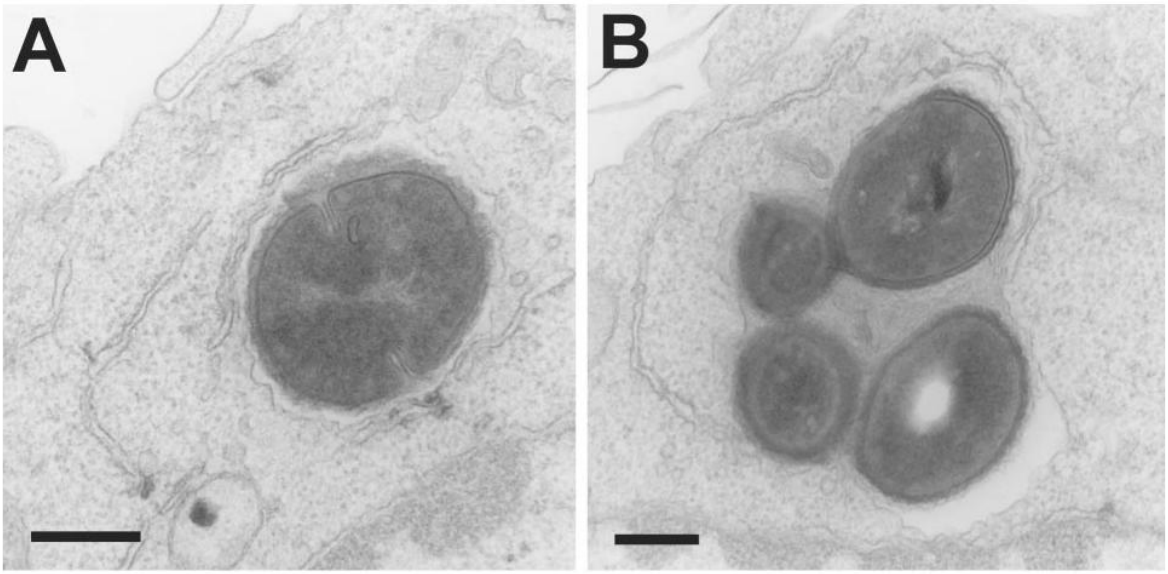

Figure 1.7: TEM images demonstrating intracellular *S. aureus* (A) engulfed within vacuoles in isolation, and (B) in clusters. Bars are 0.3 μm. Adapted with permission from Seral et al.[253]

CSLM microscopy techniques utilised for biofilm detection are readily transferrable for use in intracellular detection. Optical depth selection, when used with appropriate nucleic acid counterstains can inform the investigator whether the plane of imaging is superficial to, or within an intracellular plane of focus. When coupled with FISH probes, CSLM has been used to identify *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in epithelial cells harvested through buccal swabs[254] and to identify intracellular pathogens in the middle ear mucosa of children with recurrent otitis media.[255] The ability to image whole mucosal specimens in addition to its validity for imaging biofilms makes CSLM and FISH a useful tool that may be adapted to investigate our patient population.

Immunohistochemistry (IHC) techniques can also be performed on both mucosal biopsies and cell culture specimens for the identification of intracellular pathogens. After mucosal samples are fixed either in paraffin or a frozen medium such as optimal cutting medium (OCT) they are sectioned into thin slices (down to 3 μm) and mounted onto glass slides. Cells may be fixed directly onto culture chamber slides for experimentation and imaging. Specific antibodies are then used to target protein epitopes such as those expressed on the surface of *S. aureus* or by components of the cellular structure. Samples may be stained with dual antibodies as well as nucleic acid counterstains for accurate localisation of bacteria (Figs 1.8 and 1.9). Imaging may be with either CSLM or traditional upright immunofluorescence microscopy (UIFM).

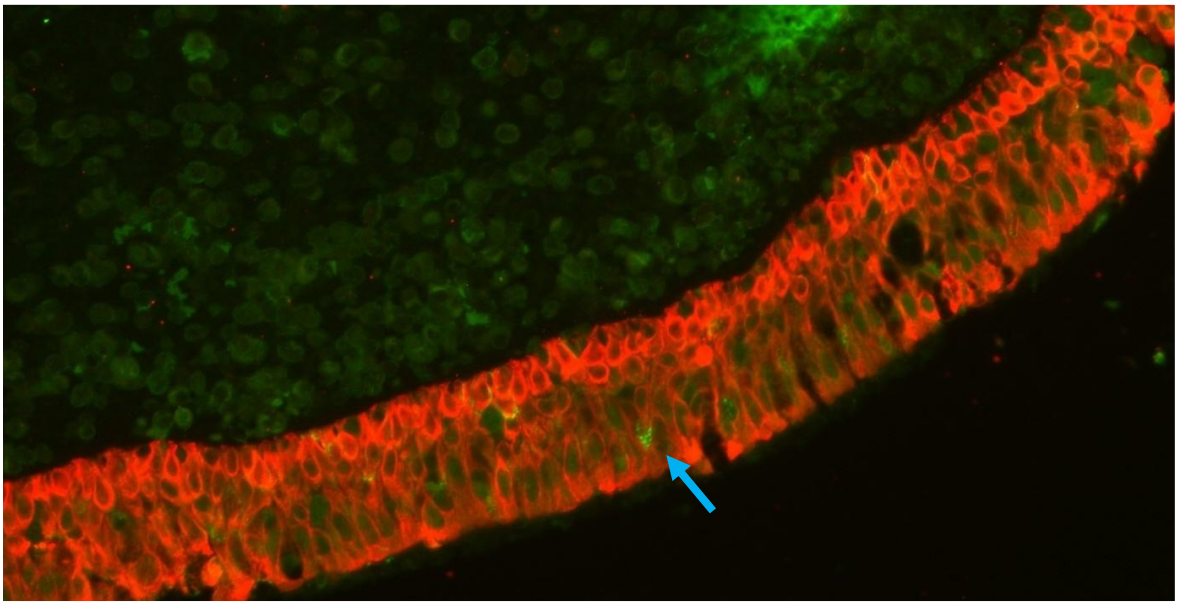

Figure 1.8: Detection of *S. aureus* in human sinonasal tissue section by IHC demonstrating clusters of intraepithelial microcolonies (blue arrow). Tissue sections were double labelled with antibodies specific for *S. aureus* (green) and cytokeratin (red).

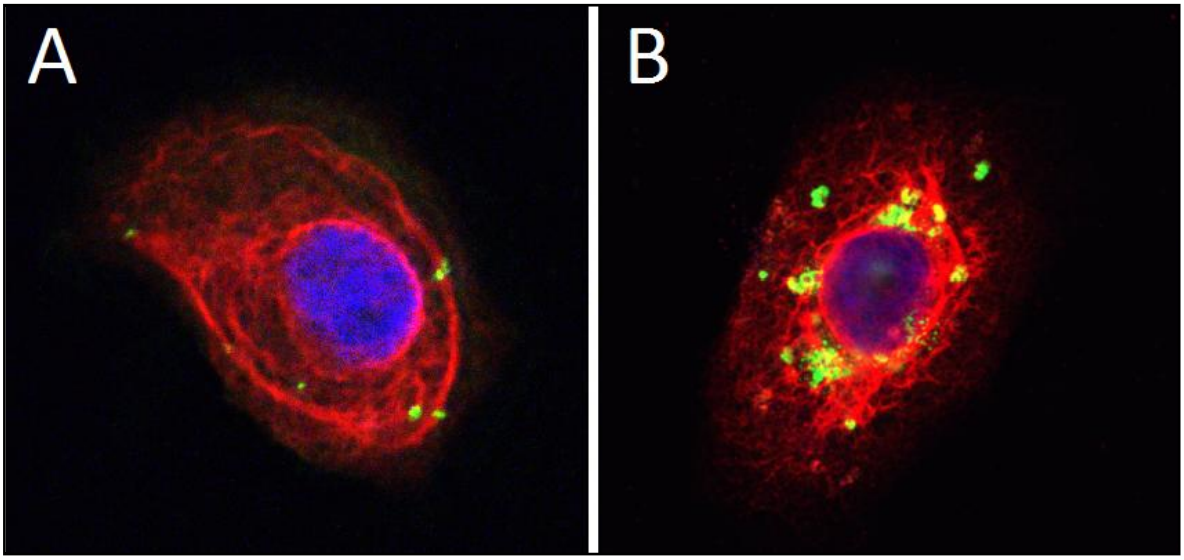

Figure 1.9: CSLM images of Primary Human Nasal Epithelial Cells incubated with *S. aureus* for; (A) 0.5h, demonstrating early invasion of bacteria around the periphery of the cell; (B) 6h demonstrating increased numbers of intracellular bacteria. Cells were double labelled with antibodies specific for *S. aureus* (green) and cytokeratin (red), as well as a nucleic acid counterstain (DAPI, blue).

Regardless of the techniques used to identify intracellular organisms, validation is required before they may be applied to cohorts of patients. Chapter 2 focuses on applying two different imaging techniques in order to assess the optimal technique for our specific research purposes.

1.4.5 INTRACELLULAR ORGANISMS IN HUMAN DISEASE

The ability for pathogens to survive and cause disease in the intracellular environment is dependent on their ability to subvert the normal immunologic processes that would otherwise lead to their destruction. Pathogens use different mechanisms in order to survive and produce their disease-forming characteristics in numerous organ-types. Table 1.6 summarises a number of intracellular genera and their route of entry into the human body.

Table 1.6: Intracellular pathogens in human disease

Organism	Disease	Route of entry
Mycobacterium tuberculosis	Tuberculosis	Lung
Mycobacterium leprae	Leprosy	Lung
Legionella pneumophila	Legionellosis	Lung
Listeria monocytogenes	Listeriosis	Gut
Chlamydia trachomatis	Chlamydia	Genitourinary

1.4.5.1 RESPIRATORY DISEASE

Mycobacterium tuberculosis and *Legionella pneumophila* are two organisms that cause respiratory disease with markedly different time-frames and characteristics. *M. tuberculosis,* the causative organism of tuberculosis (TB), enters the lung as aerosolized droplets from an infected individual where it is efficiently phagocytosed by alvelolar macrophages.[256] Once internalized, the bacterium actively prevents phagosome fusion with macrophage lysosomes[257] that would otherwise lead to its destruction. Subversion of the innate immune system therefore provides the bacterium with a protective niche in which it can lay dormant leading to the characteristic latent phase of TB that can be years or even decades before activation.[258]

The pathogenesis of Legionnaire's disease is markedly different to that of TB. Although *L. pneumophila* is also phagocytosed by alveolar macrophages, once the bacterium is internalised it actively recruits host cell endoplasmic reticulum derived vesicles and mitochondria to provide itself with the necessary machinery and resources to replicate.[259] When the host nutrient supply is exhausted, the bacteria begin to express virulence factors that ultimately lyses the host cell[260] and releases the bacteria within to infect new cells. The incubation period from bacterial exposure to clinical disease presentation is usually between 2-10 days.[261] Thus, Legionnaire's disease has a much more acute disease onset and mortality rates range from 5-30%.

1.4.5.2 GASTROINTESTINAL DISEASE

Listeria monocytogenes (L. monocytogenes) is a facultative intracellular pathogen that is well known for causing Listeriosis, a gastrointestinal disease with a mortality exceeding *Salmonella* or *Clostridium botulinum* infections.[262] Listeriosis is the most widely

understood disease that is mediated by an intracellular pathogen and is remarkable due to its ability to survive and multiply in both professional and non-professional phagocytic cells, as well as crossing numerous human barriers such as the intestinal barrier, the blood-brain barrier and the foeto-placental barrier.[263] The organism itself is rod shaped and possesses motile flagella at room temperature, and although gene expression may be downregulated at 37°C, it is still recognised that the flagella is an important factor in facilitating host-bacterial attachment.[264] Following attachment and internalisation, *L. monocytogenes* uses a number of specific mechanisms to avoid destruction by host immunity. Firstly, the bacteria secrete an enzyme listeriolysin-O (LLO) that lyses the enclosing phagosome and thus allows its escape into the cytoplasm where it is able to replicate. The next mechanism of virulence is involved in the propagation of infection at the cellular level. By commandeering the actin cytoskeleton, *L. Monocytogenes* is essentially pushed out of the cell within a slim projection until it touches the surface of another cell where is it engulfed and internalised.[265] Using this mechanism it is able to completely evade the extracellular space and resident innate immune cells.

1.4.5.3 GENITOURINARY DISEASE

Chlamydia is the commonest sexually transmitted disease and the organisms involved, *Chlamydia trachomatis* and *Chlamydia pneumonia,* are recognised obligate intracellular pathogens. The fundamental interest in Chlamydia as a disease mediated by an intracellular pathogen is that the organism exists in two distinct phenotypic forms; an elementary body (EB) and a reticulate body (RB). The EB is non-replicating but capable of infecting new host cells. Once internalisation into a phagosome occurs by a zipper-mechanism,[266] it remains in place and undergoes phenotypic alteration into an RB which is non-infectious but capable of replication. Around 48 hours post infection the RBs change back into EBs

and are released from the cell where they go on to infect new cells. The exit mechanisms have been studied extensively and two distinct methodologies have been described. Firstly, the organism may secrete a proteolytically active molecule that lyses both the vacuole and the cell itself.[267] Secondly, a novel exocytosis-like mechanism has been recognised to play a part in bacterial escape from these cells.[268] By harnessing the actin cytoskeleton, chlamydial EBs can be packaged into membranous protrusions that are expelled from the cell. An important feature of this process is that the organisms remain within the host cell membrane encapsulation. This may play a part in shielding these bacteria from local immunity, allow loco-regional dissemination where the membrane may rupture and release the pathogens, or even be taken directly into other cells to cause immediate re-infection.[269]

The various strategies utilised by bacteria to both enter and exit cells are fundamental aspects of their pathogenic capabilities. From subverting a "normal" process of macropinocytosis in internalisation to causing actin cytoskeleton rearrangement in disease dissemination, these intracellular bacteria have evolved a wide array of mechanisms to exploit their hosts.

1.4.6 INTRACELLULAR BACTERIA IN CRS

S. aureus has been traditionally considered an extracellular pathogen,[270] believed to produce its disease characteristics through the numerous locoregional virulence factors already discussed. In recent years, many studies have identified the non-professional phagocytic capabilities of cell types such as epithelium, endothelium and osteoblasts. The molecular mechanism of *S. aureus* internalisation into non-professional phagocytes has been studied extensively. Through expression of FnBPA and FnBPB on its cell surface, and mediated through fibronectin, *S. aureus* has the capability of adhering to α5β1-

integrin, which is expressed on the surface of eukaryotic cells[113,271-273] Once bound, this stimulates actin polymerisation and the zipper mechanism of phagocytosis as previously discussed. It has been demonstrated that coating inert beads with FnBP makes them easily phagocytosed by epithelial cells.[274] Furthermore, FnBP-deletion mutant strains of *S. aureus* have a drastically reduced capacity for internalisation into cells.[271]

The internalisation of *S. aureus* into epithelial cells, with the ability to survive and replicate suggests that the intracellular phenotype may offer an ability to evade host immunity and establish a niche for survival. It has been hypothesised that this intracellular niche may serve as a seeding reservoir for chronic or relapsing *Staphylococcal* infections and/or contribute to chronic carriage.[248] Despite this, irrefutable evidence in support of this hypothesis is sparse at present and circumstantial at best. An ideal experiment would directly visualise the presence of live bacteria *in vivo* in addition to harvesting a pure culture of intracellular bacteria, thus fulfilling the second of Koch's Postulates. Due to technical limitations in harvesting internalised bacteria without surface contamination it is difficult to prove without doubt that organisms come from the intracellular location. Furthermore, there are no recognised animal models of CRS so it would be difficult to prove the third postulate by causing the disease when introduced into a susceptible host. Putting Koch's Postulates aside, however, the debate over whether intracellular bacteria play an important role or not in CRS has been raised in recent years. A variety of techniques and modalities has been employed and are summarised in Table 1.7.

Table 1.7: Summary of studies reporting intracellular *S. aureus* detection rates in CRS and control patients using a range of imaging and processing techniques

Author	Technique	CRS	Control
Clement[252]	IHC, CSLM	3/3 (100%)	N/A
Plouin-Gaudon & Clement[275]	IHC, CSLM	17/27 (63%)	N/A
Niederfuhr et al[276]	FISH, UIFM	1/65 (1.5%)	N/A
Corriveau et al[277]	FISH, UIFM	7/21 (33%) CRSwNP 3/10 (30%) CRSsNP	3/9 (33%)
Sachse et al[240]	FISH, UIFM	17/25 (68%) CRSwNP0/5 (0%) CRSsNP	1/10 (10%)
Wood et al[278]	FISH, Gram-stain, UIFM	6/9 (67%) CRSwNP 4/9 (44%) CRSsNP	1/8 (13%)

The first effort to identify intracellular organisms in the sinonasal mucosa of CRS patients was in 2005, when Clement et al[252] found evidence for intracellular *S. aureus* within the epithelium, glandular tissue and myofibroblastic cells of three patients with severely recalcitrant CRS. The group reported a methodology that utilised IHC techniques coupled with CSLM. Antibodies used targeted *S. aureus* (anti-*S. aureus* mouse IgM), human keratin (wide-spectrum screen rabbit polyclonal antibody) and a nucleic acid counterstain (TOTO-III). The group went on to recruit 27 patients into a prospective study,[275] all of whom had failed medical therapy for CRS. They demonstrated intracellular *S. aureus* in 17 of the 27 patients (63%) and used clonal typing to identify that bacterial strains isolated at the time of surgery persisted in these intracellular positive patients. Despite early promise in this subject, the group discontinued research efforts and it was not for a few years that interest in the area was rekindled.

Niederfuhr et al[276] used *S. aureus* specific FISH probes and modified histochemistry techniques to examine sections of sinonasal mucosa, however were only able to detect *S. aureus* in 1 out of 65 patient samples. Whether this truly reflected the intracellular *S. aureus* status or a failure in methodology is debatable. However, they did report positive *S. aureus* culture in 32% of samples, suggesting the bacteria were present, albeit not identified.

Directly conflicting detection rates were subsequently published by Corriveau et al[277] who used similar techniques to examine sections of sinonasal mucosa from 31 patients undergoing ESS for CRS and 9 control patients undergoing septoplasty. Due to the use of upright fluorescent microscopy and not an imaging technique that offered 3D reconstruction, the group felt that they were unable to discern whether bacteria identified were intracellular or extracellular. Nevertheless, they reported evidence of intramucosal *S. aureus* in 7 of 21 (33%) CRSwNP, 3 of 10 (30%) CRSsNP and 3 of 9 (33%) control patients. In addition, the group reported that *S. aureus* could invade through the epithelium and reside in the submucosa.

Subsequently, another group reported their novel association between nasal polyp status and intracellular *S. aureus.* Using the same methodology, Sachse et al[240] identified intramucosal bacteria in 17 of 25 (68%) CRSwNP, 0 of 5 (0%) CRSsNP and 1 of 10 (10%) control patients, as well as infecting primary nasal epithelial cell cultures with *S. aureus in vitro*. They demonstrated the capability of intracellular *S. aureus* to stimulate a pro-inflammatory response measured by interleukin (IL)-6 and IL-13, concluding that intracellular *S. aureus* exhibits a direct immunomodulatory role in the pathogenesis of disease chronicity.

The most recent study utilised comparable techniques but combined fluorescent detection with light microscopy and gram staining in order to evaluate whether intramucosal organisms elicited an immunological response. Wood et al[278] detected *S. aureus* in 6 of 9 (67%) CRSwNP, 4 of 9 (44%) CRSsNP and 1 of 8 (13%) control patients, but they were unable to identify an associated neutrophilic or eosinophilic response, thereby suggesting that internalised organisms are able to subvert immunity and hide from the host immune system.

Research thus far has utilised novel techniques in *S. aureus* detection to further understand the role that this organism may play in the pathogenesis of CRS. All the studies were predominantly descriptive in nature. As the evidence points towards an association between intracellular *S. aureus* and CRS, research must switch focus to identify molecular or mechanistic explanations for how this bacterial phenotype affects the disease process. Furthermore, the true impact of intracellular bacteria on clinical outcomes must be assessed.

1.5 SMALL COLONY VARIANTS

1.5.1 DEFINITION

Small colony variants (SCVs) are described as bacterial sub-populations that exhibit distinct phenotypic and pathogenic traits when compared to the wild-type, parental strain. First identified in 1910 in a subspecies of *Salmonella*, their presence has been described in numerous bacterial genera including *S. aureus* , *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Eschericia coli and Vibrio cholerae*[279]. They have been associated with persistent and recurrent illnesses including CF-related pulmonary infections[280,281] and osteomyelitis.[282,283] The characteristic trait of an SCV is to grow colonies that are up to one tenth the size of normal colonies and take 2-3 times as long to culture (Fig. 1.10). SCVs are considered to be auxotrophic; unable to synthesise critical components necessary for normal growth. Defects in electron transport, specifically in menadione, haemin and thymidine biosynthesis, have been identified as mechanisms to explain this phenomenon.[279] In addition to slow metabolism, SCVs demonstrate decreased respiration, decreased pigmentation, decreased secretion of haemolytic toxins, increased aminoglycoside resistance and critically, an unstable phenotype[284] (Table 1.8). This instability has been demonstrated in the capacity for SCV phenotypes to revert back to the wild-type phenotype within 24 hours of sub-culture, with an associated reversion of virulence factors. The reduction of SCV virulence factors coupled with the resistance to bactericidal agents[285] have been suggested as persistence mechanisms that allow SCVs to survive in the face of host immunity and targeted anti-microbials.

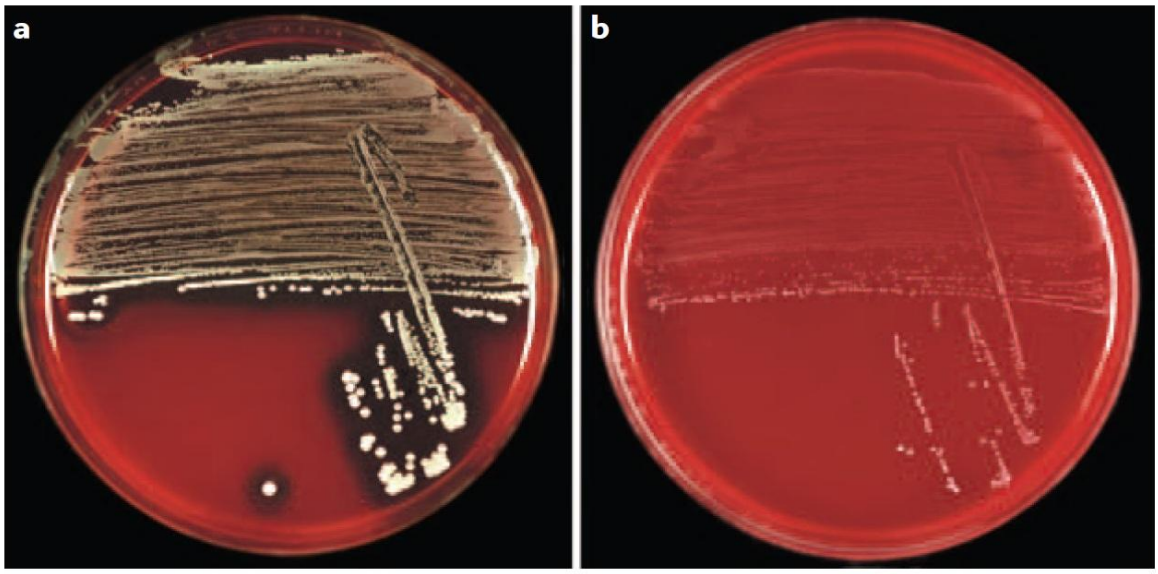

Figure 1.10: Small colony variants of *S. aureus*: Columbia blood-agar plates that show the normal (a) and the small colony variant (b) phenotypes of *S. aureus*. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology,[279] copyright 2006.

Table 1.8: Characteristic features of wild-type and SCV *S. aureus*. Adapted with permission from von Eiff et al.[286]

Characteristics	Normal *S. aureus* phenotype	SCV phenotype
Growth rate on solid media	12-18h	Delayed (48-72h)
Colony morphology	Yellow colonies ≈ 4mm	Grey-white pinpoint colonies ≈ 1mm
Auxotrophism	No	Menadione and hemin
Toxin activity; **α-haemolysin** **β-haemolysin**	 Normal Normal	 Reduced Reduced
Enzymatic activity; **Coagulase** **Catalase**	 Normal Normal	 3-4 fold delay Reduced
Biochemical reactions	Normal	Delayed and/or changed
Antibiotic sensitivity	Varying	Reduced to aminoglycosides and β-lactams

1.5.2 PATHOLOGICAL CONSEQUENCES

The properties that define a SCV are also the ones that play a part in its pathological capabilities. Their antibiotic resistance capabilities have been well documented with factors such as efflux pumps and cell-wall alterations contributing to an innate ability to counteract antimicrobials.[285] The ability to switch back to the wild-type virulent form on cessation of antibiotic therapy makes the organism highly resistant to host elimination. In addition to the inherent advantages that the SCV phenotype confers, it is recognised that *S. aureus* SCVs are more capable of internalising into, and persisting in the intracellular milieu. Through heightened expression of FnBP,[272] the SCV is more likely to adhere to epithelial surfaces and be internalised through mechanisms already discussed. Once internalised, decreased virulence such as a reduction in the production of alpha-haemolysin may play a part in explaining why SCVs are able to persist in tissues so effectively.[287] In addition to changes to the bacteria themselves, SCVs have the unique property of eliciting a smaller host response than their wild-type counterparts. Studies have demonstrated that wild-type, virulent bacteria cause the upregulation of many genes related to the innate immune system,[288] whereas SCVs do not elicit such a change.

1.5.3 IDENTIFICATION

Due to the considerably slower growth pattern of SCVs, coupled with deficiencies in biochemical reactions typically used for species identification, traditional culture techniques must be modified when attempting to identify SCVs. Two suggestions have been made to aid in the detection of *S. aureus* SCVs[279,289]; (i) inoculate specimens onto both blood agar and chromogenic agar (that identifies the activity of specific biochemical reactions e.g. α-glucosidase production), (ii) culture at 37°C for up to 72 hours, and (iii) any coagulase-negative strains suspected of being *S. aureus* should undergo molecular

confirmation of species type. Difficulties in SCV identification still exist, in part due to limitations in applying rigorous identification methodologies to standard microbiology laboratories, but moreover due to SCVs rarely existing on their own. SCVs are commonly found in mixed populations in association with wild-type strains. Thus, when standard culture techniques are applied, the SCV is often overgrown by the more metabolically active parental strain.[280]

1.5.4 SCVS IN DISEASE

The clinical recovery of SCVs have predominantly been from diseases that suffer relapsing and remitting courses. This association to conditions such as CF, chronic osteomyelitis and device related infections underlines the theory that they play an important role in recurrent acute infections on a background of an underlying chronic infectious process.

Patients suffering from CF are commonly colonized with organisms including *P. aeruginosa* and *S. aureus.* In a long-term prospective study, SCVs were identified in 46% of *S. aureus* infected patients, and more SCVs emerged with time as a consequence of repeated antibiotic therapy.[280]

Numerous studies have documented the retrieval of SCVs from orthopaedic infections.[282,283] It has been demonstrated *in vitro* that normal strains of *S. aureus* can be selectively pressured into forming a SCV phenotype after gentamicin exposure[290] and it is hypothesised that long-term, low dose gentamicin *in vivo*, such as that used in chronic osteomyelitis will cause SCVs to emerge.

1.5.5 SCVS IN CRS

The characteristics of SCV-mediated diseases draw remarkable similarities to that of CRS. The chronically relapsing - remitting course with frequent acute infective flare-ups, coupled with the failure of traditional antimicrobial therapies suggests that SCVs could contribute to disease persistence in CRS. As yet, no research exists to link SCVs to CRS, and the shift away from traditional culture techniques towards the molecular identification of bacteria may have played a part in why researchers are not recognising this bacterial phenotype. The question as to whether SCVs may be of relevance to the aetiopathogenesis of CRS is investigated in Chapter 6.

LITERATURE REVIEW SUMMARY

CRS is a common disease with a significant socioeconomic burden to both the individual and the state. The cardinal finding of sino-nasal inflammation may be related to numerous etiologies, however, no single unifying cause has thus far been identified. The traditional role of bacteria as an acute infectious agent is not believed to be a contributing factor to initial disease development and it is more likely that a multi-factorial interplay between host and surrounding environment may predispose certain patients to developing symptoms of CRS. Ear, Nose and Throat Surgeons are recognising a subgroup of patients with recalcitrant symptoms that persistently fail both medical and surgical treatment. In these patients it has been identified that altered bacterial phenotypes in the form of biofilms, specifically those involving *S. aureus,* may have an impact on disease persistence and symptomatology.

S. aureus is a remarkable organism, capable of residing as an innocuous coloniser or as a potent pathogen with significant morbidity and mortality, especially in an immune compromised host. Studies thus far have identified that CRS patients who harbour *S. aureus* biofilms have significantly worse preoperative symptomatology and a higher chance of postoperative relapse than those who are *S. aureus* negative. Although research into the treatment of biofilms is still in its infancy, the use of anti-biofilm agents that have shown *in vitro* promise has not translated a satisfactory impact on clinical outcomes when used *in vivo.* Thus, the question as to whether bacteria may be utilising other protective mechanisms to evade topical treatments has been raised.

Whilst previously considered an extracellular pathogen, *S. aureus* has demonstrated the capacity to invade, persist and replicate in both professional phagocytes such as

macrophages or neutrophils, and non-professional phagocytes such as epithelial or endothelial cells. The intracellular location is suggested to provide a protective niche that allows internalised bacteria to evade systemic antimicrobial therapy and topical treatments. The concept of intracellular *S. aureus* in CRS has emerged in recent years and so far, early evidence is accumulating to describe their presence in tissue samples of CRS patients. However, little research has identified associative factors that may play a part in allowing internalisation to occur, and what specific changes may occur to bacteria and host as a result of their exposure to the intracellular milieu.

After reviewing the available evidence for intracellular *S. aureus* in CRS, it is obvious that a number of gaps in our understanding exist;

1) Detection of intracellular *S. aureus* has been reported with a variety of processing and imaging techniques, however, no single methodology has been recognised as the ideal. In particular, current techniques do not allow for simultaneous biofilm detection co-localised to the same piece of tissue tested. Thus, novel techniques are required to image for both biofilm and intracellular bacteria in whole mucosal samples.

2) While evidence exists identifying the presence of intracellular *S. aureus* in CRS patients undergoing endoscopic sinus surgery, it has not been defined whether this influences the postoperative course, and the true clinical relevance of this bacterial phenotype is unknown.

3) Altered regulation of *S. aureus* cytolytic toxins such as alpha-toxin has been suggested as a mechanism of how the bacteria can decrease its pathogenicity to host cells. In CRS it is recognised that superantigenic exotoxins may play an impact on disease severity, however, little is known as to the regulation of these specific factors, and whether exposure to the intracellular milieu can cause such phenotypic alterations to virulence regulation.

4) The association between SCVs and CRS is as yet, unknown. Whether this bacterial phenotype is detectable in CRS patients is important in understanding specific mechanisms of how the bacterium preferentially situates itself in the intracellular environment.

These knowledge gaps form the basis behind the research contained within this PhD thesis.

STUDIES TO BE CONDUCTED

1) Develop a FISH protocol that allows for the detection of *S. aureus* intracellular and surface biofilm statuses simultaneously in CRS mucosal samples, and validate the protocol in a direct comparison to commonly used IHC techniques within the same patient cohort.

2) Develop and validate an IHC methodology to prevent false positive binding of antibodies in *S. aureus* infected tissues believed to be due to Protein A – IgG Ab interactions.

3) Utilise the FISH protocol in a wider cohort of CRS and control patients to further understand the interplay between biofilm and intracellular status and any potential synergistic relationship.

4) Examine the impact of the intracellular phenotype by performing a prospective cohort study to define whether there is an association between intracellular *S. aureus* and disease recalcitrance.

5) Determine whether the phenotype alteration of virulence factors is a concept applicable to intracellular and intramucosal infections using *in vitro* culture experiments as a possible explanation of intracellular persistence. Secondly, to identify whether SCVs may be harvested from sinonasal mucosa specimens of CRS patients.

CHAPTER 2

IDENTIFYING INTRACELLULAR *STAPHYLOCOCCUS AUREUS* IN CHRONIC RHINOSINUSITIS: A DIRECT COMPARISON OF TECHNIQUES

Conducted in the Department of Otolaryngology – Head and Neck Surgery

The University of Adelaide, Adelaide, Australia

Financial assistance provided by The University of Adelaide and the European Rhinologic Society.

Identifying intracellular *Staphylococcus aureus* in chronic rhinosinusitis: A direct comparison of techniques

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ABSTRACT

Background: *The emerging concept of intracellular pathogens such as* Staphylococcus aureus *playing a role in chronic rhinosinusitis (CRS) has led to the development of numerous imaging techniques for their identification. Traditional methods of bacterial culture are not effective at localizing bacteria to the surface or within tissue samples. The aim of this study was to develop and validate a novel imaging technique using confocal scanning laser microscopy (CSLM) coupled with a fluorescence* in situ *hybridization (FISH) probe and nucleic acid counterstain (propidium iodide [PI]) that allows for simultaneous analysis of* S. aureus *intracellular status and surface biofilm within whole mucosal samples.*

Methods: *A prospective study was performed including 17 patients undergoing endoscopic sinus surgery for CRS. Tissue samples were analyzed with both CSLM-FISH/PI and immunohistochemistry (IHC) for intracellular* S. aureus *status.*

Results: *Using CSLM-FISH/PI intracellular* S. aureus *was identified in 9/17 (47%) patients and in 7/17 (39%) using IHC. Surface biofilm can be identified with CSLM-FISH/PI in the same piece of tissue; however, deeper imaging to the submucosa is impossible. IHC showed submucosal bacteria in three patients.*

Conclusion: *Both CSLM-FISH/PI and IHC are complementary techniques that can be used to identify intracellular* S. aureus. *CSLM-FISH/PI allows for the simultaneous detection of intracellular status and surface biofilm within the tissue analyzed. IHC has a role in the identification of intracellular and submucosal* S. aureus *within these tissues. Additional investigation is required to identify the true pathogenic nature of intracellular organisms as well as any relationship to surface biofilm status.*

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Chronic rhinosinusitis (CRS) remains a complex, poorly understood disease characterized by relapsing episodes of inflammation within the sinonasal cavities. Although medical treatment and endoscopic sinus surgery (ESS) can be successful in eradicating the disease burden,[1] a significant number of cases fail such treatment and develop recalcitrant disease. *Staphylococcus aureus* has been identified as a prominent pathogen in the disease process.[2] In particular, patients with *S. aureus* biofilms at the time of ESS have significantly worse preoperative symptoms, intraoperative surgical findings, and a higher rate of recalcitrant disease.[3] Identification techniques for *S. aureus* biofilm status has been optimized within our department by using confocal scanning laser microscopy (CSLM) coupled with peptide nucleic acid–fluorescence *in situ* hybridization (PNA-FISH) probes in both the CRS patients[4] and in an animal model of sinusitis.[5]

Despite recent interest in the role of bacterial biofilms in CRS, it is but one virulence mechanism adopted by organisms such as *S. aureus* in chronic disease. Intracellular detection of *S. aureus* has also been reported in CRS patients and may represent a bacterial phenotype with clinical relevance. It is recognized that nonprofessional phagocytic cells such as epithelium can internalize live *S. aureus*,[6] and recent studies have identified its role in certain chronic disease processes including osteomyelitis,[7] mastitis,[8] and CRS.[9] The first study identifying intracellular *S. aureus* in CRS patients used immunohistochemistry (IHC) on paraffin-embedded tissue sections[9] with intracellular bacteria were found in 17/27 (63%) CRS patients. The authors followed a cohort of intracellular *S. aureus*–positive patients who also cultured the organism from the middle meatus at the time of surgery. They noted a higher clinical relapse rate with persistent infection at 1 year, which was confirmed to be the same bacteria by clonal typing. PNA-FISH probes have been used in paraffin-embedded tissue sections reporting intracellular-positive rates of between 32 and 57%.[10,11] Despite this evidence for intracellular organisms, the true pathogenic nature still remains to be proven and should be a focus for further research.

Both IHC and FISH techniques provide useful information on intracellular bacterial localization when analyzed; however, a major limitation is that it is difficult to accurately assess the biofilm status of a patient from single tissue slices. This is in comparison with whole mucosal samples taken at the time of surgery where biofilm status and potentially intracellular detection can be simultaneously achieved. In achieving a holistic understanding of microbial interactions with the host in CRS it is likely that the interplay of surface biofilm and intracellular residence will hold pathogenic importance. Their codetection in sinus mucosal specimens will be an important step in understanding the link between the two.

Therefore, the aim of this study was to validate a new method of intracellular *S. aureus* detection that has the capability for simultaneous biofilm analysis using CSLM coupled with PNA-FISH and a nucleic acid counterstain, propidium iodide (PI), and compare it with a recognized technique of IHC in tissue sections within the same cohort of patients as well as highlight the technical aspects of each modality.

METHODS

Study Design and Patient Population

This prospective, blinded study was completed in the tertiary rhinologic practice of the senior author (P.J.W.). The institution's Human Research Ethics Committee gave their approval for the study to be performed and all subjects provided informed consent to participate in the study. The study group consisted of 17 patients undergoing ESS with a diagnosis of CRS as per the Rhinosinusitis Task Force[12,13]

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By signing this document, I (the co-author) hereby acknowledge these to be accurate descriptions of the contribution I made to this paper and give permission for it to be included in the candidate's thesis.

2.1 ABSTRACT

Background: The emerging concept of intracellular pathogens such as *S. aureus* playing a role in CRS has led to the development of numerous imaging techniques for their identification. Traditional methods of bacterial culture are not effective at localising bacteria to the surface or within tissue samples.

Objectives: The aim of this study was to develop and validate a novel imaging technique using CSLM coupled with a FISH probe and nucleic acid counterstain (PI) that allows for simultaneous analysis of *S. aureus* intracellular status and surface biofilm within whole mucosal samples.

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Conclusion: Both CSLM-FISH/PI and IHC are complementary techniques that can be used to identify intracellular *S. aureus*. CSLM-FISH/PI allows for the simultaneous detection of intracellular status and surface biofilm within the tissue analysed. IHC has a role in the identification of intracellular and submucosal *S. aureus* within these tissues. Additional investigation is required to identify the true pathogenic nature of intracellular organisms as well as any relationship to surface biofilm status.

2.2 INTRODUCTION

CRS remains a complex, poorly understood disease characterised by relapsing episodes of inflammation within the sinonasal cavities. Although medical treatment and ESS can be successful in eradicating the disease burden,[3] a significant number of cases fail such treatment and develop recalcitrant disease. *S. aureus* has been identified as a prominent pathogen in the disease process.[31] In particular, patients with *S. aureus* biofilms at the time of ESS have significantly worse pre-operative symptoms, intraoperative surgical findings and a higher rate of recalcitrant disease.[202] Identification techniques for *S. aureus* biofilm statushave been optimised within our department by using CSLM coupled with PNA-FISH probes in the both CRS patients[291] and in an animal model of sinusitis.[292]

Despite recent interest in the role of bacterial biofilms in CRS, it is but one virulence mechanism adopted by organisms such as *S. aureus* in chronic disease. Intracellular detection of *S. aureus* has also been reported in CRS patients and may represent a bacterial phenotype with clinical relevance. It is recognised that non-professional phagocytic cells such as epithelium can internalise live *S. aureus,*[248] and recent studies have identified its role in certain chronic disease processes including osteomyelitis,[144] mastitis[271] and CRS.[252] The first study identifying intracellular *S. aureus* in CRS patients used IHC on paraffin embedded tissue sections[252] with intracellular bacteria were found in 17/27 (63%) CRS patients. The authors followed a cohort of intracellular *S. aureus*-positive patients who also cultured the organism from the middle meatus at the time of surgery. They noted a higher clinical relapse rate with persistent infection at 1 year which was confirmed to be the same bacteria by clonal typing. PNA-FISH probes have been used in paraffin embedded tissue sections reporting intracellular positive rates of between 32 and 57%.[240,277] Despite this

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Both IHC and FISH techniques provide useful information on intracellular bacterial localization when analyzed, however, a major limitation is that it is difficult to accurately assess the biofilm status of a patient from single tissue slices. This is in comparison with whole mucosal samples taken at the time of surgery where biofilm status and potentially intracellular detection can be simultaneously achieved. In achieving a holistic understanding of microbial interactions with the host in CRS it is likely that the interplay of surface biofilm and intracellular residence will hold pathogenic importance. Their co-detection in sinus mucosal specimens will be an important step in understanding the link between the two.

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2.3 METHODS

Study Design and Patient Population

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Data Collection

Preoperative data were collected on the day of surgery including age, gender, number of previous sinus surgeries, history of asthma or smoking, allergy status, and preoperative CT scores (Lund-Mackay). Clinical symptom scores for five major symptoms of CRS (nasal obstruction, rhinorrhoea, postnasal drip, headache or facial pain and sense of smell) were also recorded on a 0-5 scale and a total out of 25 recorded. No patient had taken any antibiotic, antifungal, systemic steroids or other immunosuppressant medication in the 3 weeks before surgery.

Perioperative data was collected during the subject's surgery for polyp status, presence of mucopus or eosinophilic mucin and condition of mucosa (normal, oedematous or polypoid). All patients had mucosal samples sent for histological analysis as well as samples for routine microbiological and fungal culture analysis. Bacterial culture was performed using standard laboratory techniques (Adelaide Pathology Partners, SA).

Tissue Collection and Transport

All sinus mucosa specimens were collected at the time of surgery and immediately placed into Dulbecco's modified eagle's medium (Gibco, Invitrogen Corp., Carlsbad, CA) and transported to our laboratory. Under sterile conditions, samples were washed thoroughly in MilliQ water (Millipore, Billerica, MA) and dissected free of any underlying bone. Mucosal samples for CSLM-FISH/PI analysis were frozen to -80°C, and samples for IHC were placed into 5 ml of 10% buffered formalin (Sigma-Aldrich, St. Louis, MI) and embedded in paraffin.

CSLM –FISH/PI Protocol

The bacterial species-specific FISH probe used in this study has been validated by our department as an effective method of *S. aureus* biofilm detection.[291] Briefly, approximately 4 x 4 mm whole mucosal samples were washed in MilliQ water and heat fixed to glass microscope slides (Superfrost;Thermo Fisher, Waltham, MA) for 20 minutes in a 55°C incubator, dehydrated in 90% ethanol and air-dried. The *S. aureus* FISH probe (AvanDX, Woburn, MA) was applied to the mucosa and hybridisation occurred for 90 minutes at 55°C. Slides were washed in the manufacturer's wash solution for 30 minutes. Subsequently, tissue was counterstained with 70 µl PI (Sigma-Aldrich) in glycerol at a concentration of 50 µg/ml to clearly identify cellular nuclei and prepared for microscope analysis.

Immunohistochemistry

Paraffin embedded tissue was prepared into 5 µm sections and mounted on glass slides (Superfrost, Thermo Fisher). The sections were deparaffinised and rehydrated using graded xylene and ethanol. Slides were immersed in 10 mM Sodium Citrate and antigen retrieval

was performed using a microwave technique. Once the slides had cooled they were transferred into phosphate buffered saline (PBS), marked with a PAP pen (Abcam, Cambridge, U.K.) and subsequently into a Tris-Buffered Saline and Tween-20 buffer (TBST). Serum free protein block (Dako, Glostrup, Denmark) was applied for 1 hour at room temperature and slides were incubated simultaneously with mouse anti-*S. aureus* (Millpore) at a concentration of 1/50 and rabbit antipancytokeratin antibodies (Invitrogen Corp) at a concentration of 1/100 overnight at 4°C. Slides were washed in TBST four times on a horizontal shaker and secondary antibodies (goat anti-mouse IgM-Alexa488 (1/100; Invitrogen Corp); Sheep anti-rabbit IgG-Cy3 (1/250; Sigma-Aldrich)) were applied for 1 hour at room temperature in a darkened moisture chamber. Finally, slides were washed in TBST five times on a horizontal shaker and coverslips were mounted in 90% glycerol for microscope analysis. Positive control sections were prepared by incubating 5 x 5mm pieces of nasal mucosa harvested from non-CRS patients with *S. aureus* American Type Culture Collection (ATCC) 25923 for various time-points and fixed in formalin before being embedded in paraffin and sectioned as per the protocol. Negative control slides were prepared identically using normal control rabbit IgG (Sigma-Aldrich).

Tissue Analysis

Slides prepared for CSLM-FISH/PI were analysed with a Leica TCS SP5 Confocal Scanning Laser Microscope (Leica Microsystems, Wetzlar, Germany). Multiple consecutive images were taken in the axial z-axis of tissue samples with a slice thickness of 0.5 µm. The tissue was assessed for evidence of *S. aureus* visualised as bright green coccal structures fluorescing on stimulation with a 488 nm laser.[294] PI was stimulated with a 561 nm laser and epithelial nuclei were identified as bright red spheric areas with a size of roughly 10 µm. Furthermore, PI also stains *S. aureus* and thus when the two channels

were merged, true *S. aureus* colocalised between the two stains and appeared bright yellow. Samples were categorised as intracellular positive if *S. aureus* could be identified at least one z-plane and closely localised adjacent to intraepithelial nuclei. Analysis for biofilm status was also made as per a validated protocol[294] using the same tissue sample. Once the intracellular plane was identified through the PI stain, the microscope stage could be digitally manipulated to bring the field of focus into a z-plane that is located more superficially to the cells to identify biofilm as clusters and towers of immobile, irreversibly attached coccal shaped bacteria, approximately 0.5 - 2 μm in size.[187] IHC slides were analysed with a Zeiss Axio Vert.A1 (Carl Zeiss AG, Jena, Germany). Positive *S. aureus* cocci could be visualised as bright green fluorescing dots within nasal epithelium outlined by cytokeratin stained in red. Although nuclei were not counterstained, a cytokeratin void could clearly be seen in the correct morphology and dimensions of an epithelial nucleus. Microscope images were collated, deidentified and analyzed by three independent, blinded observers (N.T, C.J, S.V) for their surface biofilm and intracellular *S. aureus* status.

Statistical Analysis

Results of this study were analyzed using GraphPad Prism 5.0 software (GraphPad Softward, Inc., San Diego, CA) with non parametric data displayed as a median with interquartile ranges (IQR). Dichotomous data were analysed using Fischer's exact test with statistical significance accepted when $p < 0.05$. Inter-rater agreement was assessed using Minitab 16 Statistical Software (Minitab, Inc., State College, PA) with a Fleiss's κ calculated.

2.4 RESULTS

Demographics and Preoperative Data

Seventeen patients undergoing ESS for CRS were enrolled in this study (nine men and eight women). Mean age was 45.5 years (IQR, 30 – 57). Six patients had a history of asthma and none were current smokers. Median symptom score was 18 (IQR, 13.5 – 20) and median Lund-Mackay score was 10 (IQR 4 – 15)

Intraoperative Data

Five patients underwent minor surgery in the form of mini-ESS (middle meatal antrostomy and anterior ethmoidectomy) and 12 underwent major surgery classified as either modified endoscopic Lothrop/Draf 3/frontal drillout procedure or complete sphenoid-ethmoidectomy and frontal sinusotomy. Seven patients had nasal polyposis.

Microscope Analysis

Images of all patients were de-identified and assessed by blinded observers who categorised the sample as either intracellular positive or negative. Interobserver variability in both CSLM-FISH/PI and IHC was assessed using Fleiss's κ which gave results of 0.72 and 0.63 respectively, which showed substantial agreement between the three observers.[295]

Using the CSLM-FISH/PI technique there were 9/17 (53%) patients that showed evidence of intracellular *S. aureus*. In these cases, *S. aureus* could be seen fluorescing on both the green FISH and red PI channels, colocalised to show bright yellow cocci closely related to red counterstained nuclei when viewed in the merged channel (Fig. 2.1). Consecutive z-plane images show the cellular nuclei with *S. aureus* fluorescing only at certain planes of focus, indicating that the organism is at a z-plane within the cell itself (Fig. 2.2). Three-

dimensional reconstruction of highly magnified z-stacks is possible using the CSLM. This gives an even more vivid presentation of the presence of bacteria closely localised to the nuclei of epithelial cells.

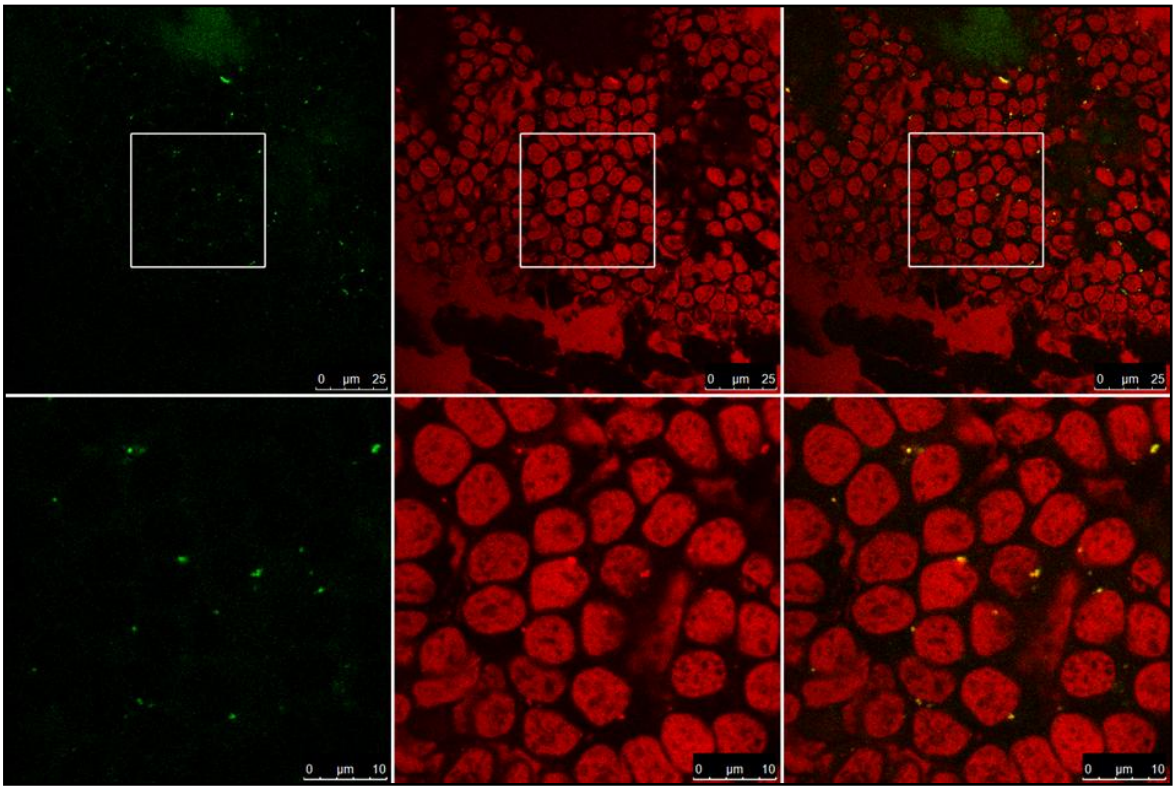

Figure 2.1: Detection of intracellular *S. aureus* by CSLM-FISH/PI (green, left column) in conjunction with PI (red, middle column). The organism is identified by co-localisation of PNA-FISH with PI (yellow, merged channels). High magnification of the boxed area (bottom) reveals cocci closely related to cellular nuclei (red only).

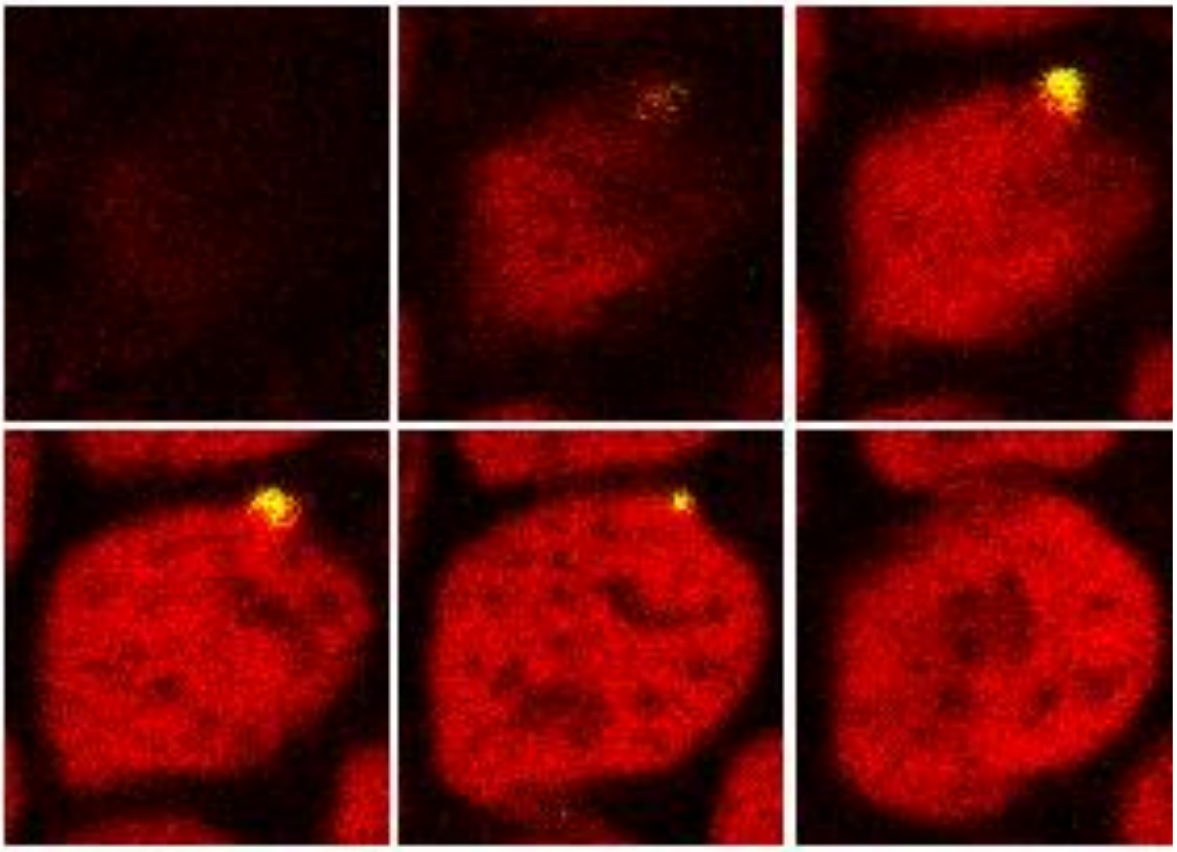

Figure 2.2: CSLM-FISH/PI appearance of consecutive z-plane images demonstrating a single cellular nucleus (red) with a focus of intracellular *S. aureus* (yellow) at consecutive planes of focus suggesting intracellular presence.

Using IHC, intracellular *S. aureus* was identified in 7/17 (41%) patients. IHC gives excellent cellular morphology and the ability to clearly identify green fluorescing *S. aureus* located within the boundaries of epithelial cells as outlined by the red cytokeratin stain (Fig. 2.3). In three cases it was possible to identify *S. aureus* within the subepithelial layer. IHC analysis of tissue explants incubated with *S. aureus ex vivo* showed evidence of organisms within an hour of infection and by 13 hours there were cocci within both the epithelium and the subepithelial glandular tissue (Fig. 2.4).

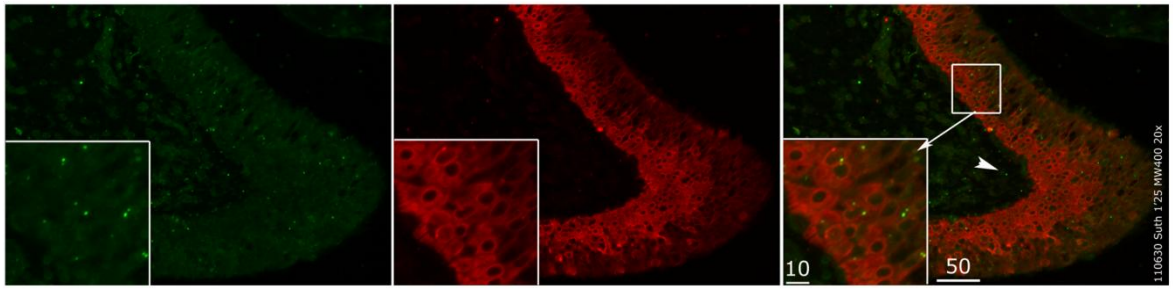

Figure 2.3: Detection of *S. aureus* in paraffin sections of sinus mucosa tissue by IHC; Left: Green fluorescence label of *S. aureus*; Middle: Red fluorescence of epithelium-specific cytokeratin; Right: Merged channels. Scales are in micrometers and applied for all panels.

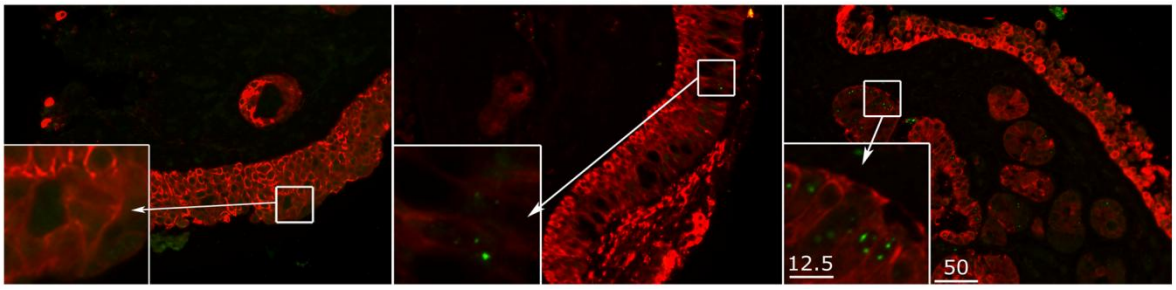

Figure 2.4: Immunofluorescence of tissue explants experimentally infected with *S. aureus*. Green: fluorescence bound to the IgM monoclonal antibody to *S. aureus*; Red: fluorescence of epithelia-specific cytokeratin. Left: Prior to infection; Middle: 1h post-infection; Right: 13h post-infection. Scales are in micrometers and applied for all panels.

Comparing both techniques, agreement in intracellular *S. aureus* status (either positive or negative) was found in 13/17 (76%) patients (Table 2.1). There were three patients deemed intracellular positive on FISH but negative on IHC and one patient positive on IHC but not FISH. Fischer's exact test indicated no significant difference in the detection rates of *S. aureus* (p=0.73).

Table 2.1: CSLM-FISH/PI versus IHC for identification of intracellular *S. aureus*

Patient no.	CSLM-FISH/PI	IHC	Microbiological Culture
1	✓	×	*S. aureus*
2	×	×	No growth
3	✓	✓	*S. aureus*
4	✓	✓	*S. aureus*
5	×	✓	*S. aureus*
6	×	×	*S. aureus*
7	×	×	No growth
8	✓	×	*S. aureus*
9	✓	✓	MRSA
10	×	×	*S. aureus*
11	✓	×	No growth
12	×	×	No growth
13	✓	✓	No growth
14	✓	✓	*S. aureus*
15	✓	✓	*S. aureus*
16	×	×	Respiratory flora
17	×	×	*Moraxella catarrhalis*

Microbiological Outcomes

Of the patients who were categorised as intracellular positive by CSLM-FISH/PI, 7/9 (78%) cultured *S. aureus* from middle meatal swabs taken at the time of surgery. In the IHC-positive group 6/7 (86%) were culture positive (Table 2.1).

2.5 DISCUSSION

To our knowledge this is the first study that reports the use of CSLM coupled with a dual probe technique of PNA-FISH and PI for intracellular *S. aureus* detection in whole mucosal samples of sinonasal epithelium, in addition to using a recognised technique of IHC within the same cohort of CRS patients. Intracellular localization of *S. aureus* was consistently detected by both methods with an equivalent efficacy. The sets of results obtained by the two independent methods are in a relative accordance with each other and this study allows for comparative highlighting of technical issues associated with detection and imaging of the organism infection by these two modalities. The significance of this study is in highlighting a modality of intracellular *S. aureus* detection that allows for simultaneous biofilm detection colocalised to the same piece of tissue and may further our understanding of the interplay between surface biofilm and intracellular residence in the chronic inflammatory state present in CRS sufferers.

In terms of technical ease, the protocol for FISH/PI analysis takes 4 hours to complete with image analysis immediately after this. Whole mucosal samples can be frozen on the day of harvest for later analysis, a process validated within our department[294] where a side-by-side analysis of frozen versus immediately processed tissue was performed showing no difference in reported outcomes. For IHC, tissue harvested on the day of surgery is soaked in 10% formaldehyde overnight, paraffin blocked and sectioned, followed by our protocol that takes 24 hours to complete. Multiple tissue sections can be processed simultaneously with both techniques and although the FISH protocol is faster, both can be performed in the laboratory setting with relative ease. Indeed it should be noted that our IHC protocol was optimised to include an overnight incubation with the primary antibody, however, it is

entirely feasible that this could be altered to a shorter primary incubation step at room temperature or 37°C.

When examining our images and technical protocols, the benefits and drawbacks of each method can be identified. IHC gives more accurate cellular morphology and an advantage of this technique is the ability to image a whole section of tissue from surface epithelium to submucosa, thereby allowing deeper structures and organisms to be identified. We noted subepithelial fluorescence in three patients, mirroring the findings of Corriveau et al[277] who first recognised submucosal bacteria in CRS. The same group went on to report that macrophages derived from CRS patients were less capable of phagocytosing *S. aureus,*[296] a factor that may well play a part in persistent disease. It remains unknown whether submucosal bacteria are a continuum of intracellular infection or a different pathogenic entity and clearly this is an area of research that would benefit from further scrutiny. A significant weakness of this technique, however, is the inability to obtain accurate biofilm data using the same tissue section, potentially caused by tissue processing techniques or sampling error. It is not possible to examine whole mucosal samples using an upright microscope because of the inability of the illuminating source to penetrate large pieces of tissue and produce an accurate in-focus view. Furthermore, the biofilm represents a three-dimensional structure on the exterior surface of mucosa, and paraffin blocking and sectioning appeared to disrupt the biofilm such that we were unable to identify extramucosal surface *S. aureus* in any patient. Because sections of tissue are only 5 µm in thickness there is a significant chance that truly adherent biofilm may be missed within the examined section.

A key feature of CSLM is its ability to select depth within a sample via a process known as optical selection. Whole mucosal samples can be imaged using specialised optical filters to block out light from areas that are out of the ultrafine focal z-plane. Thus, multiple slices from adjacent levels within a sample can be imaged with a slice thickness of as little as 0.3 µm and a true three-dimensional appreciation of the tissue obtained. Therefore, it is possible to identify whether fluorescent *S. aureus* cocci are superficial to the intraepithelial z-plane, or at an equivalent plane closely localised to epithelial nuclei. Although CSLM-FISH/PI does not give as accurate tissue morphology as IHC, it does allow for examination of larger pieces of tissue, as much as 5 x 5 mm. Under low power magnification, this allows a significantly larger area to be assessed for biofilm presence in addition to the identification of intracellular *S. aureus*. There still remains the possibility for sampling error; however, in our opinion this is greatly reduced because of the ability of CSLM-FISH/PI to examine mucosal samples with wider surface areas. The use of CSLM has already been established as an effective method of detecting biofilms from multiple pathogenic species including *S. aureus, Pseudomonas aeruginosa, Haemophilus influenzae* or fungal sources[187,291,297] and importantly, it allows for the simultaneous analysis and detection of surface biofilm attached to the same piece of tissue in which the intracellular bacteria are identified. A major weakness of the CSLM is that the laser beam is unable to penetrate deeper than the epithelial layer and thus cannot identify the presence or absence of submucosal bacteria. As previously discussed, this may be an important pathogenic factor that use of this technique will overlook.

Both methods appear capable of identifying intracellular *S. aureus* with neither seemingly better than the other. In our patient cohort we found agreement between the two techniques in 13/17 (76%) samples tested. In a number of cases we were able to identify organisms

using CSLM-FISH/PI but not IHC. Our IHC protocol uses formalin fixed tissue specimens, and although we have confirmed our protocol with positive controls, there does remain the possibility that the antigen retrieval process does not wholly allow binding of the primary antibody. A benefit of CSLM-FISH/PI is the ability of the PNA-FISH probe to accurately target the 16S sequence of *S. aureus*. When used in combination with nonspecific DNA targeting PI, this means that the two fluorescing channels can be merged and true *S. aureus* can be identified by colocalisation of both stains within the images. It should also be noted that our analysis was performed in CRS patients only; therefore further validation of CSLM-FISH/PI would be beneficial in undiseased mucosal samples. However, the fact that in some patient samples no organisms were identified with either technique coupled with negative microbiological swabs suggests that these patients were truly negative of *S. aureus*. An additional weakness is that although both techniques are able to identify *S. aureus* within the tissue of these patients, they are unable to answer an important question as to whether they represent live or dead bacteria. This question can only be concluded with further investigation. Recent literature has recognised that *S. aureus* infection can be responsible for an altered Th2 cytokine response, as shown *ex vivo* by Wang et al[298] and *in vivo* by Foreman et al.[205] Whether biofilm or intracellular infection is responsible for these complex immunologic changes still unknown. In addition to their active pathogenic role, these bacteria may represent a reservoir of infection that exists within the tissue of CRS patients that is resistant to medical and surgical treatment. An important step will be to apply these techniques to a cohort of CRS and control patients and ascertain whether biofilm plays a role in intracellular infection, as well as establish the prevalence of intracellular *S. aureus* and identify its true pathogenic nature

2.6 CONCLUSION

CSLM-FISH/PI and IHC are complementary techniques, capable of detecting intracellular *S. aureus* within sinonasal epithelial tissue samples with neither one appearing superior to the other. CSLM-FISH/PI allows for the simultaneous detection of surface biofilm within the tissue analysed with a protocol that is less time consuming and labour intensive. IHC has a role in the identification of intracellular and sub-mucosal *S. aureus* within these tissues. Additional investigation is required to identify the true pathogenic nature of intracellular *S. aureus* as well as its relationship to surface biofilm status.

CHAPTER 3

PREVENTION OF FALSE POSITIVE BINDING DURING IMMUNOFLUORESCENCE OF *STAPHYLOCOCCUS AUREUS* INFECTED TISSUE BIOPSIES

Conducted in the Department of Otolaryngology – Head and Neck Surgery

The University of Adelaide, Adelaide, Australia

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Research paper

Prevention of false positive binding during immunofluorescence of *Staphylococcus aureus* infected tissue biopsies ☆,☆☆

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ABSTRACT

Immunofluorescence is a fundamental tool used to analyse tissue and cell samples with a wide variety of available antibodies targeting specific proteins or molecules. Staphylococcal surface protein A is used both in clinical, research and industrial settings for its ability to bind mammalian immunoglobulin G. Spurious binding between protein A and IgG antibodies can lead to false-positive fluorescence and misleading results. Here we demonstrate this occurring in formalin-fixed patient samples that harbour *Staphylococcus aureus* infection, and characterise methods to overcome this issue. Specifically the use of F(ab') fragment antibodies or blocking with human IgG is shown to prevent antibody–protein A interaction in formalin-fixed *S. aureus* smears, biopsies obtained from infected patients, and experimentally infected tissue samples.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a facultative anaerobic Gram-positive coccal bacterium that plays a role in numerous disease processes ranging from acute infections such as cellulitis, bacteremia or sepsis to chronic conditions such as osteomyelitis and chronic rhinosinusitis (Ellington et al., 2003; Corriveau et al., 2009). In recent years it has been recognised that *S. aureus* has the capacity to invade, persist and even replicate within cells and tissues (Sachse et al., 2010). Within its peptidoglycan cell wall are components such as protein A, which belong to the family of microbial surface components recognising adhesive matrix molecules (MSCRAM's). Since its identification in the 1960s, protein A has been utilised in the purification of antibodies through its high affinity to the constant fragment crystallizable region (Fc region) of IgG antibodies (Lindmark et al., 1983). Similar applications are found with immunoglobulin-binding proteins identified in other bacteria, such as protein G in *Streptococcus sp.* (Bjorck and Kronvall 1984), and protein L in *Peptostreptococcus magnus* (Bjorck 1988).

During routine indirect immunofluorescence of formalin-fixed sino-nasal mucosal biopsies obtained from patients chronically infected with *S. aureus*, false-positive fluorescence associated with both primary antibodies and their negative control was occurring, therefore confusing and potentially invalidating any results. We hypothesised this was due to protein A originating from the bacterial cell wall which survived tissue processing and continued to exhibit its capacity to bind IgG with high affinity. This study provides evidence of such interactions and describes various methods to prevent this from occurring.

2. Methods

2.1. Tissue samples

Sinonasal epithelium was obtained as part of a clinical study within the tertiary otolaryngology referral practice of the

Abbreviations: F(ab'), fragment antigen-binding region of antibody; Fc, fragment crystallizable region of antibody; MSCRAM, microbial surface components recognising adhesive matrix molecules; TBS-T, Tris-buffered saline and 0.05% tween-20 buffer; SFB, serum free protein block; DAPI, 4',6-diamidino-2-phenylindole.

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☆☆ Conflict of interest: PJW receives royalties from Medtronic for instrument design and is a consultant for Neilmed Pty Ltd.

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STATEMENT OF AUTHORSHIP

Prevention of false positive binding during immunofluorescence of *Staphylococcus aureus* infected tissue biopsies

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3.1 ABSTRACT

Immunofluorescence is a fundamental tool used to analyse tissue and cell samples with a wide variety of available antibodies targeting specific proteins or molecules. Staphylococcal surface protein A is used both in clinical, research and industrial settings for its ability to bind mammalian immunoglobulin G. Spurious binding between protein A and IgG antibodies can lead to false-positive fluorescence and misleading results. Here we demonstrate this occurring in formalin-fixed patient samples that harbour *S. aureus* infection, and characterise methods to overcome this issue. Specifically the use of F(ab′) fragment antibodies or blocking with human IgG is shown to prevent antibody–protein A interaction in formalin-fixed *S. aureus* smears, biopsies obtained from infected patients, and experimentally infected tissue samples.

Highlights

Spurious binding between antibodies and *S. aureus* occurs in infected tissues.

F(ab′) antibody use and blocking with human IgG prevents false-positive fluorescence.

Blocking concentration of human IgG demonstrates a dose-response curve.

3.2 INTRODUCTION

S. aureus is a facultative anaerobic Gram-positive coccal bacterium that plays a role in numerous disease processes ranging from acute infections such as cellulitis, bacteremia or sepsis to chronic conditions such as osteomyelitis and CRS.[144,277] In recent years it has been recognised that *S. aureus* has the capacity to invade, persist and even replicate within cells and tissues.[240] Within its peptidoglycan cell wall are components such as protein A, which belong to the family of MSCRAMMs. Since its identification in the 1960s, protein A has been utilised in the purification of antibodies through its high affinity to the constant fragment crystallizable region (Fc region) of IgG antibodies.[299] Similar applications are found with immunoglobulin-binding proteins identified in other bacteria, such as protein G in *Streptococcus sp.,*[300] and protein L in *Peptostreptococcus magnus.*[301]

During routine indirect immunofluorescence of formalin-fixed sino-nasal mucosal biopsies obtained from patients chronically infected with *S. aureus,* false-positive fluorescence associated with both primary antibodies and their negative control was occurring, therefore confusing and potentially invalidating any results. We hypothesised this was due to protein A originating from the bacterial cell wall which survived tissue processing and continued to exhibit its capacity to bind IgG with high affinity. This study provides evidence of such interactions and describes various methods to prevent this from occurring.

3.3 METHODS

Tissue samples

Sinonasal epithelium was obtained as part of a clinical study within the tertiary otolaryngology referral practice of the senior author (P.J.W.). Details of the patient cohort have been previously reported[302] and ethical approval was granted from the local institution Human Research and Ethics Committee. Subjects underwent ESS for CRS and had sinus and turbinate tissue resected as part of their routine operation. Tissue samples were immediately placed in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen Corp., Carlsbad, CA) and transported to our laboratory where 5 mm x 5mm mucosal sections were placed in 10% formaldehyde (Sigma-Aldrich, St. Louis, MI) overnight and embedded in paraffin. Normal control tissue was obtained from patients undergoing endoscopic transnasal pituitary surgery that had no clinical history or radiological evidence of rhinosinusitis, and processed in an identical manner. An experimentally infected control was prepared by incubating a 5mm x 5mm piece of control nasal mucosa with *S. aureus* ATCC 25923 overnight and fixed in 10% formaldehyde before being embedded in paraffin and sectioned as per the protocol.

Antibodies

The primary antibodies used in indirect immunofluorescence were a mouse IgM monoclonal antibody to *S. aureus* (Millipore, Billerica, MA), a rabbit polyclonal anti-human pan-cytokeratin (Invitrogen Corp., Carlsbad, CA), and a rabbit polyclonal IgG anti-caspase-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Negative antibody controls were prepared with normal rabbit IgG (Sigma-Aldrich, St. Louis, MI). Secondary antibodies were goat anti-mouse IgM-Alexa 488 (Invitrogen Corp., Carlsbad, CA) and sheep IgG

F(ab′) anti-rabbit IgG-Cy3 (Sigma-Aldrich, St. Louis, MI). DAPI (Invitrogen) was used as a counterstain for DNA localisation.

Conjugated antibodies used in direct fluorescence of fixed slides of *S. aureus* were donkey anti-goat IgG FITC (Abcam, Cambridge, UK); F(ab′) donkey anti-goat IgG Alexa 488 (Jackson Immunoresearch, West Grove, PA); rabbit anti-goat IgG Cy3 (Sigma-Aldrich); F(ab′) rabbit anti-mouse IgG Alexa 594 (Bethyl Laboratories, Montgomery, TX); goat anti-mouse IgG Alexa 488 (Invitrogen); F(ab′) goat anti-mouse IgG FITC (Sigma-Aldrich); sheep anti-mouse IgG FITC (Bethyl Laboratories) and F(ab′) sheep anti-rabbit IgG Cy3 (Sigma-Aldrich). Two preparations of purified human IgG used in blocking experiments were kindly donated by Dr Michael Jackson (Department of Immunology, Flinders Medical Centre and Flinders University).

Indirect Immunofluorescence of Tissue Sections

Indirect immunofluorescence of paraffin embedded tissue sections was carried out following a previously described protocol.[303] Briefly, from paraffin tissue blocks, 5 µm sections were prepared and mounted on glass microscope slides (Superfrost, Thermo Fisher, Waltham, MA). Tissue sections were deparaffinised and rehydrated using xylene and graded ethanol. Slides were immersed in 10 mM sodium citrate and antigen retrieval performed using a microwave technique. Slides were cooled then marked with a PAP pen and transferred into a TBST. Serum free protein block (SFB) (Dako, Glostrup, Denmark) was applied for 1h at room temperature and slides were incubated simultaneously with mouse anti-*S. Aureus* and rabbit anti-pan-cytokeratin antibodies overnight at 4°C. Slides were washed in TBST four times on a horizontal shaker and secondary antibodies (goat anti-mouse IgM-Alexa 488 and sheep anti-rabbit IgG-Cy3) were applied for one hour at

room temperature in a darkened moisture chamber. Slides were washed in TBST five times on a horizontal shaker and coverslips mounted in 90% glycerol for microscope analysis with a Zeiss Axio Vert.A1 (Carl Zeiss AG, Jena, Germany).

Fixed slides of S. aureus

Fixed slides of *S. aureus* were prepared using a reference strain ATCC 25923. *S. aureus* cultures were prepared using previously described techniques.[304] Briefly, the bacterial strain was cultured overnight in cerebrospinal fluid broth (Oxoid Australia, Thebarton, SA) at 37°C in a shaking incubator. Subsequently, a single loop of bacteria was streaked onto a horse blood agar plate (Oxoid, Australia) and incubated for 18 to 24 h. A number of colonies were mixed into 2 ml 0.45% sodium chloride and diluted as necessary to create a 1 McFarland unit solution. This solution was streaked onto a glass microscope slide (Superfrost, Thermo Fisher, Waltham, MA) using a cotton tip applicator and air dried overnight in a laminar flow hood. Slides of *S. aureus* were fixed with 2.5% formalin in phosphate-buffered saline for 10 min before air drying and storage in at 4°C.

Direct immunofluorescence of fixed slides of S. aureus

Prepared slides of *S. aureus* were washed in TBST then marked with a PAP pen. Paired conjugate antibodies of whole molecule heavy IgG and cleaved F(ab′) fragment antibodies were applied for 1 h at room temperature. Slides were then washed 3 times in TBST, incubated with 200 ng/ml DAPI for 15 minutes and washed in TBST. Slides were then mounted in 90% glycerol for microscope analysis. Fluorescence intensity of antibody-bound cocci was semi-quantitatively scored by two independent researchers on 40× magnification microphotos, taken blinded to antibody binding by selection of optical fields and focusing in the DAPI channel.

Blocking of fixed slides of S. aureus with human IgG

Blocking buffer containing human IgG at various concentrations (12.5, 25, 50, 100, 200 µg/ml) was prepared in 10% SFB/TBST. As no effect of increased temperature (37°C) on the blocking process was observed in preliminary experiments, fixed slides of *S. aureus* were incubated with human IgG for 2h at room temperature, followed by incubation with normal rabbit IgG at a concentration of 20 µg/ml overnight at 4°C. Binding of rabbit IgG was detected with sheep IgG F(ab′) anti-rabbit Cy3 (Sigma-Aldrich, 1/200 dilution). From each slide, 6 microphotos at 40× magnification were taken blinded to antibody fluorescence in the DAPI channel for assessment of rabbit IgG binding. The mean fluorescence intensity of antibody-bound cocci was then measured by ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA). Statistical analysis was performed with Graphpad Prism 5.0 software (San Diego, CA) using a paired t-test and statistical significance was obtained when $p < 0.05$.

Blocking of tissue sections with human IgG

Tissue sections were prepared identically as previously described. Human IgG blocking buffer with a concentration of 200 µg/ml was prepared in 10% SFB/TBST. Prior to incubation with rabbit IgG (20 µg/mL), slides were blocked with either SFB alone or 200 mg/ml human IgG diluted in SFB for 2h at room temperature. The remainder of the protocol was identical previously described.

3.4 RESULTS

False positivity in indirect immunofluorescence of tissue sections obtained from patients having chronic infection with S. aureus.

False positive fluorescence was sporadically observed in tissue sections obtained from *S. aureus*-infected patients but not control patients when rabbit IgG preparations were used as either a primary antibody (1/50 rabbit polyclonal IgG anti-caspase-1; Santa Cruz, CA) (Fig. 3.1A) or negative control (20 μg/ml Rabbit IgG, Sigma) (Fig. 3.1B). This fluorescence was localised to the same tissue area, characterised by cytoplasmic clusters of dots sizing around 1 μm or less.

Serial sections were stained with antibodies specific for *S. aureus* and pan-cytokeratin demonstrating clusters of green fluorescent dots in infected areas within the cytoplasm (Fig. 3.1C–E). When a negative control rabbit IgG preparation was used, false positive fluorescence could be clearly seen (Fig. 3.1F–H) that could be caused by interaction of rabbit IgG with bacterial component(s) in fixed tissue.

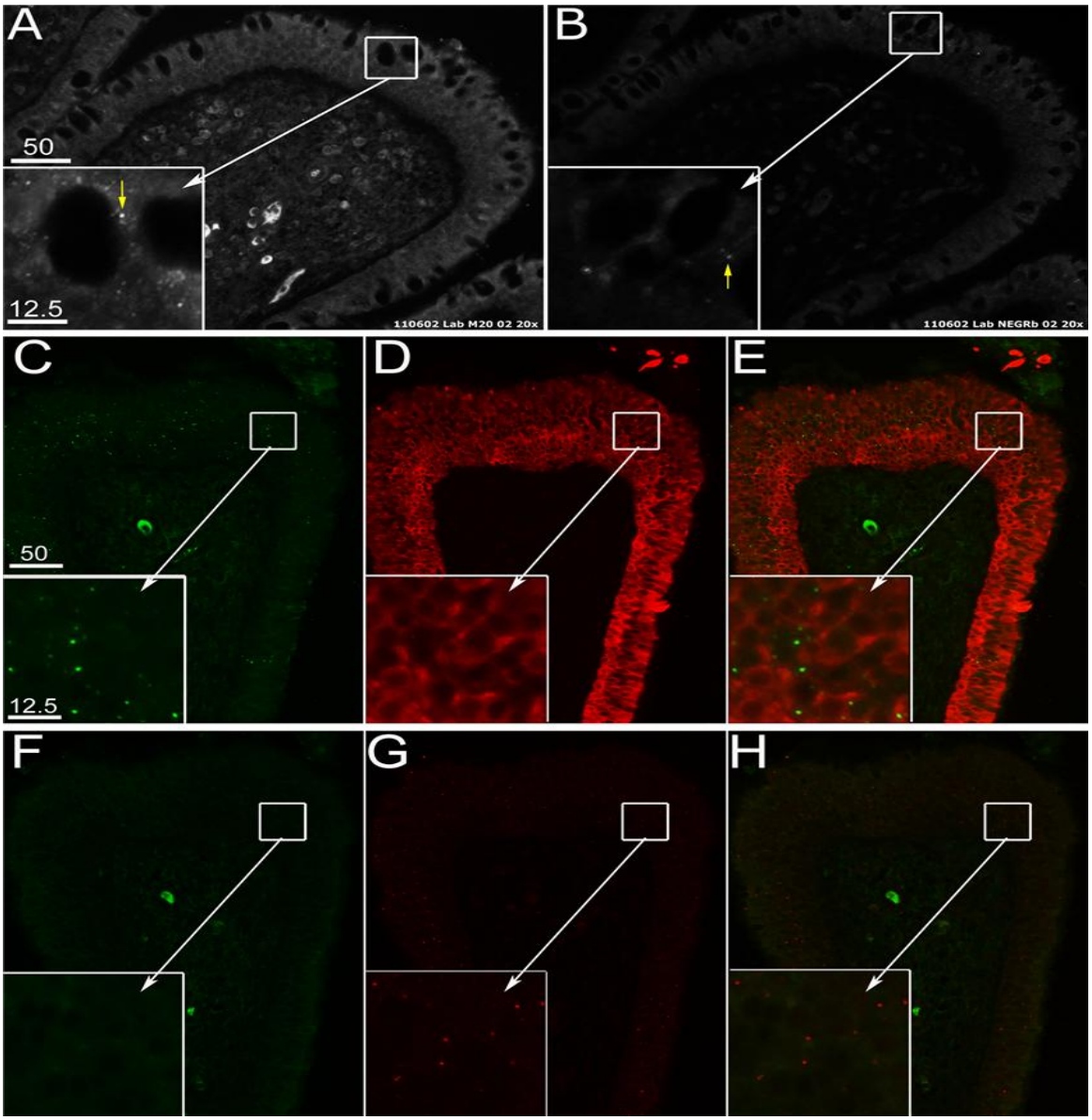

Figure 3.1: False-positive fluorescence of formalin-fixed patient biopsies in association with *S. aureus* infection.

A and B: Indirect immunofluorescence of sinonasal mucosa obtained from a patient with *S. aureus* infection. Boxed areas are magnified in the insets, demonstrating similar clusters of fluorescence in the same location of adjacent serial sections (yellow arrow) incubated with either a primary antibody (rabbit anti-caspase-1, A) or negative control (normal rabbit IgG, B). The secondary antibody in A and B was sheep IgG F(ab′)-Cy3.
C to E: Tissue sections of another patient were double labelled with antibodies specific to *S. aureus* (C, green) and cytokeratin (D, red). Conjugate antibodies used were goat anti-mouse IgM Alexa 488 (C) and sheep F(ab′) anti-rabbit IgG Cy3 (D), with the images merged. Numerous bacteria (green) were detected in cytokeratin-labelled epithelia.
F to H: In an adjacent section, normal rabbit IgG served as a negative control; the conjugate goat anti-mouse Alexa 488 did not demonstrate' any specific binding (F), however, sheep anti-rabbit Cy3 gave clusters of false-positive fluorescence (G, Red) with similar patterns of staining and being localised to the same area of infected epithelia. Scale bars in micrometers.

Elimination of IgG antibody binding due to protein A in formalin-fixed S. aureus slide smears

If the above described false positive fluorescence was due to protein A originated from the infecting *S. aureus*, it should be eliminated by replacing whole molecule IgG with F(ab′) fragment antibodies, or reduced by using antibodies raised in species known for low IgG affinity to protein A. To test this hypothesis, a panel of paired whole molecule IgG and F(ab′) fragment conjugated antibodies were compared in a direct immunofluorescence assay (Table 1). Formalin-fixed *S. aureus* smear slides were employed in this part of the project as substitute for patient samples. Smear slides stained with conjugated antibodies of whole molecule IgG raised in rabbit and donkey origin showed strong fluorescence associated with cocci of *S. aureus*, confirmed by co-localisation of the fluorescence with DAPI. F(ab′) fragments of conjugate antibodies also raised in rabbit and donkey did not demonstrate any binding to *S. aureus* with images demonstrating DAPI fluorescence alone. Whole molecule IgG raised in sheep and goat demonstrated very weak or no binding to *S. aureus*, whereas the F(ab′) fragments demonstrated no binding at all (Table 3.1).

Table 3.1: Direct immunofluorescence of *S. aureus* slide smears by IgG conjugates

Species	Whole molecule IgG	F(ab′) fragment IgG
Rabbit	+++	-
Donkey	+++	-
Goat	+/-	-
Sheep	+/-	-

Next, as human IgG is the species known for having the highest affinity to protein A, a series of experiments were carried out using normal human IgG preparations as a blocking reagent to prevent binding of rabbit IgG to *S. aureus* slide smears. An F(ab′) sheep anti-rabbit conjugate was employed to detect rabbit IgG binding which importantly did not demonstrate any *S. aureus* binding in previous experiments. Thus, 40 to 80% removal of rabbit IgG binding to cocci required at least 2h pre-incubation with human IgG, blocking effects of which became saturated between 5 and 10 fold concentration (Fig. 3.2). A statistically significant difference in fluorescence intensity was noted with application of all concentrations of human IgG. Although detection of 200μg/ml HuIgG preparation was possible using automated imaging (ImageJ), this signal was not resolved by eye (Fig. 3.3).

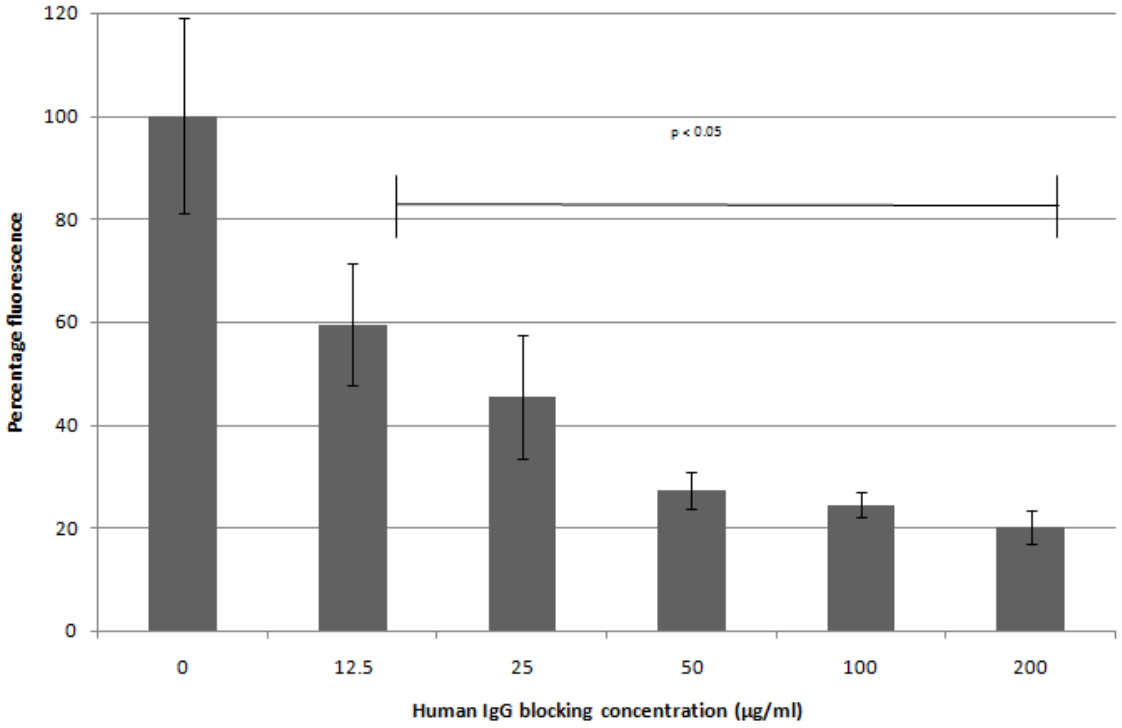

Figure 3.2: Blocking effects of normal human IgG on binding of normal rabbit IgG to cocci of *S. aureus*. Following 2h incubation at room temperature with preparations of normal human IgG slide smears of *S. aureus* were allowed to bind with 10ug/mL normal rabbit IgG (Sigma-Aldrich, St. Louis, MO) overnight at 4°C. Binding of rabbit IgG was detected in red fluorescence by sheep IgG F(ab′) anti-rabbit IgG Cy3 (Sigma-Aldrich). Cocci were photographed under 40× objective, using DAPI fluorescence channel for selection of optical fields and focusing. At each concentration of applied human IgG, particles' mean fluorescence intensity in red channel was quantitated from 6 optical fields by ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA), then plotted in y-axis against concentration of normal human IgG on x-axis. Results were representative of 2 experiments.

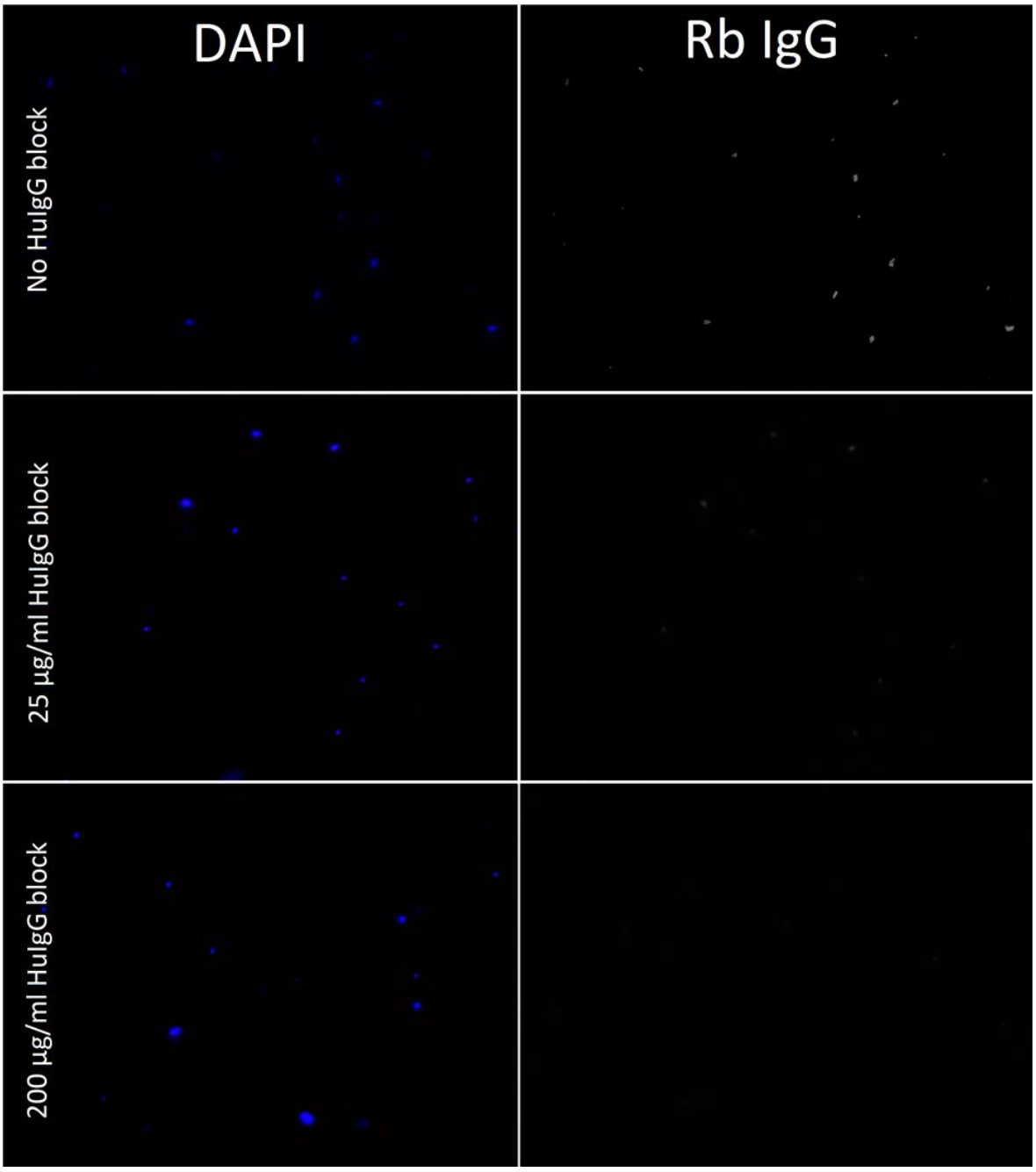

Figure 3.3: Representative images of *S. aureus* slide smears pre-treated with no block (top), 25µg/mg human IgG block (middle) and 200ug/ml human IgG block (bottom) for 2 hours prior to incubation with DAPI (left) and Rabbit IgG (right), as well as sheep IgG F(ab′) anti-rabbit Cy3 as a conjugate. Cocci are clearly visible in the rabbit IgG channel when no blocking was performed compared to being barely preceptible when pre-treated with 200 µg/ml human IgG.

Prevention of false positivity due to S. aureus infection in immunofluorescence of human tissue

The methods described of avoiding antibody binding to *S. aureus* on formalin-fixed smear slides were translated back to human tissue samples. In indirect immunofluorescence of tissue samples that were positively stained using a *S. aureus* IgM antibody, false positive fluorescence in the negative control could be efficiently removed by 2h pre-incubation with normal human IgG at 200µg/mL (Fig.4). Furthermore, tissue from a control patient experimentally infected *ex vivo* with *S. aureus* showed both positive fluorescence by anti-*S. aureus* antibody and false positive fluorescence in the negative control. As expected, the latter false positive fluorescence was completely removed by blocking of tissue sections with human IgG (Fig. 3.4).

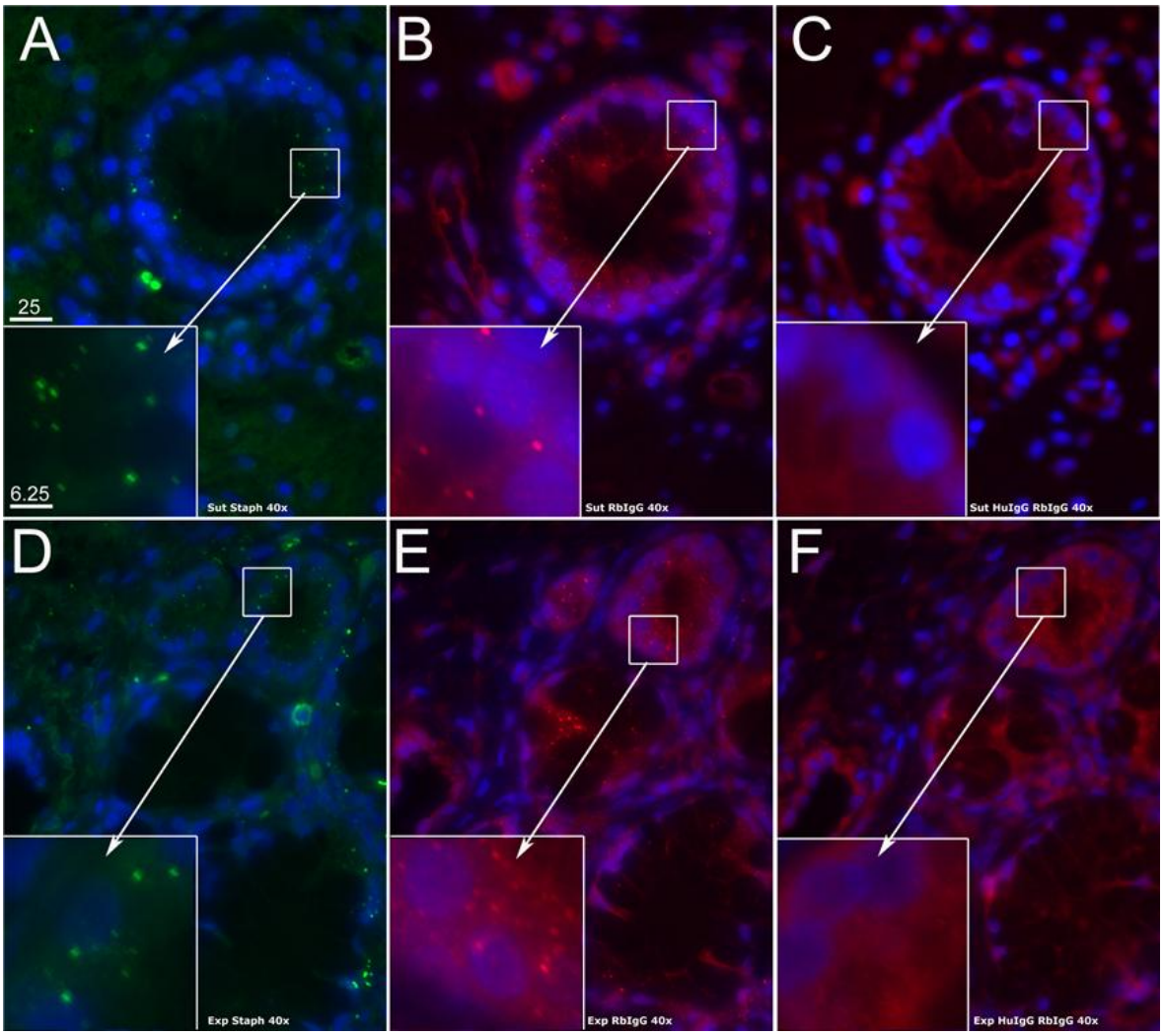

Figure 3.4: Blocking of rabbit IgG binding to *S. aureus*-infected human tissue sections in indirect immunofluorescence assay with human IgG.

A-C: Biopsy section obtained from a patient with *S. aureus* infection stained with an IgM antibody specific for *S. aureus* (A) and normal rabbit IgG (B) demonstrating clusters of bacteria within the tissues (green and red). A serial section pre-blocked with 200 µg/ml normal human IgG for 2 hours prior to incubation with normal rabbit IgG shows an absence of false-positive fluorescence (C).
D-E: Healthy control tissue explants experimentally infected with *S. aureus* stained specifically for *S. aureus* (D) and with normal rabbit IgG (E) also demonstrating clusters of bacteria within the tissues (green and red). A serial section pre-blocked with 200 µg/ml normal human IgG for 2 hours also shows an absence of false-positive fluorescence (F). Secondary antibodies were goat IgG anti-mouse IgM AF488 (green), and sheep IgG F(ab′) anti-rabbit IgG Cy3 (red). Blue was for DAPI. Insets are 4 time-magnification of the boxed areas. Scale bars in micrometers in microphoto A and its inset are applied for the whole panel.

3.5 DISCUSSION

Invasion of tissues by the pathogenic *S. aureus* bacteria has been noted in a broad range of both acute and chronic diseases.[240,248,252] In particular it has been suggested as a possible mechanism of pathogenicity in chronic osteomyelitis[144,305] and chronic rhinosinusitis[240,252,277] in which the bacteria may sequestrate inside the cell to evade immune responses. In this context, the protein A of the intracellular bacteria may be mostly free from opsonisation with the patient IgG and therefore be available for binding IgG *ex vivo*, for example during various immunoassays.

Here we provide the first evidence that tissue samples containing. *S. aureus* may give false positive results when performing indirect immunofluorescence of samples, even when processed into paraffin blocks. Such unintentional binding of IgG antibodies to *S. aureus* within tissues can produce misleading results. Through a number of experiments we have demonstrated that this type of false positivity is likely associated with specific binding of protein A to the Fc region of IgG antibodies. Based on this concept, approaches have been developed for prevention of false positivity in immunofluorescence of tissues that have the possibility of infection with *S. aureus*. These include the use of F(ab′) fragment antibodies, and selection of species IgG with low protein A affinity such as goat and sheep, which is in concordance with previous reports.[306] Use of donkey and rabbit whole molecule IgG antibodies on infected tissues should be carried out with caution. For this purpose a simple but efficient method of blocking has been developed using human IgG which has the highest affinity for protein A amongst all species.[307]

3.6 CONCLUSION

Researchers should be vigilant when performing immunofluorescence assays on tissues that have the possibility of infection with *S. aureus,* due to binding of antibodies to bacterial components such as protein A. Recommended antibodies to be used in this circumstance are cleaved F(ab′) fragments or IgG of species having low affinity to protein A. A simple but efficient method of blocking with purified human IgG has been validated to prevent false positivity due to antibody binding to *S. aureus* in infected tissues.

CHAPTER 4

THE MULTIPLICITY OF *STAPHYLOCOCCUS AUREUS* IN CHRONIC RHINOSINUSITIS: CORRELATING SURFACE BIOFILM AND INTRACELLULAR RESIDENCE

Conducted in the Department of Otolaryngology – Head and Neck Surgery

The University of Adelaide, Adelaide, Australia

Financial assistance provided by The University of Adelaide and the European Rhinologic Society.

The Laryngoscope

The Multiplicity of *Staphylococcus aureus* in Chronic Rhinosinusitis: Correlating Surface Biofilm and Intracellular Residence

Neil C.-W. Tan, MBBS, MRCS; Andrew Foreman, BMBS(Hons), PhD; Camille Jardeleza, MBChB; Richard Douglas, MBChB; Hai Tran, PhD; Peter John Wormald, MD, FRCS

Objectives/Hypothesis: The biofilm paradigm of chronic rhinosinusitis (CRS) is increasingly understood to play a key role in the pathophysiology of this disease. The role of intracellular infection of sinonasal epithelial cells has been suggested as a potential reservoir of pathogenic organisms that can lead to recalcitrant disease despite maximal medical and surgical treatment. Could a surface biofilm play a role in allowing intracellular infection to occur, and what are the factors associated with potential intracellular infections? The aim of this study was to investigate these questions.

Study Design: A prospective study including 36 CRS patients undergoing endoscopic sinus surgery and five control patients undergoing endonasal pituitary surgery.

Methods: Sinonasal mucosa harvested at the time of surgery was examined with a *Staphylococcus aureus* fluorescence in situ hybridization probe and propodium iodide counterstain using the confocal scanning laser microscope for both biofilm status and evidence of intracellular organisms.

Results: Intracellular *S aureus* was identified in 20/36 (56%) CRS patients compared to 0/8 (0%) control patients. CRS patients with intracellular infection were significantly more likely to harbor surface biofilm (20/20, $P = .0014$) and have a *S aureus*-positive culture swab (12/20, $P = .0485$).

Conclusions: This study gives further evidence supporting a role of intracellular *S aureus* in CRS. In all cases intracellular infection was associated with surface biofilm, suggesting a potential relationship between the two. Further work is required to delineate the true mechanisms of intracellular persistence and also the role that it plays in the recalcitrant nature of CRS.

Key Words: Chronic rhinosinusitis, intracellular infection, biofilms, Staphylococcus aureus.

Level of Evidence: 2c

***Laryngoscope*, 122:1655–1660, 2012**

INTRODUCTION

Recent research has implicated Staphylococcus *aureus* in patients who have a poor outcome after endoscopic sinus surgery.[1,2] In this research, patients with *S aureus* found at the time of surgery had an increased likelihood of persistent postoperative symptoms and frequent recurrent infections. *S aureus* may exist in the planktonic, biofilm, small colony variant, or intracellular form among others, each of these associated with unique disease-producing characteristics. Since 2004, biofilm presence has been consistently demonstrated on the mucosa of CRS patients using a variety of techniques, with a general prevalence of 40% to 80%.[2] More recently, species-specific fluorescence in situ hybridization (FISH) probes have been used to accurately identify *S aureus* biofilms in over 50% of patients, with a direct association to more severe disease parameters.[1]

Despite our further understanding of the pathophysiology of CRS, there are still unanswered questions. We see a subset of patients with detectable *S aureus* biofilms who, despite maximal medical therapy and repeated operations, have significantly worse postoperative outcomes and a higher incidence of recurrent infection.[1]

It is increasingly recognized that *S aureus* can evade the host's immune defenses. The role of intracellular *S aureus* has been suggested as a mechanism for how the bacterium can evade traditional therapies including surgery, antibiotics, and topical washes.[3,4] *S aureus* has been recognized as having the capacity to invade numerous human tissue types including osteoblasts, bronchial epithelium, keratinocytes, and sinonasal epithelium[5–9] through the nonprofessional phagocytic capacity of epithelial cells.

A number of studies have demonstrated that *S aureus* can reside intracellularly within the sinonasal mucosa of CRS patients[10–13]; however, there has been no

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The multiplicity of *Staphylococcus aureus* in chronic rhinosinusitis: Correlating surface biofilm and intracellular residence

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4.1 ABSTRACT

Objectives/Hypothesis: The biofilm paradigm of CRS in increasingly understood to play a key role in the pathophysiology of this disease. The role of intracellular infection of sinonasal epithelial cells has been suggested as a potential reservoir of pathogenic organisms that can lead to recalcitrant disease despite maximal medical and surgical treatment. Could a surface biofilm play a role in allowing intracellular infection to occur, and what are the factors associated with potential intracellular infections? The aim of this study was to investigate these questions.

Study Design: A prospective study including 36 CRS patients undergoing endoscopic sinus surgery and 8 control patients undergoing endonasal pituitary surgery.

Level of evidence: 2C

Methods: Sinonasal mucosa harvested at the time of surgery was examined with a *S. aureus* FISH probe and PI counterstain using the CSLM for both biofilm status and evidence of intracellular organisms.

Results: Intracellular *S. aureus* was identified in 20/36 (56%) CRS patients compared to 0/8 (0%) control patients. CRS patients with intracellular infection were significantly more likely to harbour surface biofilm (20/20, p=0.0014) and have a *S. aureus* positive culture swab (12/20, p=0.0485).

Conclusions: This study gives further evidence supporting a role of intracellular *S. aureus* in CRS. In all cases intracellular infection was associated with surface biofilm suggesting a potential relationship between the two. Further work is required to delineate the true mechanisms of intracellular persistence and also the role that it plays in the recalcitrant nature of CRS.

4.2 INTRODUCTION

Recent research has implicated *S. aureus* in patients who have a poor outcome after ESS.[31,202] In this research, patients with *S. aureus* found at the time of surgery had an increased likelihood of persistent postoperative symptoms and frequent recurrent infections. *S. aureus* may exist in the planktonic, biofilm, small colony variant or intracellular form among others, each of these associated with unique disease producing characteristics. Since 2004, biofilm presence has been consistently demonstrated on the mucosa of CRS patients using a variety of techniques with a general prevalence of 40% to 80%.[31] More recently, species specific FISH probes have been used to accurately identify *S. aureus* biofilms in over 50% of patients with a direct association to more severe disease parameters.[202]

Despite our further understanding of the pathophysiology of CRS there are still unanswered questions. We see a subset of patients with detectable *S. aureus* biofilms who, despite maximal medical therapy and repeated operations have significantly worse postoperative outcomes and a higher incidence of recurrent infection.[202]

It is increasingly recognised that *S. aureus* can evade the host's immune defences. The role of intracellular *S. aureus* has been suggested as a mechanism for how the bacterium can evade traditional therapies including surgery, antibiotics and topical washes.[308,309] *S. aureus* has been recognised as having the capacity to invade numerous human tissue types including osteoblasts, bronchial epithelium, keratinocytes and sinonasal epithelium[144,221,248,310,311] through the non-professional phagocytic capacity of these cell types.

A number of studies have demonstrated that *S. aureus* can reside intracellularly within the sinonasal mucosa of CRS patients,[240,252,275,277] however, there has been no correlation between surface biofilm and the presence of intracellular bacteria. Could a surface biofilm play a role in allowing intracellular infection to occur, and what are the factors associated with potential intracellular infections? The aim of this study was to investigate these questions.

4.3 METHODS

Study Design and Population

This prospective study was completed in the tertiary referral practice of the senior author (P.J.W.) based in Adelaide, South Australia. The local institution human research ethics committee gave their approval for the study and all patients provided informed consent to participate in the study. Patients who met the American Academy of Otolaryngology-Head and Neck Surgery diagnostic criteria for CRS[1] and were undergoing ESS were recruited into the study as well as a control group consisting of patients undergoing pituitary surgery. Patients were excluded from the study if they were under 18 years of age, suffered from any form of immunodeficiency or had clinical or radiological evidence of invasive disease.

Data Collection

Preoperative demographic and clinical information including age, sex, medical history (including history of smoking, asthma and immunodeficiency), any previous ESS procedures and preoperative CT (Lund-Mackay) scores were recorded. As part of clinical history a scoring system was used that recorded 5 major symptoms of CRS (nasal obstruction, rhinorrhoea, postnasal drip, headache or facial pain and sense of smell).

Perioperative Data Collection

Patients included in the study either underwent minifunctional ESS (maxillary antrostomy and ethmoidectomy), full functional ESS (complete sphenoid ethmoidectomy and frontal sinusotomy) or modified endoscopic Lothrop/Draf 3/frontal drill-out procedure. Intraoperative findings were recorded including presence of mucopus, fungus or nasal

polyposis. Endoscopically guided swab cultures and fresh mucous were taken from any areas suggesting infection at the time of surgery.

Tissue Collection

All CRS patients had pieces of sinonasal mucosa harvested from the nasal cavity during their procedure then immediately stored in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen Corp., Carlsbad, CA) and transported to our laboratory. Tissue was washed thoroughly in MilliQ water (Millipore, Billerica, MA) to remove planktonic bacteria and frozen for later analysis. Our previous studies have demonstrated that freezing tissue at -80°C does not adversely affect subsequent analysis.[294]

Fluorescence in situ Hybridization Protocol

This study utilised a *S. aureus* specific FISH protocol that has been validated as an effective method of intracellular[302] and biofilm detection.[291] Briefly, single 1 mm x 1 mm pieces of sinus mucosal tissue were washed and heat fixed to glass slides for 20 minutes in a 55°C incubator, dehydrated in 90% alcohol and air dried. The FISH probe was applied to the tissue and hybridisation occurred at 55°C for 90 minutes. Slides were washed in the manufacturer's wash solution at 55°C for 30 minutes. Tissue was then counterstained with PI (70 µl of 50 µg/ml PI in glycerol) for DNA and a glass coverslip was fixed into place. The *S. aureus* FISH probe used in this study is labelled with an Alexa-488 (Molecular Probes, Eugene, OR) fluorophor that can be stimulated by lasers at a wavelength of 488 nm and detected by photomultipliers at a peak of 519 nm. Conversely, PI labelled DNA can be stimulated by lasers at a wavelength of 561 nm and detected at 617 nm, thereby allowing us to differentiate between the two labelled structures within our tissue sample.

Tissue Analysis

The prepared tissue was immediately transported to Adelaide Microscopy for analysis with the Leica TCS SP5 CSLM (Leica Microsystems, Wetzlar, Germany). The tissue was analysed for the presence of *S. aureus* biofilm using techniques previously described by our department.[294] Following detection of *S. aureus* the PI counterstain was identified using a different frequency laser, revealing both spheric nuclei of around 10 μm size and bacterial DNA as bright coccal structures of less than 3-4 μm. When the green fluorescence of FISH and the red fluorescence of PI are merged, the *S. aureus* cocci would appear yellow, having been fluorescing on both channels. Intracellular detection of *S. aureus* is defined as two or more fluorescent coccal structures adjacent to or between epithelial cell nuclei. It was recorded whether there was presence of *S. aureus* as a surface biofilm only, present within the intranuclear plane only or present both as a surface biofilm and intracellularly. The presence of *S. aureus* surface biofilm is defined as visible coccal clusters of microcolonies on the mucosal surface.[291] The entire thickness of the specimen was imaged by means of z-stacks at 0.5 μm slices and assessed using the guidelines above by a second investigator blinded to the clinical profile and surgical findings, to ensure reproducibility of results.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA). Non parametric data are displayed as median and IQR. Dichotomous data were analysed using Fischer's exact test with statistical significance accepted when $p < 0.05$.To assess for inter-rater agreement, data were analysed using Minitab 16 Statistical Software (Minitab, Inc., State College, PA) with a Cohen's kappa calculated.

4.4 RESULTS

Demographic and Preoperative Data

A total of 41 patients were enrolled into this study with the details summarised in Table 4.1. The control group contained eight patients (3 male and 5 female) undergoing trans-sphenoidal pituitary surgery with no evidence of CRS with a mean age of 48.2 years (IQR, 33-64). The CRS group contained 36 patients (20 male and 16 female) undergoing ESS for CRS with a mean age of 46.4 years (IQR, 38-55).

Table 4.1: Characteristics of CRSwNP, CRSsNP and control group

	CRSwNP	CRSsNP	Control Group
Number	21	15	8
Age	49.9 (34-81)	43.5 (20-71)	50.5 (30-78)
Gender	15M : 6F	5M : 10F	3M : 5F
Symptom Score	18.62 (17-21)	17.1 (15-19)	0
Previous Sinus surgery	16 (76%)	7 (47%)	0
Presence of biofilm	18 (86%)	10 (67%)	0
Presence of intracellular *S. aureus*	13 (62%)	7 (47%)	0
Positive *S. aureus* bacterial culture	11	4	0

Preoperative Data

Of the CRS group, 23 patients had undergone previous endoscopic sinus surgery, 14 reported history of asthma and four were current cigarette smokers. The mean symptom score of the CRS group was 18.0/25 (IQR,16-20.75) with the most significant symptom being nasal obstruction, scoring 3.8 out of 5. The number of patients undergoing revision

surgery was 23/36 (64%) reflecting the tertiary nature of our clinical practice. There were 12 patients who reported allergies to medications. Two patients reported sensitivity to aspirin or salicylates and both suffered from asthma with associated nasal polyposis noted at surgery, therefore suffering from aspirin-exacerbated respiratory disease (AERD). Nineteen patients had a positive radioallergosorbent test (RAST) sensitivity to common aeroallergens with mould being the most frequent (n=12) followed by dust mite (n=10) and grass (n=9). Average Lund-Mackay CT scores were 15.13 (IQR, 12-21).

In comparison, none of the patients in the control group reported any subjective symptoms or had any objective evidence of CRS at the time of surgery. One patient complained of severe headache that was non-sinogenic and the symptom that led to her pituitary adenoma diagnosis. Two patients suffered from asthma, and there was one current smoker.

Intraoperative Data

All control subjects were undergoing transsphenoidal pituitary adenoma resection with no previous history of CRS. Of the 36 patients in the CRS group, six patients underwent minor surgery classified as mini-FESS and 30 had major surgery classified as either modified endoscopic Lothrop/Draf 3/frontal drill-out procedure or complete sphenoid ethmoidectomy and frontal sinusotomy. Twenty patients (56%) had evidence of nasal polyposis at the time of surgery.

Confocal Laser Microscope Findings

There were no control samples that showed the presence of *S. aureus* at an intracellular or surface level (Table 4.1). In the CRS group overall, 20 out of the 36 patients (56%) showed evidence of *S. aureus* present at an intracellular plane whereby counterstained nuclei were

seen surrounded by multiple *S. aureus* cocci (Fig. 4.1). In accordance with our previous data [291] these organisms were also detected in surface biofilms, which were identified in this study as *S. aureus* surface staining showing no PI-positive nuclei (Fig. 4.2) at a level superficial to the intracellular plane.

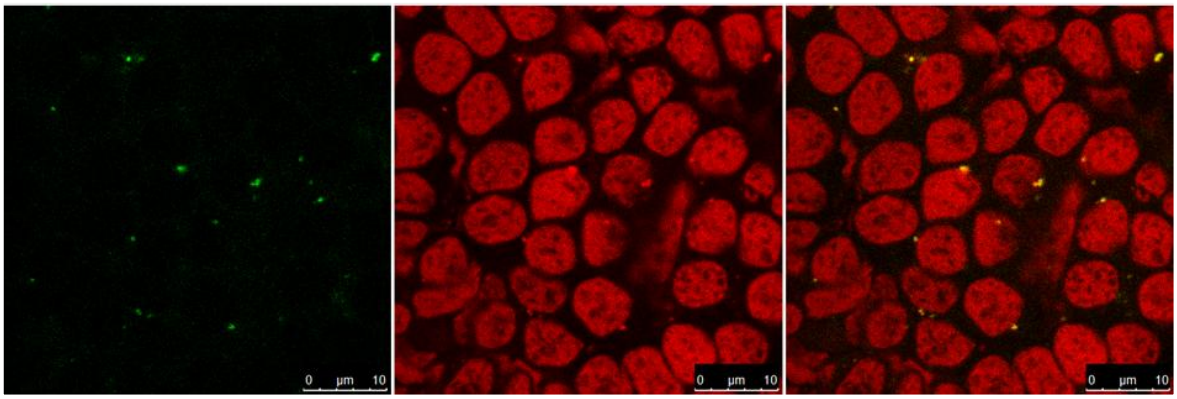

Figure 4.1: CSLM images of intracellular *S. aureus*; l – r: *S. aureus* PNA-FISH channel (green), PI channel (red), merged images demonstrating cell nuclei (red) and *S. aureus* cocci (yellow).

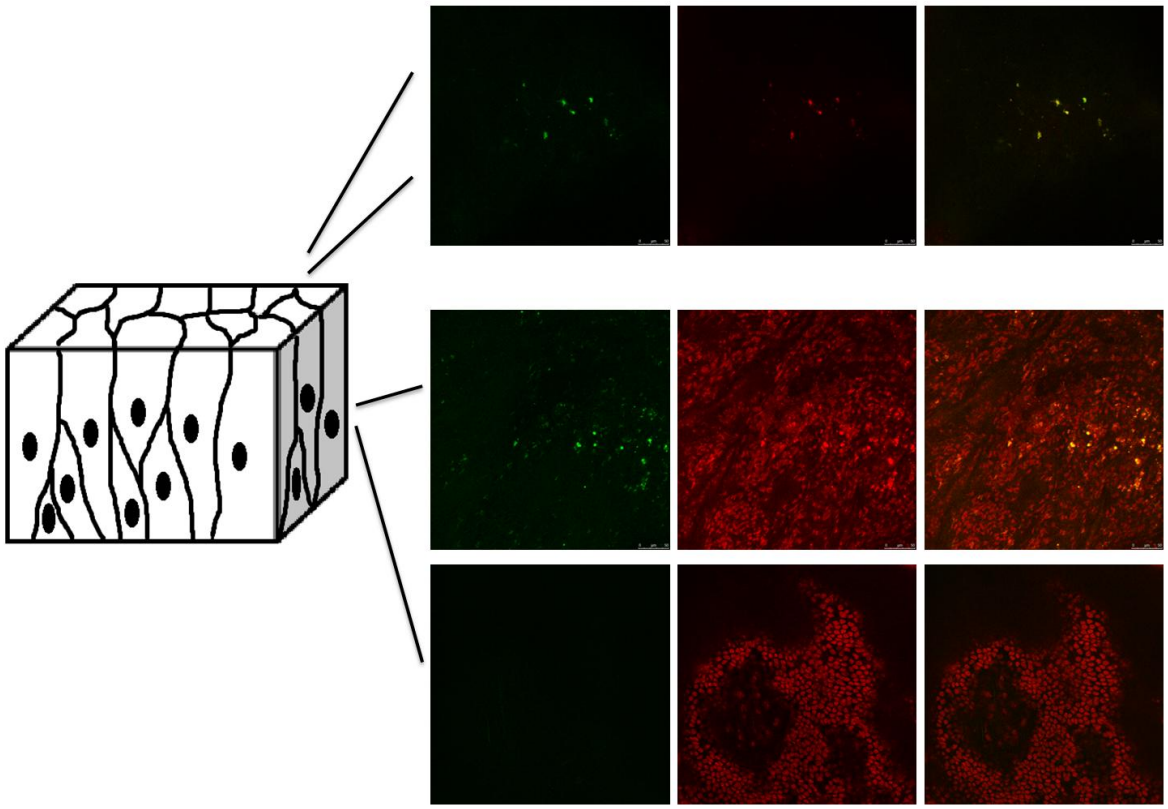

Figure 4.2: CSLM Images taken at multiple z-planes within a piece of tissue; *Top:* surface biofilm demonstrating fluorescent on both channels with absence of cell nuclei. *Middle:* Intracellular *S. aureus* fluorescing clearly with adjacent cell nuclei on the same z-plane. *Bottom:* Cell nuclei seen with an absence of *S. aureus* fluorescence.

S. aureus biofilms were detected in 20/20 (100%) of cases with intracellular infection and 9/16 (56%) cases that had no intracellular infection, with a statistically significant difference between the two subgroups (Table 4.2). In no cases were we able to identify intracellular *S. aureus* without coexisting surface biofilm in the same piece of tissue (Table 4.3). In the 13 patients undergoing primary surgery the intracellular infection rate was 6/13 (46%) compared to 15/23 (65%) in the revision cohort. There was no statistically significant difference between the two groups noted.

Table 4.2: Comparison of the CRS Intracellular *S. aureus* positive and negative groups

	CRS with intracellular *S. aureus*	CRS without intracellular *S. aureus*	p values
number	20	16	
biofilm	20 (100%)	8 (50%)	***0.0004****
symptom score	18.25 (17.0 - 19.50)	17.69 (16.36 - 19.01)	0.3759
previous sinus surgery	15	8	0.1691
positive bacterial culture	12 (60%)	4 (25%)	***0.0485****
Nasal Polyposis	13 (65%)	8 (50%)	0.4996

* = statistically significant difference ($p < 0.05$, Fischer's exact test)

Table 4.3: Overall biofilm and intracellular *S. aureus* results

	Biofilm positive	*Biofilm negative*
Intracellular *S. aureus* +ve	20	0
Intracellular *S. aureus* -ve	8	8

Agreement in *S. aureus* positivity between the two assessors was found in 93.9% of cases. With dichotomous data there is a random chance of obtaining the same reading in 50% of cases; therefore a Cohen's kappa statistic was calculated to take this into account. Using this test the inter-rater agreement was 0.87, a result that demonstrates very good agreement between the two assessors.[295]

All patients had endoscopically guided microbiological swabs or mucin taken at the time of surgery. Care was taken to avoid contamination from the nasal vestibule. *S. aureus* was the most commonly cultured organism (Table 4.4) with a methicillin resistant strain cultured in one patient. A number of polymicrobial cultures were also noted. Patients who cultured *S. aureus* at the time of surgery were significantly more likely to be harbouring an intracellular infection than those who were culture negative (p=0.0485) (Table 4.5).

Table 4.4: Microbiological results of swabs

Species	Number of Patients
Staphylococcus aureus	16
No growth	11
Streptococcus pneumoniae	3
Haemophilus influenzae	2
Pseudomonas aeruginosa	2
Moraxella	1
Proteus mirabilis	1

Table 4.5: Intracellular infection rates compared to culture status

	***S. aureus* positive culture**	***S. aureus* negative culture**
Intracellular *S. aureus* +ve	12	4
Intracellular *S. aureus* -ve	8	12

4.5 DISCUSSION

This study provides further demonstrable evidence that *S. aureus* can be identified inside the epithelium of sino-nasal mucosa using a species specific PNA-FISH probe. Intracellular *S. aureus* was related to the presence of a surface biofilm in all cases. Control tissue specimens did not harbour staphylococcal infection in either intracellular or surface biofilm form ($p = 0.0216$).

The overall intracellular detection rate of 20/36 (56%) is comparable to previously published studies. Plouin-Gaudon and Clement[275] found evidence of intracellular infection in 17/27 (63%) CRS patients using IHC and noted a higher clinical relapse in intracellular positive patients. More recently, Corriveau et al[277] used FISH and DAPI counterstaining with a semi-quantitative scoring system to assess the relative amounts of bacteria within tissue samples. Although they found evidence of *S. aureus* within tissue samples, they were unable to detect a significant difference in the amount of bacteria between CRS or control patients. Sachse[240] found conflicting results to this when using similar techniques in identifying intracellular *S. aureus* in 17/30 (57%) CRS patients compared to 0/10 control patients. They went on to demonstrate that *S. aureus* has the capacity to invade a nasal polyp epithelial cell culture.

Use of CSLM and FISH/PI counterstaining gives us confidence in the validity of our results. This technique has been validated for the identification of intracellular *S. aureus* in a side-by-side comparison of CSLM-FISH/PI versus a recognised technique of immunohistochemistry.[302] The importance of this technique is that it also allows for the simultaneous assessment of biofilm status using identification methods already validated by our department.[291] Furthermore, images at multiple z-planes within an entire block of

tissue can be digitally compiled into three-dimensional reconstructions giving an excellent appreciation and confirmation of both surface biofilm and intracellular bacterial location.

There is now little doubt that *S. aureus* can be internalised by non-professional phagocytic cells such as nasal epithelium.[244,311] The major mechanism of bacterial-epithelial cell interaction involves fibronectin-binding proteins expressed on the bacterial surface, fibronectin and integrins within the host cell membrane.[248] After attachment, subsequent pseudopod extension engulfs the bacterium into a membrane lined vacuole in what is termed the zipper-type mechanism of non-professional phagocytic cells.[270]

Multiple intracellular outcomes have been suggested following internalisation of *S. aureus*, broadly separated into two groups: clearance of bacteria or persistence within host tissues. Bacteria may be eradicated from host tissues by fusion of lysosomes to the bacteria containing vacuole and subsequent destruction of *S. aureus,* or alternatively host cell apoptosis may occur. Studies have demonstrated that following lysis of cells infected with *S. aureus,* viable pathogenic bacteria are released that are capable of infecting other cells,[144] although whether bacteria are viable after apoptosis is unknown. Conversely, bacteria may persist inside cells either within its vacuole or after escaping into the cytosol. To promote intracellular survival, radical changes to bacterial gene regulation have been described.[222] Phenotypically, intracellular *S. aureus* bacteria have been found to be significantly more resistant to antibiotic therapy compared to their extracellular counterparts. This was demonstrated in an osteoblast cell culture model[221] where it was found that intracellular *S. aureus* cell walls were significantly thicker when compared to extracellular *S. aureus*, thereby conferring additional the resistance to antibiotics.

There appears to be a clear relationship between intracellular infection and biofilms. In this study we identified a subgroup of patients demonstrating surface biofilm only; however, we were unable to identify any cases of intracellular infection that did not have evidence of surface biofilm. Could there be a temporal relationship between the biofilm and subsequent intracellular infection? As internalisation of *S. aureus* is so dependent on bacteria - host cell attachment, the biofilm may offer a synergistic environment for this to occur. It is recognised that biofilms are significantly associated with CRS patients. Foreman et al[205] examined sinonasal mucosal samples to provide evidence for a direct immunological consequence of biofilm infection in CRS. Furthermore, a link between biofilms and superantigen-specific IgE was noted within the CRS patient cohort. The role of intracellular bacteria in these aspects of the disease pathogenesis, however, is somewhat less clear. Whether intracellular *S. aureus* or is an independent disease modifying factor or has the capacity to secrete superantigens is an area that needs further clarification.

Intracellular infection could well be a cause of recalcitrance in CRS. We know that patients with biofilms have more severe disease preoperatively and suffer from worse postoperative outcomes.[201,312] Despite treatment with antibiofilm agents including topical antibiotics such as mupirocin,[213] we still see a significantly high clinical and microbiological relapse rate.[313] The intracellular bacterium may represent a reservoir of infection that is protected from host immunity and standard medical therapy but continues to act as a stimulus for ongoing inflammation. Furthermore, if viable bacteria escape from the epithelial cell or are released following cell lysis/apoptosis, this could reinfect the surface of the sinonasal mucosa leading to clinical relapse. It would be important to investigate the pathogenic capacity of these intracellularly identified bacteria.

4.6 CONCLUSION

This study uses a validated FISH protocol combined with a nucleic acid counterstain to demonstrate that intracellular infection occurs in a significant number of patients undergoing surgery for CRS. In all cases it was related to a surface *S. aureus* biofilm indicating a relationship between the two. There is little doubt that *S. aureus* can be internalised by non-professional phagocytes such as epithelial cells, although further work needs to be done delineate the true mechanisms of intracellular persistence as well as the role that it plays in the recalcitrant nature of CRS.

CHAPTER 5

INTRACELLULAR *STAPHYLOCOCCUS AUREUS:* THE TROJAN HORSE OF RECALCITRANT CHRONIC RHINOSINUSITIS?

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Financial assistance provided by The University of Adelaide and the European Rhinologic Society.

Intracellular *Staphylococcus aureus*: the Trojan horse of recalcitrant chronic rhinosinusitis?

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Background: **Despite recent evidence suggesting that *Staphylococcus aureus* exists within the sinonasal epithelium of chronic rhinosinusitis (CRS) patients, certain questions remain. The intracellular environment may provide a protective niche for pathogenic bacteria to evade host immunity and yet provide a reservoir for reinfection. To date, no studies have examined the impact of this bacterial phenotype; therefore, this study was designed to evaluate the role of intracellular *S. aureus* on postsurgical outcomes.**

Methods: **This study included 51 patients undergoing endoscopic sinus surgery (ESS) for medically-recalcitrant CRS. Sinonasal mucosa harvested at the time of surgery was dually stained with fluorescent molecular probes and imaged using confocal scanning laser microscopy for biofilm and intracellular status. Patients were followed in their early and late postoperative course for evidence of ongoing disease and signs of clinical relapse.**

Results: **Intracellular *S. aureus* was identified in 20 of 51 (39%) patients, and all were associated with surface biofilm. Biofilm alone was found in 16 of 51 (31%) patients and 15 of 51 (29%) patients had no evidence of *S. aureus*. Intracellular positive patients had a significantly higher risk of late clinical and microbiological relapse ($p = 0.014$). In this study, biofilm status without coexisting intracellular bacteria did not appear to impact on outcomes.**

Conclusion: **Clinical and microbiological relapse of disease following ESS is significantly associated with intracellular *S. aureus*. Evidence suggests that this disease association is independent to surface biofilm status. Intracellular bacteria should be taken into consideration when designing novel treatment strategies to lessen the chance of reinfection.**

Key Words:
chronic rhinosinusitis; *Staphylococcus aureus*; intracellular infection; biofilms; microbiology; clinical outcome

How to Cite this Article:
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Recalcitrant chronic rhinosinusitis (rCRS) has been linked to the Gram-positive facultative anaerobic organism *Staphylococcus aureus* in a plethora of different phenotypes, including planktonic, biofilm, intracellular or small colony variant forms. This bacteria uses a variety of pathogenic factors including superantigens[1–3] and bacterial cell wall membrane proteins[4] among others, to induce acute and chronic inflammatory changes to infected tissues. Recently, the paradigm of intracellular invasion into sinonasal epithelium has been suggested as a mechanism by which the organism can exploit an environment in order to protect itself from traditional treatments such as topical douches and antibiotics. To take advantage of the intracellular milieu, the concept of phenotype switching[5] has been recognized as a method by which the organism can alter the regulation of certain factors including bacterial metabolism or virulence in order to evade host immune responses and persist within tissues.

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Intracellular *Staphylococcus aureus:* The Trojan horse of recalcitrant chronic rhinosinusitis?

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5.1 ABSTRACT

Background: Despite recent evidence suggesting that *S. aureus* exists within the sinonasal epithelium of CRS patients, certain questions remain. The intracellular environment may provide a protective niche for pathogenic bacteria to evade host immunity and yet provide a reservoir for reinfection. To date, no studies have examined the impact of this bacterial phenotype; therefore this study was designed to evaluate the role of intracellular *S. aureus* on postsurgical outcomes.

Methods: This study included 51 patients undergoing ESS for medically-recalcitrant CRS. Sinonasal mucosa harvested at the time of surgery was dually stained with fluorescent molecular probes and imaged using CSLM for biofilm and intracellular status. Patients were followed in their early and late postoperative course for evidence of ongoing disease and signs of clinical relapse.

Results: Intracellular *S. aureus* was identified in 20 of 51 (39%) patients, and all were associated with surface biofilm. Biofilm alone was found in 16 of 51 (31%) patients and 15 of 51 (29%) patients had no evidence of *S. aureus*. Intracellular positive patients had a significantly higher risk of late clinical and microbiological relapse ($p = 0.014$). In this study, biofilm status without coexisting intracellular bacteria did not appear to impact on outcomes.

Conclusions: Clinical and microbiological relapse of disease following ESS is significantly associated with intracellular *S. aureus*. Evidence suggests that this disease association is independent to surface biofilm status. Intracellular bacteria should be taken into consideration when designing novel treatment strategies to lessen the chance of reinfection.

5.2 INTRODUCTION

Recalcitrant chronic rhinosinusitis (rCRS) has been linked to the Gram-positive facultative anaerobic organism *S. aureus* in a plethora of different phenotypes including planktonic, biofilm, intracellular or small colony variant forms. This bacteria uses a variety of pathogenic factors including superantigens[137,314,315] and bacterial cell wall membrane proteins[108] among others to induce acute and chronic inflammatory changes to infected tissues. Recently, the paradigm of intracellular invasion into sinonasal epithelium has been suggested as a mechanism by which the organism can exploit an environment in order to protect itself from traditional treatments such as topical douches and antibiotics. To take advantage of the intracellular milieu, the concept of phenotype switching[251] has been recognised as a method by which the organism can alter the regulation of certain factors including bacterial metabolism or virulence in order to evade host immune responses and persist within tissues.

Until now, the true clinical significance of intracellular *S. aureus* in CRS has not been fully explored with research mainly limited to descriptive studies on the presence of this highly pathogenic organism in sinonasal mucosa.[240,252,277,316] These studies used a variety of detection techniques including immunohistochemistry and molecular FISH probes as well as different imaging techniques such as inverted immunofluorescence and CSLM.

Without robust evidence for the pathogenic capacity of intracellular *S. aureus*, it is difficult to conclude that this bacterial phenotype is a factor in the disease process. Therefore, the aim of this study was to apply techniques of intracellular bacterial detection to a wide cohort of patients followed in their postoperative course in an attempt to clarify whether

intracellular infection of sinonasal tissue in CRS is a mere bystander or an independent risk factor for clinical relapse when compared to the biofilm alone.

5.3 METHODS

Study Design Population

This study was performed in the tertiary rhinologic practice of the senior author (P.J.W.) based in Adelaide, South Australia. Approval was granted from the local human research ethics committee (#2011054) and all patients gave informed consent to be included in the study. Patients who fulfilled the updated American Academy of Otolaryngology-Head and Neck Surgery diagnostic criteria for CRS[293,317] and were undergoing ESS were invited to participate. Patients were excluded from the study if they were under 18 years of age, suffered from any form of immunodeficiency, or had clinical or radiological evidence of invasive fungal disease. Any interstate patients that were unavailable for postoperative follow-up by the senior author were also excluded from this study.

Preoperative Data Collection

Preoperative data was collected on the day of surgery including demographic information (age, gender), clinical information (history of asthma, smoking, immunodeficiency or previous surgery) and preoperative radiological Lund-Mackay scoring. A 5 point clinical symptom scoring system was recorded that scored the symptoms of nasal obstruction, rhinorrhoea, postnasal drip, headache or facial pain and sense of smell from 1 to 5. The sum of the individual symptom scores was recorded as a total symptom score out of 25. This 5-point symptom scoring system has been validated against other objective scoring systems such as the 22-item Sino-Nasal Outcomes Test (SNOT-22), Lund-Mackay and Lund-Kennedy scores (Quality of life assessment in chronic rhinosinusitis using the Adelaide Disease Severity Score; Naidoo, Tan et al, J Laryngol Otol, *in press*).

Perioperative Data Collection

During the surgical procedure the presence of mucopus, polyposis and eosinophilic mucous was documented. In all patients, samples were sent for bacterial and fungal culture and mucosal biopsies sent for routine histopathological examination.

Tissue Collection

Multiple pieces of mucosa were harvested from the sinonasal cavity and immediately placed into Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen Corp., Carlsbad, CA) for transportation to our laboratory. Tissue was thoroughly washed in MilliQ water (Millipore, Billerica, MA) to remove planktonic bacteria and cryofrozen to -80°C[294] for subsequent analysis.

Tissue Analysis

Dual staining of 3mm × 3mm mucosal specimens was performed with a *S. aureus* species-specific FISH probes and a nucleic acid counterstain (PI) using a protocol that has been previously validated within our department.[302] The prepared tissue specimens were analysed using the Leica TCS SP5 CSLM (Leica Microsystems, Wetzlar, Germany) and multiple representative image stacks taken from each piece of tissue. Patients were categorised as to the presence of *S. aureus* biofilm and evidence of intracellular organisms as per previously reported criteria using blinded investigators (C.J., S.V.).[316] Representative images can be seen in Figure 5.1.

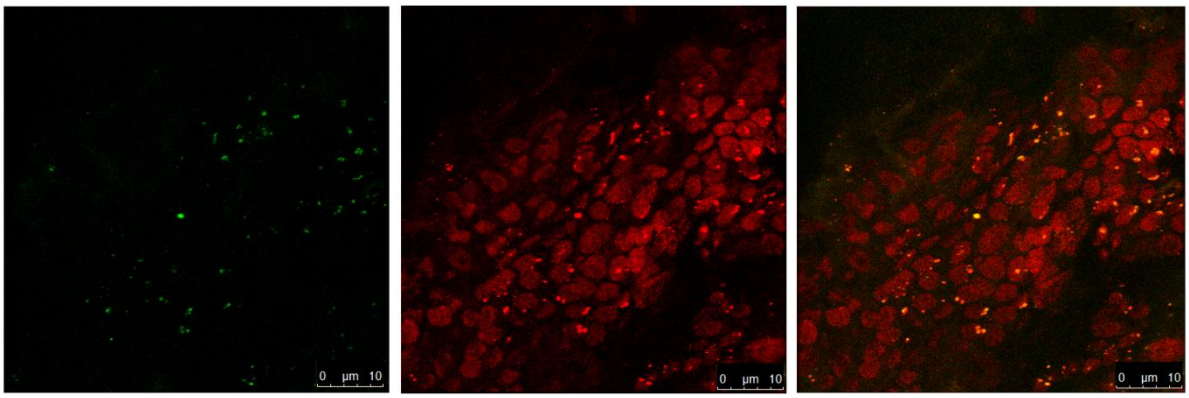

Figure 5.1: Representative CSLM image of intracellular *S. aureus*; *left*: *S. aureus* green FISH probe demonstrating multiple clusters of coccal shaped fluorescence; *middle*: PI counterstain demonstrating cellular nuclei and *S. aureus* DNA in red; *right*: merged images showing co-localisation of the *S. aureus* fluorescence demonstrated in yellow closely related to cellular nuclei.

Postoperative Data Collection

Following their surgery, all patients underwent a routine postoperative regime of care by the senior surgeon. The medical treatment included a 2-week course of amoxycillin & clavulanic acid if not allergic, mometasone spray and topical saline douches. Patients were assessed at 2 weeks, 6 weeks, and 6 months post-surgery. During these appointments, rigid endoscopic surveillance was performed in order to assess for evidence of persisting disease or clinical relapse. This was considered to be the presence of abnormal features in the sinonasal mucosa (oedema, polypoid change, granulations, crusting or frank mucopus or discharge). If signs of infection were present, then clinically directed endoscopic swabs were taken and processed as per standard microbiological techniques for bacterial identification and antibiotic sensitivities.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (San Diego, CA). Non parametric data are displayed as median and IQR. Dichotomous data was analysed using Fischer's exact test and nonparametric data was analyses using the Mann-Whitney test with statistical significance accepted when $p < 0.05$.

5.4 RESULTS

Demographics

Sixty-seven patients were enrolled in the study; however, 16 were lost to follow-up or did not have an adequate follow-up period and were thus excluded. This left 51 patients (26 male: 25 female) who reported a median symptom score of 17 out of 25 (IQR, 15-20) and median Lund-Mackay score of 14 out of 24 (IRQ, 10–19.25). Mean follow-up was 9.4 months (range, 6-17). Nineteen patients were asthmatic, 4 reported sensitivity to salicylates and 4 were current cigarette smokers. None of these factors showed any significance in subgroup analysis

S. aureus FISH results

Intracellular *S. aureus* was identified in 20 patients giving an overall detection rate of 39%. Surface biofilm was consistently detected in the same piece of mucosa when intracellular infection was present and in a further 16 patients with no intracellular infection. 15 patients demonstrated no sign of *S. aureus* in either biofilm or intracellular form. Thus, the subcategories of our patient population were 15 of 51 (29%) biofilm-negative, intracellular-negative (B-IC-); 16 of 51 (31%) biofilm positive, intracellular-negative (B+IC-); and 20 of 51 (39%) biofilm-positive, intracellular-positive (B+IC+).

Preoperative data analysis

Table 5.1 contains data for preoperative symptom scores, Lund-Mackay scores and other demographic data broken down as per the subcategories. There were no statistical differences in symptom scores, asthma status or allergy between any of the groups. Lund-Mackay score was significantly higher in the B+IC+ group when compared to the B-IC- group ($p = 0.0261$, Mann-Whitney test), as was the likelihood of previous surgery ($p =$

0.0038, Fisher's exact test). Interestingly, we found a statistically significant association between nasal polyposis and intracellular *S. aureus*, with 15 of 29 (52%) CRS with nasal polyposis (CRSwNP) patients being IC+ compared to only 5 of 22 (23%) of CRS without nasal polyposis (CRSsNP) patients (p = 0.0461, Fisher's exact test).

Table 5.1: Demographic details of patient cohorts

	Number	Age	Symptom Score	Lund-Mackay Score	Previous sinus surgery
Biofilm negative, Intracellular negative	15	54 (29-76)	17 (15-19)	10 (6.5-15)	5 (33%)
Biofilm positive, Intracellular negative	16	52 (21-71)	17.5 (16-20)	13.5 (12-16.75)	11 (67%)
Biofilm positive, Intracellular positive	20	50 (20-81)	17 (15-20)	18 (12.5-21)	17 (85%)

Primary vs revision

In the overall cohort of patients, 18 underwent primary and 33 underwent revision surgery. When subdividing by intracellular status, we found that 17 of 20 patients (85%) in the B+IC+ group underwent revision surgery. This was significantly different to the 5 of 15 (33%) in the B-IC- group (p = 0.0038, Fisher's exact test) but not when compared to the B+IC- group (11/16, 69%; p = 0.4217).

Intraoperative infection rate

Intraoperative microbiological samples cultured any bacteria in 7 of 15 (47%) B-IC- patients, 9 of 16 (56%) B+IC- patients and 14 of 20 (70%) B+IC+ patients. Intraoperative *S. aureus* was cultured in 4 of 15 (27%) B-IC- patients, 5 of 16 (31%) B+IC- patients and 12 of 20 (60%) B+IC+ patients. No significant difference was found between any groups.

Microbiological failure rate

Patients were categorised as having either an early (0-2 months) or late (2+ months) postoperative failure if they were noted to have abnormal features of mucosa and a positive microbiological swab. In the B-IC- category, 1 patient cultured *S. aureus* early in the postoperative course but did not develop any signs of late failure. Four of 16 (25%) B+IC- patients suffered early *S. aureus* infections and in only 1 of these did the infection persist. In patients who were B+IC+ there were 10 of 20 (50%) who suffered an early infection with a significant difference compared to the B-IC- group ($p = 0.0095$, Fisher's exact test). Moreover, there were 11 of 20 patients (55%) who developed a late failure with a statistically significant difference noted between both B+IC- vs B+IC+ (Fisher's exact test, $p = 0.014$) and B+IC+ vs B-IC- ($p = 0.0005$, Fisher's exact test) groups (Fig. 5.2).

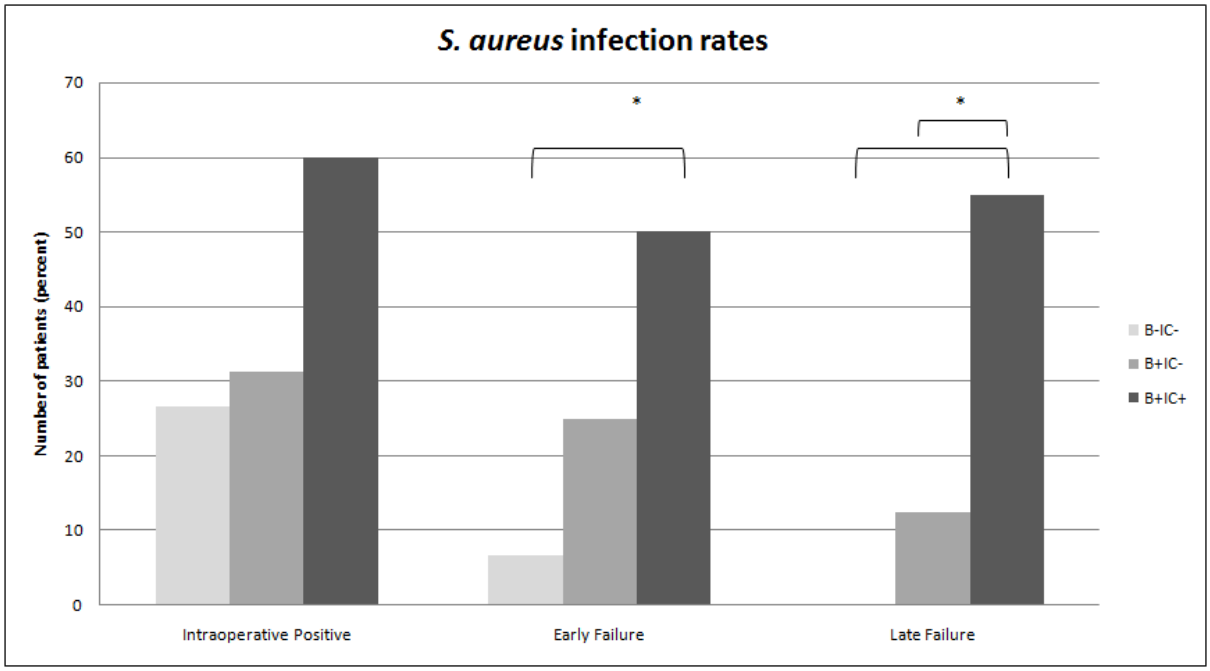

Figure 5.2: *S. aureus* intraoperative culture, early and late relapse rates in the postoperative course. Light gray: B-IC- patients; gray: B+IC- patients; black: B+IC+ patients. * $p < 0.05$. B-IC- = biofilm negative, intracellular-negative; B+IC- = biofilm-positive, intracellular-negative; B+IC+ = biofilm positive, intracellular-positive

5.5 DISCUSSION

Despite the advancements in both technical and surgical aspects of ESS with application of more rigorous postoperative treatment regimes, the modern rhinologist will continue to see a distinct subset of patients who fail to improve following surgery. In this study we used a CSLM-FISH protocol that has been validated[302] for simultaneous detection of biofilm and intracellular *S. aureus* within the same piece of tissue. This helps to further understand the interplay between the biofilm and intracellular bacteria, as well as to identify if the 2 factors are synergistic or independent disease modifying factors. We report that patients with evidence of intracellular *S. aureus* at the time of surgery are more likely to suffer a late clinical and microbiological relapse suggesting that intracellular location of the organism offers a protective niche where it can avoid host immune clearance and topical or systemic treatments.

Recent research has concentrated on the role of the biofilm, in particular *S. aureus* unimicrobial or polymicrobial biofilms, as having a negative prognostic impact on post-surgical outcomes.[202,297,312] Such patients have been found to have more significant pre-operative symptoms as well as poorer outcomes following surgery.[31,202,318,319] However, the concept of intracellular uptake by non-professional phagocytes such as epithelial cells has generated interest in recent years. Certain chronic diseases such as osteomyelitis are well known to be associated with *S. aureus*. *In vitro* experimentation has demonstrated that a wide variety of cells such as osteoblasts,[144,221] fibroblasts, endothelial cells[320] and epithelial cells[240] are capable of internalising and harbouring live organisms within the intracellular milieu. Indeed live, culturable bacteria can be harvested even 28 days post *in vitro* infection into epithelial cell cultures.[251] Although the evidence for bacterial persistence in cell culture models is robust, this has not fully translated into clinically appreciable

research with only few studies describing the presence of intracellular bacteria in human and animal clinical specimens.[252,321] No studies have, as yet, harvested viable bacteria that are capable of causing further infection, and this is an area that no doubt requires further scientific investigation.

The technique used for intracellular detection has previously been validated using multiple blinded investigators in previous departmental studies. We first validated this technique against immunohistochemistry,[302] and then we reported on a series of patients[316] in which we first noted the association between biofilm and intracellular *S. aureus*. Hence, we feel confident in the use of this detection methodology for our cohort characterisation.

In patients who were biofilm and intracellular negative, 4 cultured *S. aureus* at the time of surgery. Interestingly, none of these patients went on to develop postoperative infections. This suggests that these intraoperative bacteria may have existed in a planktonic phenotype and that effective surgery and postoperative medical treatment were successful in eradicating the pathogen or reducing the bacterial load to clinical insignificance. Conversely, 2 patients who did not demonstrate any bacterial growth on intraoperative culture went on to suffer early *S. aureus* infections. It has been suggested that postoperative *de novo* infections may be a cause in these situations[216,322] with factors such as mucosal injury coupled with postsurgical ciliary denudation or dysfunction[21] playing a part in allowing pathogenic bacteria to grow. The fact that both these patients suffered polymicrobial infections with the additional presence of *K. pneumonia* and *E. aerogenes* reinforces the opportunistic infection hypothesis in these cases.[322]

When considering the cohort with biofilm alone, 5 patients cultured intraoperative *S. aureus* and of these, 2 continued to culture early postoperative *S. aureus*. Early failure may be caused by persistent bacteria or represent part of the natural life cycle of a mature biofilm in which planktonic bacteria are released with the potential of causing reinfection.[216,323]

When analysing patients who were biofilm and intracellular positive, a very different picture emerges. Although not a statistically significant difference, the intraoperative culture rate of 60% was higher than the other cohorts (B-IC- 27% and B+IC- 31%). Risk of early failure was significantly higher comparing B-IC- and B+IC+, although not when comparing biofilm alone. When looking at the late relapse rate in these patients and controlling for biofilm status, we found a statistically significant association between intracellular status and disease severity, with 55% of B+IC+ patients suffering a treatment clinical and microbiological failure compared to 14.2% of B+IC- and 0% of B-IC- patients. Early studies made suggestions that intracellular *S. aureus* plays a role in recurrent and persistent disease;[275] however, to our knowledge this is the first study to report this finding in a robust cohort of patients.

It is recognised that *S. aureus* is capable of altering its phenotype after internalisation into epithelial cells in the *in vitro* setting.[222] In this elegant study, the authors demonstrate significant transcriptional changes in gene regulation after exposure to the intracellular environment. Specific alterations include genes controlling metabolic pathways including cell division and nutrient transport being significantly downregulated, so as to allow the bacteria to adopt a "dormant state".[222,251,324] Further phenotypic changes include the resistance of internalised bacteria to antibiotics, as demonstrated in an osteoblast cell

culture model[221] where it was found that the cell walls of intracellular *S. aureus* were significantly thicker than their extracellular counterparts. This correlated to a resistance against the bactericidal activity of rifampicin when applied to the culture 12 hours post infection. In addition, it is understood that internalised bacteria are capable of evading certain components of the innate immune system; *S. aureus* is almost completely resistant to the action of lysozyme, a human enzyme that has potent antimicrobial properties. O-acetyltransferase is a bacterial enzyme that is readily expressed in *S. aureus* which acts to confer resistance to lysozyme through acetylation of the peptidoglycan cell wall.[133] This has been suggested as a mechanism of bacterial persistence once internalisation has occurred.[248]

We do recognise certain methodological limitations that have occurred within our study. In this study we report on microbiological outcomes coupled with clinical assessment. A full data set of objective and subjective postoperative symptom scores was not obtained in this study, hence the relationship between intracellular bacteria and symptomatic relapse can only be inferred. Nevertheless, we feel that our criteria of clinical relapse as being endoscopic signs of disease such as mucosal cobble-stoning, crusting or mucoid discharge coupled with positive microbiology is sufficient to draw conclusions on the risk of developing recurrent postsurgical infections.

We have focused on the specific role of *S. aureus* and not examined or controlled for the impact of other bacteria and it is believed that geographical variability in microbiome ecologies exists. However, it has been reported in the literature that using culture independent techniques such as 16S rRNA analysis, a high incidence of *S. aureus* has been found in North America (50%)[325] and the United Kingdom (~50%).[326] Although the *S.*

aureus detection rates are usually lower using culture-dependant technique; reported at between 15.4% and 24.1% in CRS patients,[327] they have been found to be as high as 63.2% in allergic fungal rhinosinusitis patients.[328] Therefore, as the literature suggests *S. aureus* holds a major role in the pathophysiology of chronic, and especially recalcitrant rhinosinusitis,[202,297,329] this leads us to believe that our results are representative of the true disease impact and that the association noted in our study may well be applicable to other geographical areas.

Within our cohort of patients that were IC+, we saw a significantly higher rate of revision surgery and nasal polyposis. As these factors are also known to be associated with higher rates of failure, it would be important to recruit a wider cohort of patients so that these significant confounders can be controlled for. It has previously been reported that *S. aureus* infection of CRS patients tissue cultures *ex vivo* leads to a significant increase in Th2 cytokines such as IL5, IL8, IL13, and critically those involved with tissue remodelling and fibrosis; matrix metalloproteinase (MMP) 2, MMP-9, and tissue inhibitor of MMP 1 (TIMP).[298] These data have also been confirmed *in vivo* where it was noted that *S. aureus* biofilms themselves directed a trend toward a T-helper 2 based cytokine response.[205] This suggests that *S. aureus* itself may play an important role in the aetiopathogenesis of polyps themselves, and thus it could even be that IC *S. aureus* infection is the factor that promotes polyp formation. Clearly this hypothesis requires rigorous testing and validation in further scientific experiments before it can be accepted as fact.

An additional limitation is that although the molecular probes used in our techniques specifically bind to *S. aureus,* they can potentially target organisms regardless of whether alive or dead. Thus, we are unable to conclusively state that the fluorescence seen within

our tissue sections represent viable bacteria. The difficulties in harvesting bacteria that are truly from the intracellular milieu has been previously discussed.[248] Tuchscherr et al[251] reported the ex vivo harvest of organisms from tissue specimens of *S. aureus* endovascular, soft tissue and bone infections. No mention, however, was made of any attempts to sterilise extracellular bacterial contaminants and thus it is unknown whether bacteria cultured were truly representative of those in the IC location. Further experimentation aimed at answering these questions would undoubtedly strengthen the argument for IC *S. aureus* and is a focus of further interest within our research group.

5.6 CONCLUSION

This study is the first of its kind to report on the impact of intracellular infection with *S. aureus* in the post surgical CRS patient. Evidence suggests that this disease phenotype is independent to surface biofilm status in its association for developing clinical and microbiological relapse beyond the immediate postoperative period despite maximal therapy. With the increasing evidence that intracellular *S. aureus* plays a role in CRS, this should also be taken into consideration when considering novel treatment strategies to prevent recurrent disease.

CHAPTER 6

SMALL COLONY VARIANTS AND PHENOTYPE SWITCHING OF INTRACELLULAR *STAPHYLOCOCCUS AUREUS* IN CHRONIC RHINOSINUSITIS

Conducted in the Department of Otolaryngology – Head and Neck Surgery

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Small colony variants and phenotype switching of intracellular *Staphylococcus aureus* in chronic rhinosinusitis

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6.1 ABSTRACT

Background: CRS has been linked to the gram-positive bacteria *S. aureus* in its biofilm or intracellular forms. Recent evidence suggests that *S. aureus* also exists in an SCV form as a mechanism of altering its virulence capabilities

Objective: The purpose of this study was to investigate the presence of SCVs in sinonasal mucosa of CRS patients and whether the phenomenon of phenotype switching can be applied to intracellular epithelial infections.

Methods: Sinonasal specimens were examined for the presence of intramucosal *S. aureus* and characterised to the strain level. An airway epithelial cell culture infection model was utilised to investigate whether bacteria were capable of alterations in virulence phenotype.

Results: Intramucosal organisms harvested from sinonasal biopsies demonstrate phenotypic growth patterns and lack of coagulase activity consistent with SCVs. Intracellular infection of airway epithelial cell cultures with *S. aureus* led to decreased secretion of enterotoxins and phenotypic growth alterations consistent with SCVs

Conclusion: Regulation of *S. aureus* virulence factors is a dynamic process and exposure to the intracellular environment appears to provide the necessary conditions to enable these alterations in an attempt for the bacterium to survive and persist within host tissues. Further work is required to ascertain whether SCVs in CRS hold a clinically relevant pathogenic role in recalcitrant disease.

Key Messages

- *S. aureus* SCVs exist in the sinonasal epithelium of CRS patients.
- *In vitro* internalisation of *S. aureus* causes the necessary environmental pressures to switch a wild-type planktonic bacteria into an SCV.
- Intracellular bacteria downregulate their virulence as demonstrated by reduced production of Staphylococcal enterotoxin.

6.2 INTRODUCTION

CRS is a condition characterized by mucosal inflammation affecting the nasal cavity and paranasal sinuses with an estimated prevalence of between 10-15%[317] and costs of US$5.8 billion per annum.[8] Relapses in disease are common despite ever evolving medical and surgical therapy[64,215,216] Bacterial infections, in particular those mediated by *S. aureus*, are believed to hold an important role in the recalcitrant nature of this condition with frequent relapses attributed to its numerous forms including planktonic, biofilm and intracellular phenotypes.[31,201,202,330] Previous work has demonstrated that *S. aureus* is detectable both intramucosally[240,277] and intracellularly[252,275,331] using a variety of techniques including indirect IHC[252,275] or molecular probes and CSLM[240,331]. The association between bacterial biofilms and intracellular status in CRS has been noted,[331] with significantly more frequent relapses in disease being recognised when both the biofilm and intracellular organisms were found within *ex vivo* tissue samples.[330] Similar relapsing and remitting patterns have been noted in other clinical diseases mediated by *S. aureus* such as chronic osteomyelitis,[144] endocarditis[243] and cystic fibrosis.[281]

S. aureus has the capability of adhering to α5β1-integrin, which is expressed on the surface of eukaryotic cells via the expression of FnBPA and FnBPB on its cell surface, and mediated through fibronectin.[113,271-273] Once bound, alterations in the actin cytoskeleton lead to phagocytosis and internalisation of whole bacteria making this final process passive from the bacterial point of view.[274] It has been demonstrated that coating beads with fibronectin binding protein makes them easily phagocytosed by epithelial cells.[274] Furthermore, FnBP-deletion mutant strains of *S. aureus* have a drastically reduced capacity for internalisation into cells.[271] Expression of FnBP is dynamic, and it has been identified that certain subspecies of *S. aureus* can, in the right environment, express higher than

normal amounts of FbBP, thus making them more likely to internalise than other types of *S. aureus*.[251]

SCVs are a bacterial phenotype that, by definition, grow in smaller colonies than their wild-type or planktonic counterparts.[279] They express alterations in pathogenic factors including heightened expression of FnBP[272] and reduction of alpha-toxin production,[332] which act in their favour for obtaining access and persisting within the intracellular milieu. The association between chronic diseases and SCVs is one that has been examined in recent years and related to conditions such as osteomyelitis and cystic fibrosis.[279] The conversion of bacteria from wild-type to SCV and vice versa is understood to be a highly dynamic process that can occur after days or even hours of exposure to an altered environment. Consequently, as opposed to investigating these so called wild-type SCVs which can undergo rapid phenotype alterations, recently research has focused on stable SCVs generated by deletions in their genome or through selection by repeated aminoglycoside exposure.[332]

S. aureus exhibits its virulence through a number of factors including the secretion of SEs.[137] These superantigenic toxins have the capability of bridging and bypassing the specific antibody-antigen complex between MHC Class II molecules and the receptors on T-helper cells,[137] thus stimulating a much larger, non-specific T-cell expansion and related inflammatory response. An association between SEs and CRS has been made through the detection of superantigen-specific IgE[333,334] in the serum of CRS patients. Other studies reported that SE proteins themselves could be identified in the tissue of almost 50% of CRS patients, specifically those with eosinophilic nasal polyposis.[335] It is unknown as to whether the production of these toxins is a constant or dynamic process, and thus in this

study we examined whether enterotoxin secretion can change through exposure of *S. aureus* to different environments.

There has been little research examining the role of SCVs in CRS, or whether the phenomenon of *S. aureus* phenotype switching is a concept applicable to intracellular and intramucosal infections. In the present study we used a series of *in vitro* and *ex vivo* experiments in order to identify whether SCVs exist within sinonasal mucosa from patients with CRS. Our results indicate that exposure to the intracellular environment causes an alteration from the wild-type phenotype to reduce the production of virulence factors in order to promote their existence as a SCV.

6.3 METHODS

Bacterial strains

S. aureus strains used were ATCC 13565 and 14458, being reference strains for the production of SEA and SEB respectively. Overnight liquid cultures were prepared for all experiments by inoculating 5 ml of nutrient broth with a single loop of bacteria and placed into a shaking incubator overnight at 120 rpm and 37°C. The liquid culture was then centrifuged at 4000 rpm for 10 minutes and the supernatant collected, filtered and cryopreserved at -80°C. Bacterial pellets were then used for infection experiments or RNA analysis. Clinical isolates were obtained through routine microbiological swabs taken from patients at the time of endoscopic sinus surgery. If they cultured *S. aureus*, bacteria were isolated and sub-cultured in nutrient broth before being cryopreserved at -80°C.

Harvest of intramucosal bacteria from patient tissue specimens

Multiple samples of sino-nasal mucosa were collected from a patient undergoing endoscopic sinus surgery for chronic rhinosinusitis. Ethical approval for tissue collection was granted from the local institution Human Research and Ethics Committee. Tissue samples were immediately placed in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen Corp., Carlsbad, CA) and transported to our laboratory. Samples were swabbed, then soaked in 0.03% cetylpyridinium chloride (CPC, Sigma-Aldrich) for 10 minutes, a disinfectant agent recognised to be effective against *S. aureus* in its planktonic and biofilm forms at high concentrations. After washing three times with MilliQ water, samples were swabbed and immersed in PBS and placed into a waterbath sonicator at medium intensity for 15 minutes. The sonicated tissue was swabbed, soaked again in CPC and re-swabbed. After three further washes in MilliQ water, tissue samples were homogenised using a Tissueruptor (Qiagen, GmbH, Hilden, Germany) to release intramucosal bacteria then

cultured on blood agar plates. Samples were incubated at 37°C for up to 72 hours and experiments performed in duplicate. The diameter of 25 individual colonies was measured using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA). Statistical analysis was performed with Graphpad Prism 5.0 software (San Diego, CA) using an unpaired t-test and statistical significance was obtained when $p < 0.05$.

RNA Isolation

Bacterial pellets from overnight liquid cultures were resuspended in RNALater (Ambion, Carlsbad, CA) and stored overnight at 4°C. This was centrifuged at 4000 rpm for 10 minutes and the bacterial pellet lysed in TE buffer containing lysostaphin at 500 µg/ml for 1 hour at 37°C in a rotary heating block. Into the bacterial suspension was added 350 µl of RLT buffer (Qiagen) and 250 µg of acid washed glass beads (Sigma-Aldrich, St. Louis, MI). Mechanical lysis was performed at 30 beats/second in a Tissueruptor (Qiagen) for 10 minutes. RNA was purified using RNEasy columns (RNeasy Mini Kit, Qiagen) following the manufacturer's instructions and included an on column DNAse treatment (Qiagen). RNA quantification was performed with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Australia).

qRT-PCR typing of SEA

Real-time PCR was performed with RNA isolated from the bacterial strains. Firstly, cDNA was produced using the Quantitect RT kit (Qiagen) and qRT detection was with the IQ Sybr green supermix (Biorad, Hercules, CA). Oligonucleide primer sequences were obtained and optimised from the following sources; SEA [336], SEB [337] and spa [338]. Reactions were heated for 30s at 95°C followed by 35 cycles of 30s at 95°C and 60s at 60°C. All experiments were performed in duplicate using an iQ5 Cycler (Biorad).

Cell culture infection model

Human bronchial epithelial cells (16HBE14o-HBE'014) were a kind gift from Dr Dieter Gruenert and cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% L-Glutamine and 1% penicillin / streptomycin (Gibco, Carlsbad, CA). The cellular monolayer was washed then infected with a 5.0 McFarland unit solution of *S. aureus* giving a multiplicity of infection (MOI) of 100:1. The cells were incubated for one hour to allow attachment and phagocytosis before being washed three times to remove non-adherent bacteria. Incubation with media containing 4ug/ml lysostaphin for 30 minutes was performed in order to eliminate any other extracellular *S. aureus*[339] followed by three further washes in PBS and finally MEM supplemented with 10% FCS, 1% L-Glutamine and 100 µg/ml gentamicin added. Comparison flasks of extracellular bacteria were inoculated with bacteria but lysostaphin treatment and subsequent steps were omitted in order to maintain extracellular bacteria. After 24 hours, culture supernatants were collected. Cell monolayers were washed three times in PBS and lysed in Mammalian Protein Extract Reagent (Thermo Scientific).

In order to confirm that intracellular bacteria were present in the infected flasks free of extracellular contaminants, the culture supernatant was used to inoculate a blood agar plate. After washing the monolayer twice in PBS, cells were lysed in water, scraped and inoculated onto blood agar. Culture supernatants from extracellular bacteria flasks were also plated onto blood agar.

Superantigen detection

Detection of enterotoxin was determined using the 3M TECRA SIDVIA kit (3M, North Ryde, NSW) following the manufacturer's instructions. Briefly, supernatants and cell

lysates were centrifuged at 13,300 g for 10 minutes before being passed through a 0.2 µm filter and 200 µl was added to 10 µl of buffer solution. Wells were pre-soaked with wash solution for 30 minutes followed by incubating 50 µl of supernatant or lysate for 2 hours at 37°C. Following washing, 200 µl of substrate solution was added and incubated for 1 hour and then washed again. 200 µl of conjugate was added and the plate read after 30 minutes. The TECRA kit uses a visual assessment that either classifies a sample as enterotoxin positive or negative with a sensitivity of 1 ng/ml.

Clonal typing

Swabs of *S. aureus* were sent to PathWest Laboratory (Perth, Western Australia, Australia) for molecular typing. The typing methodology employed was Pulsed field gel electrophoresis (PFGE), commonly considered the Gold standard of molecular bacterial typing.[340] Briefly, genomic DNA is extracted and purified, digested using restriction enzymes and run on a PFGE gel to visualise the band pattern. These patterns allow comparison of the various *S. aureus* isolates to determine if they are of the same strain.

Effects of intracellular S. aureus on cells

To determine whether *S. aureus* infection had any longer term effects on cells, airway epithelial cells (AEC) were seeded into 6 well plates and infected as per the previously described protocol. Culture media was changed every 24 hours and frozen to -80°C for subsequent analysis for IL-6 and lactate dehydrogenase (LDH). Comparison was made to negative controls that had daily media changes with no *S. aureus* infection. All experiments were performed in triplicate.

IL-6 ELISA

A sandwich ELISA technique was carried out to detect IL-6 protein expression in cell supernatants. Briefly, ELISA plates were coated overnight with rat anti-human capture antibody (BD Biosciences, California, USA), diluted in 0.1 M $NaHCO_3$, at 4°C. After washing with PBS containing 0.05% tween-20 (Sigma-Aldrich), plates were incubated with blocking buffer (PBS containing 2% bovine serum albumin (Sigma-Aldrich)) for 2 hours at room temperature. Samples and standard concentrations (BD Biosciences) were applied to the wells in duplicates and incubated at room temperature for another 2 hours. After washing, biotinylated rat anti-human IL-6 antibody (BD Biosciences) was added and incubated at room temperature for 30 minutes. After washing, Horseradish Peroxidase-conjugated streptavidin (Thermo Scientific, Australia) was incorporated and incubated at room temperature for 30 minutes. TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution (Thermo Scientific, Australia) was applied and the reaction stopped with 0.16 M sulphuric acid after 10 minutes. Light absorbance was measured at 450 nm using spectrometry. The values obtained were corrected by dilution factor and expressed in pg/ml. The lower limit of detection was 12.5 pg/ml. Statistical analysis was performed with Graphpad Prism 5.0 software (San Diego, CA) as previously described.

Lactate Dehydrogenase Assay

Culture supernatants were centrifuged then passed through a 0.22 µm filter before being processed by a commercially available LDH assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI) as per the manufacturer's instructions. Briefly, in a 96 well plate, 50 µl of filtered supernatant was mixed with 50 µl of substrate mix and incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 µl of stop solution and absorbance determined at 490 nm.

6.4 RESULTS

Intramucosal S. aureus harvested from sinonasal tissue samples demonstrate small colony variant growth patterns

Swabs from the surface of sinonasal tissue samples grew numerous bacterial colonies on blood agar plates after 48 hours with a normal phenotype, characterised by yellow pigmentation and weak hemolysis. After disinfecting the surface of the tissue, we were unable to culture any bacteria from further tissue swabs. However, upon homogenisation of tissue samples we noted the growth of small and very small sized non-pigmented, non-hemolytic colonies of *S. aureus* after 48 hours. Clear morphologic differences in terms of colony size and colour could be seen between the bacteria harvested from surface tissue swabs and those harvested from tissue homogenates (Fig. 6.1A-B). Colony sizes were measured using ImageJ and a statistically significant difference in size was noted ($p < 0.0001$, unpaired t-test) (Fig. 6.1D). When these colonies were immediately plated onto chromogenic agar, they demonstrated a lack of coagulase activity, a hallmark of the SCV phenotype.[289] Intramucosal colonies that were subcultured overnight in nutrient broth and then plated onto blood agar demonstrated a return to normal colony growth patterns. Swabs of surface, intramucosal and sub-cultured intramucosal colonies were sent for microbiological analysis that identified the bacterial species and genus as *S. aureus*. In order to confirm that the differing growth patterns could not be attributed to strain divergence, PFGE was performed, demonstrating that the strains of *S. aureus* cultured held an identical genotype, despite the marked difference in growth patterns (Fig. 6.1E)

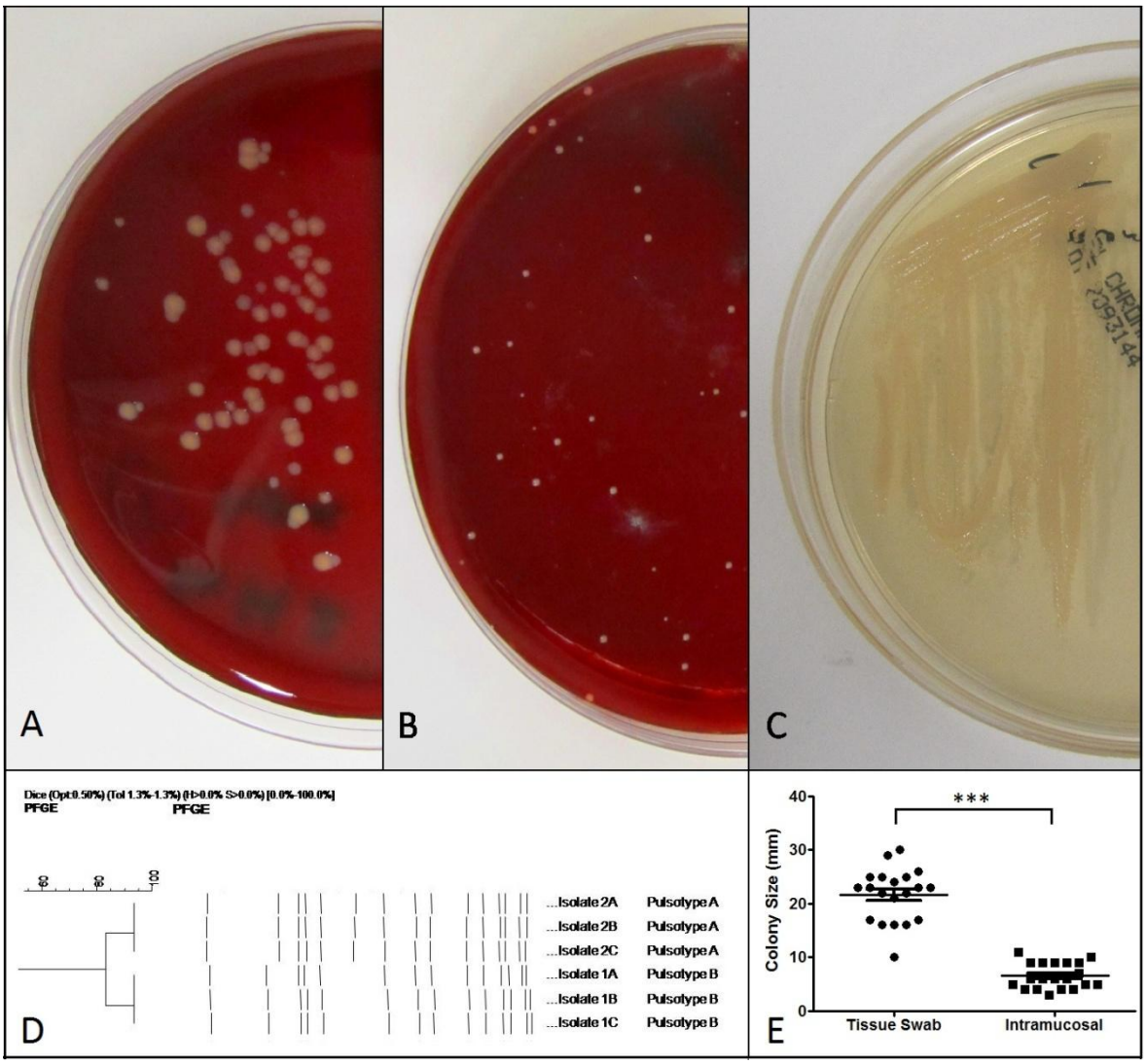

Figure 6.1: Small Colony Variants in CRS mucosal samples.

A-C: Horse blood agar plates incubated at 37°C for 48 hours and inoculated with; A: surface tissue swabs of sinonasal mucosa demonstrating a mixed population of normal sized and small colonies of *S. aureus* with the characteristic yellow colour*;* B: sinonasal mucosal tissue homogenate post disinfection of surface bacteria demonstrating a population of small and very small colonies of *S. aureus*, whitish in colour; C: intramucosal colonies subcultured onto chromagenic agar demonstrating lack of coagulase activity.
D: Comparison of colony size retrieved from tissue swab versus sinonasal mucosal tissue homogenate. Bars represent mean +/- SEM (*** $p < 0.0001$, unpaired t-test).
E: Pulse Field Gel Electrophoresis dendrogram demonstrating identical clonal types of *S. aureus* harvested from surface tissue swabs from two different CRS patients (1A/2A), intramucosal small colonies (1B/2B) and subcultured intramucosal colonies (1C/2C). Vertical bars demonstrate fragments of bacterial DNA cut by restriction enzymes and pulsotype refers to the same clonal strain.

S. aureus strains downregulate virulence factors upon internalisation into epithelial cells

Reference strains *S. aureus* ATCC 13565 and ATCC 14458 both expressed the appropriate enterotoxin gene, detected using qRT-PCR and secreted the respective SEA or SEB toxin, identified using the TECRA kit. These strains were then used to infect AEC with an MOI of 100:1. Culture supernatants from flasks of purely intracellular infected cells did not demonstrate any bacterial growth when plated onto agar (Fig. 6.2A). However, when the same cellular monolayer was lysed and plated onto agar, it grew numerous colonies of bacteria (Fig. 6.2B). These results indicate that a one hour exposure of the epithelial cells to *S. aureus* enabled the bacteria to enter the cells and that they stayed viable within the intracellular compartments. Also, the lysostaphin/gentamicin treatment could effectively eliminate all extracellular bacteria while leaving the intracellular bacteria unaffected. Control cell cultures that were infected with *S. aureus* and did not get a lysostaphin/gentamicin treatment demonstrated significant bacterial growth in the culture supernatant (Fig. 6.2C). These were clonally typed by PFGE to confirm that they were the same strain as that used to inoculate the cultures.

In the absence of any SEA-positive (ATCC 13565) extracellular bacteria, after 24 hours of intracellular infection we were unable to detect SEA in either the culture supernatant (Fig. 6.2D) or cell lysate (Fig. 6.2E) of intracellular infected cells compared to detectable SEA in the supernatant of extracellular *S. aureus* flasks (Fig. 6.2F). This suggests that the intracellular bacteria had switched off their toxin production. In order to determine if this phenotype switching was reversible, the bacteria harvested from the intracellular infected monolayer were subcultured in nutrient broth overnight. Analysis of the broth with the TECTA enterotoxin kit demonstrated positive SEA (results not shown), indication that the

secretion was switched back on by exposure to the different environment. Conversely, infection with the SEB reference strain ATCC 14458 demonstrated evidence of SEB within the cell lysate and culture supernatants of both intracellular and extracellular flasks suggesting that the bacteria were unable to downregulate their toxin production. Using the semi-quantitative scale, there was more toxin detected in the cell lysates of intracellular infected cells as opposed to the corresponding culture supernatant (Fig. 6.2G-I).

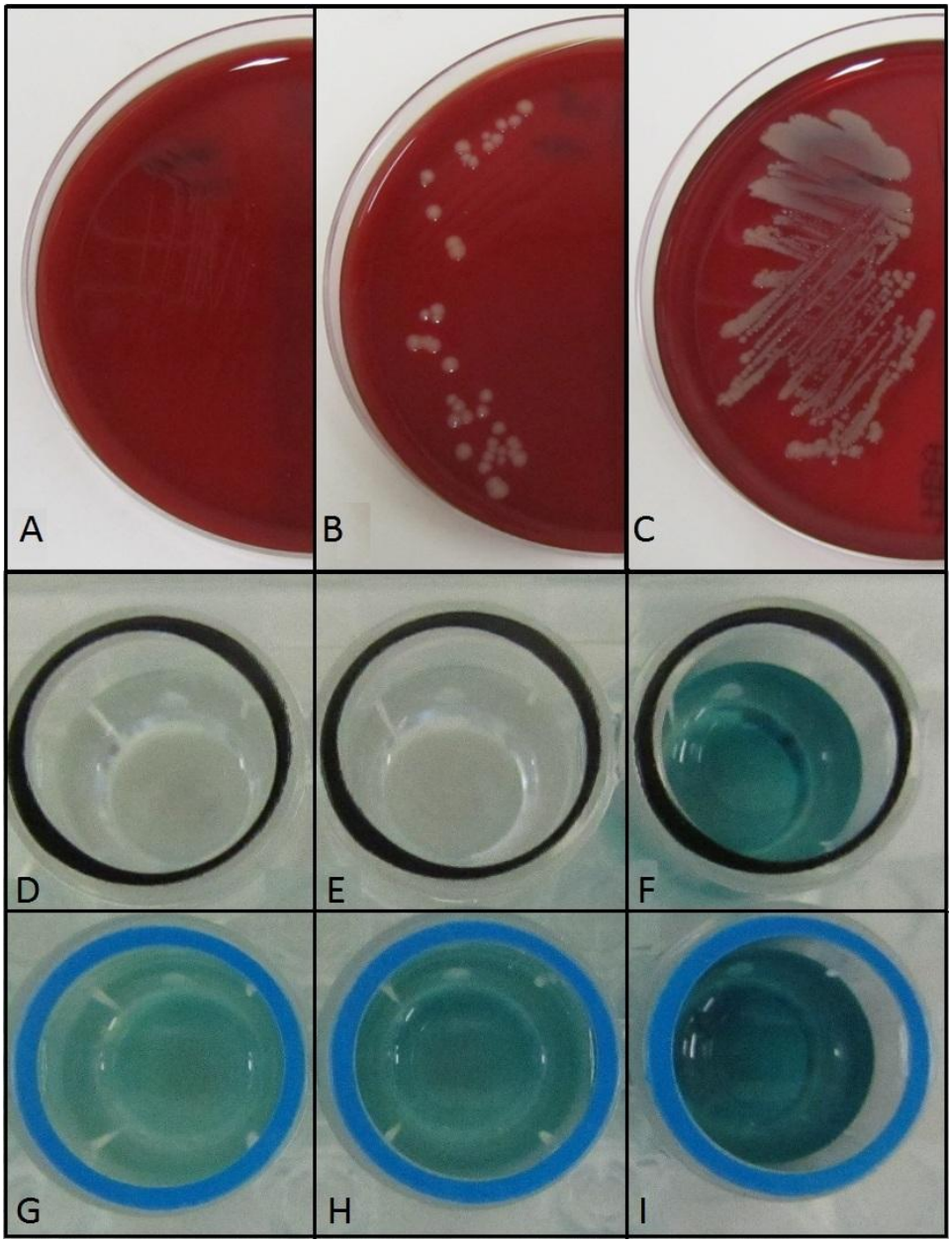

Figure 6.2: Staphylococcal entereotoxin detection from *in vitro* infection experiments.

A-C: Horse blood agar plates grown overnight at 37°C inoculated with; A: culture media from airway epithelial cell flasks intracellularly infected with ATCC 13565 demonstrating the absence of viable bacteria; B: epithelial monolayer lysed in water 24 hours post infection with *S. aureus* demonstrating presence of intracellular bacteria; C: extracellular culture media infected with *S. aureus* demonstrating significant bacterial growth.
D-I: 3M TECRA *S. aureus* enterotoxin A and B identification test; experiments using ATCC 13565 (SEA, D-F *(black*)); experiments using ATCC 14458 (SEB, G-I *(blue)*:); D/G: culture media from intracellular infected epithelial cells demonstrating reduced production of SEA but not SEB; E/H: cell lysates of intracellular infected epithelial cells demonstrating reduced production of SEA but not SEB; F/I: culture media from extracellular infected epithelial cells demonstrating evidence of SEA and SEB.

Enterotoxin production in clinical isolates

Six patients had *S. aureus* cultured at the time of surgery. Of these strains, we used qRT-PCR to identify that one strain expressed the SEA gene and another, isolated from a different patient, expressed the SEB gene. TECRA analysis demonstrated that the respective toxins were produced when the isolates were placed in liquid culture overnight at 37°C. When these strains were used to infect cellular AEC monolayers, the cell lysates or supernatants showed no detectable toxins. Conversely, detectable toxin was found in the supernatants of extracellular infected flasks. This indicates that clinical isolates that contain enterotoxin genes are capable of "switching off" their production of toxins upon entering the intracellular environment. Interestingly, both strains appeared able to switch off their toxin production, as opposed to only one of the reference strains, suggesting that these clinical isolates had adapted the capacity to downregulate their virulence as a mechanism to persist within the cellular environment.

S. aureus does not cause significant cellular toxicity after internalisation and develops a SCV-like growth pattern

After AEC were infected with *S. aureus* ATCC 13565 as previously described, cell culture media was changed daily, and IL-6 measured using ELISA. The IL-6 level in the culture supernatant of cells infected with *S. aureus* was significantly higher than control cells ($p=0.0002$, unpaired t-test) on day 1 after infection (Fig. 6.3A). However, at all subsequent time points up to 7 days after initial infection, there was no significant difference in IL-6 levels between the control and infected cell cultures. This indicates that HNECs react when challenged with *S. aureus* by secreting the pro-inflammatory factor IL6, as expected, but that the intracellular bacteria do not elicit such a reaction. In order to test for toxic effects, we determined LDH levels in the same culture supernatants. When LDH measurements

from infected cell cultures were analysed (Fig. 6.3B) against negative control cultures there were no significant differences at any time point. Cell lysates cultured on blood agar confirmed intracellular bacterial presence throughout the experiments and demonstrated phenotypic growths pattern changes from normal colony sizes at day 0-2, to SCV-like colonies identified after 3 days (Fig. 6.3C-D).

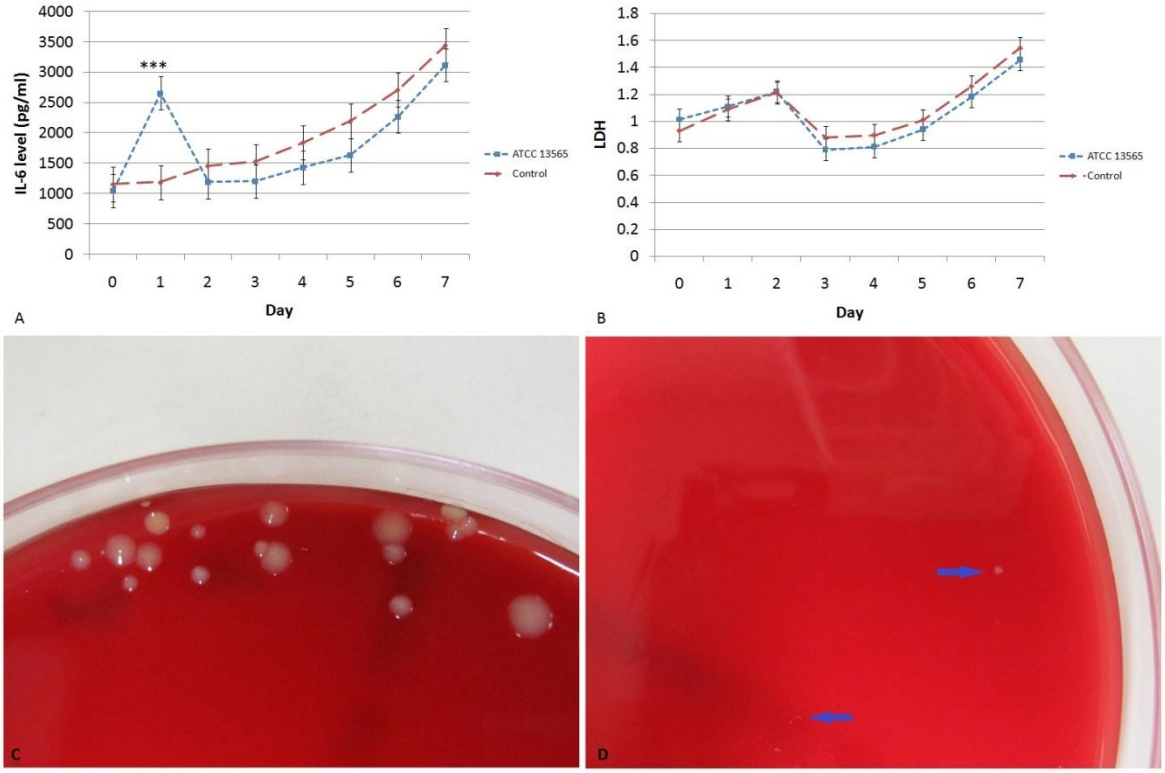

Figure 6.3: Effects of long term intracellular infection with *S. aureus* ATCC 13565 on airway epithelial cells.

A: IL-6 ELISA of culture media post intracellular infection demonstrating a statistically significant rise on day 1 (*** p = 0.0002, unpaired t-test) followed by a reduction to levels similar to non-infected cells;
B: LDH measurements of culture media post intracellular infection demonstrating no significant difference compared to non-infected cells. Bars for A/B represent mean +/- SEM;
C/D: Intracellular colonies retrieved from epithelial monolayer; C: Day 3 post infection demonstrating mixed population of normal and small colonies; D: Day 6 post infection demonstrating several small colony variants, characterized by tiny (pinpoint) non-pigmented colonies (blue arrows).

6.5 DISCUSSION

In this study, we sought to link the concept of small colony variants to that of phenotype switching and the potential relationships to recalcitrant CRS. We identified that the bacterial phenotype of intramucosal organisms harvested *ex vivo* from sinonasal tissue specimens is indicative of a SCV. PFGE demonstrated that these organisms were of an identical clonal type despite variation in growth patterns. In the second part of this study we used an *in vitro* model to demonstrate that after internalisation into epithelial cells, wild type bacteria rapidly reduce an aspect of virulence potential as represented by suppression of superantigen production. Reversion to wild-type phenotype, represented by normal growth pattern and detectable superantigen production was achieved after a single subculture step, indicating virulence suppression was readily reversible.

Through our harvesting techniques we are able to identify that different phenotypic growth patterns can be seen when comparing bacteria swabbed from the surface of mucosal specimens obtained from CRS patients and those from the mucosal homogenates. Organisms harvested from the mucosa were significantly smaller than surface ones. Through PFGE analysis we confirmed that the bacterial strains are clonally identical despite the markedly different growth patterns, perhaps suggesting altered transcription factor regulation or epigenetic changes occurred. Furthermore, bacteria from the mucosa did not demonstrate any coagulase activity when plated onto chromogenic agar, a hallmark of the SCV phenotype.[289] These harvested SCVs demonstrated their capacity to revert back to the wild type form with a single sub-culture step. Studies have described SCV growth from tissue homogenates previously;[251] however our methodology employs a series of steps that aims to eliminate surface bacterial contaminants, and demonstrated the elimination of any surface bacterial growth post-treatment. In studies that homogenise

whole tissue samples without surface cleansing, it is possible that these bacteria were cultured from surface biofilms, as decreased metabolic rate and growth is also a hallmark of the biofilm phenotype.[341] Biofilms are known to colonise vascular prosthesis,[342] cardiac devices[343] and artificial joints,[192] as well as playing a role in chronic rhinosinusitis.[202,297,319] The phenotypic form of bacteria that reside and form a biofilm could even be that resembling a SCV, a hypothesis that could account for their altered growth and antibiotic resistance patterns. This is an area that would benefit from further investigation.

We have demonstrated through our *in vitro* experiments that enterotoxin production can, in certain strains, be downregulated after only 24 hours of exposure to the intracellular environment. This may represent the bacterium altering its pathogenic capacity so as to reduce the toxic effects to the cell and thus maintain its protective environmental niche. When extracellular bacteria were left in the culture media, the fact that the monolayer was effectively lysed within 24 hours is a clear demonstration as to the pathogenic capabilities of the organism through virulence factors such as staphylococcal enterotoxins and pore-forming toxins such as alpha-hemolysin. Interestingly, after infecting cell cultures with SEA and SEB producing clinical isolates, both strains were capable of downregulating their virulence state. Conversely, one of the two reference strains was unable to reduce its enterotoxin production such that enterotoxin was detected within the cell lysate after internalisation. This suggests that the ability to alter the virulence state may be an important factor in achieving the indolent infection, and that less adaptable organisms may alert the host immune system for its eradication. However, the mechanism by which organisms sense and respond to their environmental pressures remains poorly understood. One factor that has been described as a potential mechanism is known as the "bet-hedging strategy".[344] In this, it is thought that sub-population variations in phenotypic expression

may be selectively pressured into emergence through factors such as antibiotic treatment. This may occur as a means for overall species survival in the face of hostile environments.

When we performed intracellular infections with medium-term durations of up to 7 days, it was interesting to see that although the internalisation of the organisms caused an initial inflammatory response (IL-6 production), this was rapidly reduced as a consequence of reduced virulence. The initial stimulation supports the findings of Sachse et al,[240] however, in this study the authors did not make any further supernatant recordings beyond a 12 hour time-point. The fact that there was no significant increase in LDH at any time point suggests that despite this inflammatory insult, it did not lead to any additional toxicity.

6.6 CONCLUSION

Our results strengthen the hypothesis that intracellular *S. aureus* in chronic rhinosinusitis have a small colony variant phenotype, and subvert the host immune response by different mechanisms. These include the abortion of a pro-inflammatory immune response, absence of toxic effects and a reversible switching of bacterial virulence factors. Regulation of *S. aureus* virulence factors is a dynamic process dependant on environmental factors; specifically, the intracellular milieu appears to provide the necessary pressures to cause these alterations in an attempt for the bacterium to survive and persist within host tissues. The small colony variant hypothesis is one that is applicable to bacterial persistence in chronic rhinosinusitis, strengthened by evidence from their retrieval within mucosal homogenates. Further investigation is required to ascertain whether SCVs in CRS hold a clinically relevant pathogenic role in recalcitrant disease.

CHAPTER 7

GENERAL DISCUSSION

7.1 THESIS SYNOPSIS

Research into the impact of intracellular *S. aureus* in CRS is still at its early stages. The work presented in this PhD thesis has provided novel insights alluding to a role of intracellular *S. aureus* in recalcitrant CRS. The versatility of *S. aureus* in altering its phenotypic characteristics to exploit the local environment makes it challenging to fully eradicate, and significant associations can be made between intracellular infection and recalcitrant disease. Despite this, there remains a significant body of work that is required to be performed prior to being able to attribute this as an independent risk factor in recalcitrant CRS.

In Chapter 2, a novel imaging and processing technique that utilised FISH-CSLM/PI was developed to allow the simultaneous detection of intracellular and surface biofilm *S. aureus* status within the same piece of tissue. This was validated against a recognised technique of IHC within the same cohort of patents with an assessment as to the pros and cons of each technique. IHC allows for more accurate cellular morphology, and the specific advantage of being able to image a full section of tissue through the epithelium and down to the sub-mucosa. The disadvantage of IHC is that accurate biofilm analysis is impossible due to tissue processing artefact or sampling error. On the other hand, FISH-CSLM/PI did allow for the simultaneous biofilm and intracellular analysis, a critically important function of this technique as it allowed us to co-localise the two phenotypic forms of *S. aureus* within the same piece of tissue. Furthermore, by the use of whole mucosal specimens it is possible to image a much larger area for evidence of bacteria, thus reducing the risk of sampling errors. However, a drawback of the FISH-CSLM/PI technique is that the laser beam is unable to penetrate deeper than the epithelial layer, and therefore we are unable to obtain any data as to the presence or absence of submucosal

bacteria. As yet, it is unknown whether submucosal bacterial infection is a continuum of intraepithelial infection, or represents a distinct clinical entity in itself. This would clearly benefit from further investigation.

After performing IHC on *S. aureus* infected patient tissue samples an unexpected finding was noted; that of false-positive fluorescence associated with primary antibodies and their negative isotype controls, thereby confusing and potentially invalidating any IHC results. In Chapter 3, this was hypothesised to be due to the protein A component of *S. aureus* cell walls which survived tissue processing and continued to exhibit its strong affinity for IgG type antibodies. Therefore the methodology of blocking protein A by saturation with HuIgG prior to application of primary antibodies was developed. Blocking with different concentrations of HuIgG identified a dose-related response, and the optimum conditions were found at a concentration of 200 μg/ml for a duration of 2 hours at room temperature. This was then validated threefold; (i) in formalin fixed *S. aureus* slide smears, (ii) in biopsy specimens of intracellular *S. aureus* positive CRS patients, and (iii) in biopsy specimens of healthy control patients experimentally infected with *S. aureus*, to show that false-positive Protein A – Ab binding in IHC could be effectively eliminated.

In Chapter 4, the validated FISH-CSLM/PI protocol was applied to a wider cohort of patients undergoing endoscopic sinus surgery, including both CRS patients and control patients with no evidence of sino-nasal disease. We found no evidence of *S. aureus* infection within the control group. In the CRS cohort, we found that all cases of intracellular *S. aureus* were related to surface biofilm, and furthermore there were no cases of intracellular infection without concurrent surface biofilm. This suggested that the biofilm may provide a conditioned environment to allow internalisation of bacteria into the

epithelial layer. Certainly it is well known that healthy epithelium produces a mucociliary blanket that traps planktonic bacteria in its periphery, and when the underlying cilia are functioning normally to expel mucous out of the sinus, this may provide a mechanism to prevent adherence of bacteria to the epithelial surface. By the very nature of a biofilm being attached to the mucosal surface, coupled with local destructive effects to cilia, this may provide the micro-environment that allows planktonic bacteria shed from the biofilm to adhere to, and then invade into deeper tissues.

The next step was to determine whether the intracellular phenotype had any effect on outcomes following endoscopic sinus surgery. If it could be shown that patients who harboured intracellular *S. aureus* at the time of surgery had a different outcome profile to that of negative patients or those with biofilm only it would direct our future research to focus on methods of intracellular elimination. In Chapter 5 we identified that patients with intracellular *S. aureus* infections were more likely to suffer a clinical and microbiological failure of medical and surgical therapy, and for the first time in the literature we reported the impact of intracellular *S. aureus* in the post surgical patient. As with any study we do accept the limitations of this work. Our main outcome measures during clinical follow-up were symptomatic abnormal mucosa (oedema, polypoid change, granulations, crusting or frank mucopus or discharge) associated with *S. aureus* positive microbiological culture. As a full data set of objective and subjective postoperative symptom scores was not obtained in this study, the relationship between intracellular bacteria and symptomatic relapse can only be inferred. Nevertheless, we feel that our criteria of clinical relapse as being endoscopic signs of disease coupled with positive microbiology was sufficient to draw conclusions on the risk of developing recurrent post-surgical infections.

Finally, in Chapter 6 we endeavoured to further understand the mechanisms of how bacteria interact with host cells upon internalising and we questioned the concept of bacterial phenotype switching in airway epithelial cells. For *S. aureus* to persist within tissues we hypothesised that it is capable of dynamically altering its virulence factors. By utilising reference strains of *S. aureus* known to produce superantigenic enterotoxins, we performed *in vitro* infections of airway epithelial cells and demonstrated that within 24 hours of internalisation, the bacteria reduced their production of toxins to an undetectable level. This was accompanied by alterations in growth patterns with a decreased metabolic rate to produce phenotypic colonies remarkably similar to SCVs. After intracellular bacteria were harvested from the epithelial monolayer, a single sub-culture step was all that was required to cause their reversion back to the wild-type parental strain with a return of virulence potential as noted by their enterotoxin production. When considering the medium-term effects of intracellular infection, we sought to understand the effects on the host cell. By utilising an IL-6 ELISA we identified that internalisation of bacteria caused an initial inflammatory insult, however, this rapidly reduced to comparable levels with control cells, and furthermore at no point did we notice any cellular cytotoxicity as per our LDH assay. Finally we wanted to correlate the *in vitro* findings of induced SCVs to that of bacteria within sino-nasal mucosal biopsies. We were able to identify that the bacterial phenotype of intramucosal organisms harvested *ex vivo* from sinonasal tissue specimens highly resembles that of an SCV with the characteristically small colony growth and lack of coagulase activity. By clonal typing bacteria using PFGE it was noted that the harvested SCVs were identical to bacteria on the tissue surfaces that cultured phenotypically normal colonies.

7.2 FUTURE DIRECTIONS

The work presented thus far sets a solid background for which future research can be directed in order to more completely understand the impact of the intracellular phenotype of *S. aureus* on disease progression and especially recalcitrance in CRS. The future plans are summarised as follows;

7.2.1 REFINEMENT OF INTRACELLULAR BACTERIA DETECTION AND HARVESTING TECHNIQUES

Although the studies presented in this thesis utilise state of the art molecular and imaging techniques for the detection of intracellular *S. aureus*, a limitation is that molecular FISH probes target the nucleic acid "fingerprint" left by bacterial DNA. Thus, it is difficult to definitively conclude that the fluorescence seen represents live, viable bacteria. Although we have attempted to overcome this issue with modifications of standard culture techniques in Chapter 6, there are a number of potential methodologies that could be employed to more definitively harvest intracellular/intramucosal bacteria. After enzymatic cell proteolysis of mucosal specimens it is possible to collect intact epithelial cells separately from other structures that encompass the ECM such as fibroblasts or collagen. This could be accomplished either using magnetic beads coupled to epithelial cell specific antibodies, or, alternatively by sorting epithelial cells after flow cytometry (fluorescence-activated cell sorting-FACS). From this pure epithelial cellular distillation it would be possible to lyse the cells and culture any intracellular bacteria present. If this technique was validated against FISH/CSLM samples from the same subject it would provide significant proof that the fluorescence seen in tissue samples using our protocols demonstrates live bacteria.

If these techniques are successful in conclusively harvesting intracellular organisms not only does it add credence to the work presented thus far, but also allows for the possibility of performing whole genomic analysis of bacteria harvested from patient samples *ex vivo.* Commercially available microarray kits can be used to sequence the whole genome of *S. aureus*, and have already been used to identify significant transcriptional changes that occur after *in vitro* infection experiments. To be able to confirm these results in *ex vivo* harvested bacteria would certainly be an important finding.

7.2.2 EFFECT OF INTRACELLULAR INFECTION ON SYMPTOMATIC OUTCOMES

Although we noted an association between intracellular infection at the time of surgery and clinical and microbiological failure, it would be advantageous to be able to define a true impact on outcomes from the patient perspective. Therefore, a further prospective study with validated subjective symptom scores collected pre- and post-operatively along with other objective markers of disease severity such as the Lund-Kennedy endoscopic score is underway. By collecting subjective and objective outcome data we will have a more encompassing understanding of the true impact on disease. Furthermore, sub-dividing patient categories to take into account confounding causes for clinical relapse such as the presence of nasal polyposis will allow us to identify whether intracellular *S. aureus* represents a true independent risk factor for developing recalcitrant disease.

7.2.3 CHARACTERISATION OF IMMUNOLOGICAL RESPONSES TO INTRACELLULAR INFECTION

Research thus far has not assessed the immunological impact of intracellular *S. aureus* infection. Although we have identified a mechanism by which the organism can alter its

phenotypic form so as to shield itself from detection, it is certainly possible that the organisms are in some way interacting with the host cells. The recognition that extracellular planktonic or biofilm *S. aureus* can induce an altered Th2 cytokine response leads to questions as to whether the intracellular phenotype may contribute to these changes through intracellular PRR's such as toll-like receptors (TLR) or nucleotide oligomerization domain (NOD) like receptors. Whether the activation of intracellular PRR's leads to irreversible mucosal fibrosis and scarring through the upregulation of pro-fibrotic factors such as transforming growth factor beta (TGF-β) is an interesting hypothesis, and certainly worth further investigation. The intracellular infection model used in Chapter 6 would offer an excellent model to perform such experiments in. After a set infection period, all extracellular bacteria are eliminated from the culture by the addition of lysostaphin. This model results in an epithelial monolayer infected with *S. aureus* with the exclusion of extracellular bacteria as a confounding factor.

7.2.4 THE FATE OF INTRACELLULAR *STAPHYLOCOCCUS AUREUS*

The ultimate fate of intracellular *S. aureus* remains unknown. To understand whether intracellular bacteria can be released following cell lysis or apoptosis would be critical in identifying mechanisms by which intracellular organisms *in vivo* can be responsible for relapsing infections. Although the studies included in Chapter 6 have demonstrated that intracellular bacteria can be cultured from *in vitro* cell cultures lysed in water, further experimentation to identify whether an apoptotic cell, for example, is able to release viable bacteria would add credence to the "reservoir of infection" hypothesis. By using the intracellular infection model, epithelial monolayers can be treated with agents that induce necrosis such as 5% Zinc Sulphate, or apoptosis such as etoposide or doxorubicin. Cellular supernatants can then be cultured to determine whether viable bacteria can be found.

7.3 CONCLUSION

In conclusion, this PhD thesis has provided novel insights alluding to a role of intracellular *S. aureus* in recalcitrant CRS. We have identified that intracellular *S. aureus* not only exists in the sinonasal epithelium of CRS patients, but also plays a significant role in disease relapse following surgery. The concepts of bacterial phenotype switching and SCVs in CRS have been raised as potential mechanisms by which organisms may survive and persist within epithelial cells. We have identified the presence of SCVs in *ex vivo* tissue samples and found that wild-type bacteria may be pressured into an SCV-like phenotype after exposure to the intracellular environment. Although the long term effects and the ultimate fate of intracellular *S. aureus* is not yet known, this work has provided a foundation to guide further investigation into intracellular *S. aureus* in CRS. Ultimately, we hope that this may direct investigations into potential therapeutic treatments of this complex disease.

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