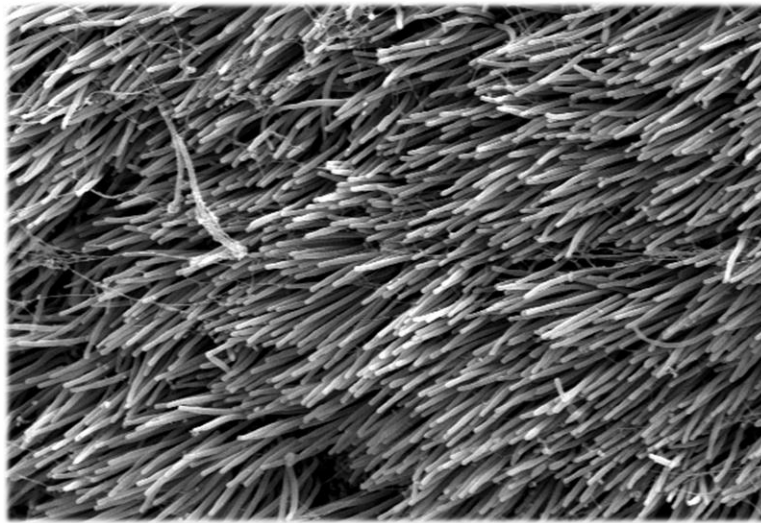


Bacteriophage therapy for application against
Staphylococcus aureus infection and biofilm
in chronic rhinosinusitis



Amanda Jane Drilling

Faculty of Health Sciences

School of Medicine

Discipline of Surgery

March 2015

The enemy of my enemy is my friend

Table of Contents

I.	Abstract	v
II.	Declaration	vii
III.	Acknowledgments.....	viii
IV.	Presentations and Awards Arising from this thesis.....	x
V.	List of tables.....	xii
VI.	List of Figures	xiii
VII.	Abbreviations	1
1	Systematic review of the Literature	3
1.1	Rhinosinusitis.....	3
1.1.1	Acute and Chronic Rhinosinusitis.....	4
1.1.2	Prevalence of Chronic rhinosinusitis.....	7
1.1.3	Economic burden of the CRS condition.....	10
1.1.4	Factors contributing to CRS.....	11
1.1.4.1	Nasal polyposis	12
1.1.4.2	Asthma	13
1.1.4.3	Allergy	14
1.1.4.4	Bacterial and fungal infection	16
1.1.4.5	Genetics.....	18
1.1.4.6	Environmental factors	20
1.1.5	Medical management of CRS	22
1.1.5.1	Systemic and topical steroids	22
1.1.5.2	Antibiotics.....	24
1.1.6	Surgical management of CRS	25
1.2	<i>Staphylococcus aureus</i>	27
1.2.1	Friend or Foe?	28
1.2.2	Epidemiology and Prevalence of <i>S. aureus</i> in CRS	29
1.2.3	<i>S. aureus</i> Virulence Factors and their role in CRS.....	30
1.2.3.1	Superantigens	31
1.2.3.2	Enzymes and non-superantigen toxins.....	32
1.2.3.3	Antibiotic Resistance	34
1.2.3.4	Immune Evasion.....	37
1.2.4	Summary of the contribution of <i>S. aureus</i> in CRS.....	38
1.3	Biofilms.....	39
1.3.1	Universal biofilm structure	39

1.3.2	Formation of Bacterial Biofilms	41
1.3.3	Mechanism of resistance	44
1.3.3.1	Biofilm resistance to antibiotics	45
1.3.3.2	Biofilm resistance to host immune response	47
1.3.4	Biofilms in CRS	48
1.3.5	Novel anti-biofilm therapies targeting <i>Staphylococcus aureus</i>	49
1.4	Bacteriophages	51
1.4.1	History of Bacteriophage	51
1.4.2	Bacteriophage Taxonomy	52
1.4.3	Myoviridae family	55
1.4.3.1	Structure of Phage K	55
1.4.4	Lifecycle of bacteriophage	57
1.4.4.1	Lytic Phage Lifecycle	59
1.4.5	Bacteriophage Therapy	60
1.4.5.1	Early History of Phage Therapy	60
1.4.5.2	Early phage therapy in Eastern Europe	61
1.4.5.3	Resurgence of Phage Therapy	62
1.4.6	Applications for bacteriophage in CRS	63
1.4.6.1	Bacteriophage versus biofilms	63
1.4.6.2	Specificity of phage	64
1.5	Summary of Literature Review	66
1.6	Project Aims	68
2	Cousins, siblings or copies: the genomics of recurrent <i>Staphylococcus aureus</i> infections in chronic rhinosinusitis	69
2.1	Statement of authorship	69
2.2	Publication Title Page	72
2.3	Article	73
2.3.2	Abstract:	74
2.3.4	Introduction	75
2.3.6	Materials and Methods:	76
2.3.8	Results	80
2.3.9	Discussion	88
2.3.10	Conclusion	90
2.3.11	Acknowledgments	90
3	Bacteriophage reduces biofilm of <i>Staphylococcus aureus ex vivo</i> isolates from chronic rhinosinusitis patients	91
3.1	Statement of Authorship	91

3.2	Publication Title Page	93
3.3	Article.....	94
3.3.1	Abstract	95
3.3.2	Introduction.....	96
3.3.3	Materials and Methods.....	98
3.3.4	Results.....	104
3.3.5	Discussion	112
3.3.6	Conclusion	115
3.3.7	Acknowledgments.....	115
4	Safety and efficacy of topical bacteriophage and EDTA treatment of Staphylococcus aureus infection in a sheep model of sinusitis	116
4.1	Statement of Authorship	116
4.2	Publication Title Page	120
4.3	Article.....	121
4.3.1	Abstract	122
4.3.2	Introduction.....	123
4.3.3	Materials and Methods.....	125
4.3.4	Results.....	130
4.3.5	Discussion	139
4.3.6	Conclusion	144
4.3.7	Acknowledgments.....	144
5	Thesis summation	145
6	Conclusion	152
7	References.....	153

I. Abstract

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

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

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

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

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

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

II. Declaration

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

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Date: _____

III. Acknowledgments

Throughout my PhD I have received indispensable help and support from many supervisors, colleagues and friends. I would like to formally recognise the contribution of these individuals.

First and foremost I would like to acknowledge Prof. PJ Wormald for the exceptional guidance, encouragement and support he has provided me throughout the three years of my PhD. His passion for research and improvement of patient care has been a huge inspiration for me, and his enthusiasm and positivity has helped me through difficult times faced during my research.

I would particularly like to mention Dr. Samuel Boase and Dr. Camille Jardeleza. Without Sam's patience and superb teaching skills I may never have developed the skills I have today. To Camille, both a colleague and good friend throughout the last three years. Her immense dedication to research and her incredible teaching skills provided me with the skills and drive to conduct this research.

To Dr. Sarah Vrugde who has been an amazing mentor during my PhD. Her guidance and encouragement has been an integral element of my PhD and I cannot thank her enough for all she has done. Furthermore to Dr. Clare Cooksley, who was always happy to help me whenever I needed advice or a helping hand. I cannot begin to express my gratitude to Clare, particularly for the way she kindly fields some of my more naïve questions. To my co-supervisor Dr. Peter Speck whose virology expertise has aided in project design and development. His guidance has been much appreciated throughout my PhD and his knowledge has been very valuable. To Dr. Alkis Psaltis whose passion for research has been inspirational and his help and guidance has been irreplaceable. I would also like to greatly acknowledge Lyn Martin, whose assistance has been invaluable. I am so very appreciative of the time and effort she has put in to help me through different situations.

To my friends and colleagues, Sukanya Rajiv, Dijana Miljkovic, Judy Ou, Shalini Nayar, Sian Nelligan, Sathish Paramasivan, Caroline Cousins, Neil Tan, Daniel Cantero, Vikram Padhye, Ahmed Bassiouni and Irene Zinonos and the entire ENT department who have shared the highs and

lows of my PhD and been an amazing support base. Their friendship and support throughout my PhD has meant the world to me.

I would also like to acknowledge my co-contributors to this research. To Dr. Craig James for the help and expertise he provided with histological interpretation. To Dr. Stuart Howell and Dr. Tom Sullivan for their help and expertise with statistical analysis. To Geoffrey Coombs for his advice, expertise and assistance with *Staphylococcus aureus* molecular typing. Also to Dr. Sandra Morales and Dr. Tony Smithyman for their amazing advice, expertise and guidance in the area of bacteriophage.

On a more personal note I would like to thank my amazing external support base. To my partner Michael (Mox) White whose unconditional support has helped me through the last year and a half of my PhD. His understanding nature and grammar skills have been essential for the completion of this PhD. Also to my Family Jane and Simon Drilling and Brother Jack/John Drilling who have provided me with a huge amount and love and encouragement over the years and without this I may have never undertook, or completed my PhD.

IV. Presentations and Awards Arising from this thesis

Presentations:

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

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

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

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

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

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

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

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

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

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

Awards:

Finalist in Poster Presentation,

TQEH Research Foundation Research Day, Adelaide Australia, 2012

Best Presentation Senior PhD researchers

TQEH Research Foundation Research Day, Adelaide Australia, 2013

V. List of tables

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

Table 1.2: Summary of Literature discussing the Prevalence of CRS

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

Table 2.1: Patient Demographics

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

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

Table 2.4: Antimicrobial susceptibility profile changes.

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

Table 3.1: Article Abbreviations Reference Table

Table 3.2: Summary of CRS *S. aureus* clinical strain susceptibility to phage infection

Table 3.3: Frequency of emergence of bacteriophage-insensitive mutants (BIM)

Table 3.4: Mean optical density_{600nm} of the media present in the MBEC assay plates after 24 and 48hrs of treatment

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

VI. List of Figures

Figure 1.1: Computed tomography scan of paranasal sinus region.

Figure 1.2: Factors contributing to the pathogenesis of Chronic Rhinosinusitis.

Figure 1.3: Structure of *Staphylococcus aureus* cells

Figure 1.4: Mechanism of bacterial resistance to antibiotics.

Figure 1.5: Generalised Biofilm Structure

Figure 1.6: General, simplified stages of biofilm development.

Figure 1.7: Mechanisms of antimicrobial resistance in Biofilms.

Figure 1.8: Diagram of Phage K structure proposed in 1954.

Figure 1.9: Electron Microscopy image of Phage K.

Figure 1.10: Bacteriophage lifecycle.

Figure 2.1: Guarded culture swabs

Figure 2.2: Dendogram representing the relationships of *S. aureus* isolates harvested from patients experiencing recurrent *S. aureus* infection.

Figure 3.1 Susceptibility of *S. aureus* to phage lysis.

Figure 3.2: Biofilm biomass levels following 48hrs of CT-SA treatment.

Figure 3.3 Compressed image stacks of bacterial biofilms observed on treated MBEC assay pegs.

Figure 3.4: Planktonic cell analysis of MBEC assay wells.

Figure 3.5: Phage counts of MBEC assay plates assessed after 24 and 48hrs of treatment

Figure 4.1 Representative images of the histology of the sinus mucosa harvested from sheep in the safety arm.

Figure 4.2: Comparison of the chronic inflammatory cell infiltrate in the sub-epithelial layer of sheep in the safety arm.

Figure 4.3: Representative images of scanning electron micrographs of sinus mucosa harvested from sheep in the safety arm.

Figure 4.4: Representative images of the histology of the sinus mucosa harvested from sheep in the efficacy arm.

Figure 4.5: Comparison of the acute inflammatory cell infiltrate in the sub-epithelial layer of sheep in the efficacy arm.

Figure 4.6: COMSTAT2 computation of biofilm mass levels present on sheep sinus mucosa.

VII. Abbreviations

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

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