# Bacteriophage therapy for chronic rhinosinusitis: targeting Pseudomonas aeruginosa biofilms

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# Contents

# Abstract

Chronic rhinosinusitis (CRS) is a chronic inflammatory condition involving the paranasal sinuses, affecting roughly 10.5% of the population. Current theories suggest that the aetiology of CRS is multifactorial, with evidence that microbial dysbiosis and bacterial biofilms may both play a role. *Pseudomonas aeruginosa* is a gram-negative bacterium, which is associated with poorer disease-specific quality of life and patients requiring revision sinus surgeries for CRS. *P. aeruginosa* is also a key pathogen in cystic fibrosis (CF), where it may cause both sinus and lower respiratory tract infections.

Unfortunately, *P. aeruginosa* frequently displays intrinsic and acquired resistance to antibiotics. The use of lytic bacteriophages has been proposed as an alternative treatment for infections caused by antibiotic-resistant bacteria. Lytic bacteriophages are bacterial viruses that can infect, replicate within, and lyse bacteria, killing the host bacteria through lysis. This thesis examines the suitability of anti-*P. aeruginosa* lytic bacteriophages as a treatment for *P. aeruginosa* infections in CRS and CF-associated CRS.

An *in vitro* study of the efficacy of a mixture of anti-*P. aeruginosa* bacteriophages (referred to as CT-PA) against a panel of 40 clinical *P. aeruginosa* respiratory isolates, from CRS and CF patients across 3 continents, was performed. CT-PA was found to have a broad host range, including activity against multidrug-resistant isolates. CT-PA was able to significantly reduce biofilm biomass after 24 and 48 hours exposure to biofilms.

The sheep rhinosinusitis model was then adapted to simulate *P. aeruginosa* sinusitis. The growth of viable *P. aeruginosa* biofilms within sheep frontal sinuses was confirmed using fluorescence in situ hybridisation and LIVE/DEAD BacLight staining. This animal model was then used to assess the safety and efficacy of CT-PA sinus flushes *in vivo*. The Safety arm confirmed a good safety profile, with no signs of local or systemic toxicity observed after 3 weeks of twice-daily CT-PA sinus flushes. The Efficacy arm of the study showed a statistically significant

reduction in sinus biofilm biomass in sheep treated with CT-PA sinus flushes, twice daily for 7 days, compared to sheep who received 0.9% saline flushes instead.

The emergence of bacteriophage insensitive mutants (BIMs) in *P. aeruginosa* biofilms exposed to CT-PA was also explored in an *in vitro* biofilm model, using whole genome sequencing and antibiotic susceptibility testing. This revealed changes in antibiotic susceptibility in BIMs compared to their parent strains. Whole genome sequencing revealed several genetic variations between the BIM and phage-sensitive isolate of each strain, most notably in the prophage Pf1 region of the genome.

In summary, bacteriophage therapy holds promise for treating *P. aeruginosa* infections in CRS and CF. Specifically, the CT-PA phage cocktail appears to be safe and effective in treating *P. aeruginosa* sinus infections in an *in vivo* animal model. Further investigations are required to determine whether the development of BIMs will hamper the efficacy of lytic bacteriophage therapy, or conversely whether the emergence of phage resistance may create evolutionary trade-offs that can be exploited to decrease resistance to conventional antibiotics.

# Declaration

I, Stephanie Anne Fong, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I acknowledge 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 Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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# Publications arising from thesis

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Fong SA, Drilling AJ, Ooi ML, Paramasivan S, Finnie JW, Morales S, Psaltis AJ, Vreugde S, Wormald PJ. Safety and efficacy of a bacteriophage cocktail in an in vivo model of Pseudomonas aeruginosa sinusitis. Transl Res. 2019 April;206:41-56.

Fong SA, Bassiouni A, Morales S, Psaltis AJ, Wormald PJ, Vreugde S. Genomic variation and changes in antibiotic susceptibility in *Pseudomonas aeruginosa* clinical respiratory isolates with acquired resistance to a bacteriophage cocktail [unpublished manuscript]. Adelaide: University of Adelaide; 2020.

# Presentations arising from thesis

21st October 2016

Bacteriophage therapy for CRS: targeting *Pseudomonas aeruginosa* biofilms.

The Queen Elizabeth Hospital Research Day 2016

24th March 2017

Bacteriophage therapy for treating *Pseudomonas aeruginosa* infections in chronic rhinosinusitis.

Australian Society of Otolaryngology Head and Neck Surgery Annual Scientific Meeting 2017

8th September 2017

Activity of bacteriophages in removing biofilms of *Pseudomonas aeruginosa* isolates from chronic rhinosinusitis patients.

American Rhinologic Society Annual Meeting 2017

20th October 2017

Efficacy and safety of a *Pseudomonas aeruginosa* bacteriophage cocktail in a sheep model of rhinosinusitis.

The Queen Elizabeth Hospital Research Day 2017

10th March 2018

Efficacy and safety of a *Pseudomonas aeruginosa* bacteriophage cocktail in a sheep model of rhinosinusitis.

Australian Society of Otolaryngology Head and Neck Surgery Annual Scientific Meeting 2018

14th November 2019

Bacteriophage therapy for *Pseudomonas aeruginosa* infections in chronic rhinosinusitis and cystic fibrosis.

Australian Society of Otolaryngology Head and Neck Surgery South Australian state section Gristwood Medal dinner 2019

# Awards arising from thesis

14th November 2019

Bacteriophage therapy for *Pseudomonas aeruginosa* infections in chronic rhinosinusitis and cystic fibrosis.

Australian Society of Otolaryngology Head and Neck Surgery South Australian state section Gristwood Medal dinner 2019

- The Gristwood Medal for research by a South Australian Otolaryngology Head and Neck Surgery trainee

# List of abbreviations

| | |
|---|---|
| 15-HETE | 15-hydroxyeicosatetraenoic acid |
| 5-LO | 5-lipoxygenase |
| AAOHNS | American Academy of Otolaryngology – Head and Neck Surgery |
| Abi | abortive infection system |
| ADP | adenosine diphosphate |
| AERD | aspirin-exacerbated respiratory disease |
| AFRS | allergic fungal rhinosinusitis |
| AHL | N-acylhomoserine lactone |
| ALI | air-liquid interface |
| AMP | adenosine monophosphate |
| AR | allergic rhinitis |
| AST | aspartate transaminase |
| BAFF | B cell activating factor |
| BPI | bactericidal/permeability-increasing protein |
| CD | cluster of differentiation |
| CDC | United States Centers for Disease Control and Prevention |
| CF | cystic fibrosis |
| CFTR | cystic fibrosis transmembrane conductance regulator |
| COX | cyclooxygenase |
| CRISPR | clustered regularly interspaced short palindromic repeats |
| CRS | chronic rhinosinusitis |
| CRSsNP | chronic rhinosinusitis without nasal polyps |
| CRSwNP | chronic rhinosinusitis with nasal polyps |
| CT | computed tomography |
| C-terminal | carboxyl-terminal |
| cys-LT | cysteinyl-leukotrienes |
| DNA | deoxyribonucleic acid |
| dsDNA | double-stranded deoxyribonucleic acid |
| EBI2 | Epstein-Barr virus-induced protein 2 |
| EPOS 2012 | European Position Paper on rhinosinusitis and nasal polyps 2012 |
| EQ-5D | European Quality of Life – 5 Dimensions |
| ESBL | extended-spectrum beta-lactamase |
| ESS | endoscopic sinus surgery |
| FISH | fluorescence in situ hybridisation |
| GGT | gamma-glutamyl transpeptidase |
| GTPase | guanosine triphosphate |
| hCAP18 | human cationic antimicrobial peptide 18kDa |

| | |
|---|---|
| ICTV | International Committee on Taxonomy of Viruses |
| IFN | interferon |
| IFNγ | interferon gamma |
| Ig | immunoglobulin |
| IL | interleukin |
| INCS | intranasal corticosteroids |
| INFORM | International Network for Optimal Resistance Monitoring program |
| KPC | *Klebsiella pneumoniae*-type carbapenemase |
| LBP | lipopolysaccharide binding protein |
| LNA | locked nucleic acid |
| LPLUNC | long palate, lung, and nasal epithelial clone |
| LPS | lipopolysaccharide |
| $LTC_4$ | leukotriene $C_4$ |
| MBC | minimum bactericidal concentration |
| MBEC | minimum biofilm eradication concentration |
| MccJ25 | microcin J25 |
| MDR | multidrug-resistant |
| MGE | mobile genetic element |
| MIC | minimum inhibitory concentration |
| MMP | matrix metalloproteinase |
| MOI | multiplicity of infection |
| NERD | non-steroidal anti-inflammatory drug-exacerbated respiratory disease |
| NFκB | nuclear factor-kappa B |
| NHANES | National Health and Nutrition Examination Survey |
| NSAID | non-steroidal anti-inflammatory drug |
| N-terminal | amino-terminal |
| OmpA | outer membrane protein A |
| PBMCs | peripheral blood mononuclear cells |
| PFU | plaque forming units |
| $PGE_2$ | prostaglandin $E_2$ |
| PLUNC | palate, lung, and nasal epithelial clone |
| Poly(I:C) | polyinosinic-polycytidilic acid |
| QoL | quality of life |
| RCT | randomised controlled trial |
| RM | restriction-modification system |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| SEA | staphylococcal enterotoxin A |
| SEB | staphylococcal enterotoxin B |
| Sec | general secretory pathway |

| | |
|---|---|
| SEM | scanning electron microscopy |
| SENTRY | SENTRY Antimicrobial Surveillance Program |
| SF-36 | Medical Outcomes Study 36-item Short Form Health Survey |
| Sie | superinfection exclusion |
| SLPI | secretory leukoprotease inhibitor |
| SNOT-20 | 20-item Sinonasal Outcome Test |
| SNOT-22 | 22-item Sinonasal Outcome Test |
| SPLUNC | short palate, lung, and nasal epithelial clone |
| ssDNA | single-stranded deoxyribonucleic acid |
| T1SS | type 1 toxin secretion system |
| T2SS | type 2 toxin secretion system |
| T3SS | type 3 toxin secretion system |
| T5SS | type 5 toxin secretion system |
| T6SS | type 6 toxin secretion system |
| TER | transepithelial resistance |
| $TH_2$ | type 2 helper T cell |
| TLO | tertiary lymphoid organ |
| TLR | toll-like receptor |
| TNFα | tumour necrosis factor alpha |
| TSLP | thymic stromal lymphopoietin |
| URTI | upper respiratory tract infection |
| USA | United States of America |
| XDR | extended drug resistant |

# Chapter 1: Literature review

## 1.1 Chronic rhinosinusitis

### 1.1.1 Definition of chronic rhinosinusitis

Chronic rhinosinusitis (CRS) is an inflammatory condition involving the paranasal sinuses and lining of the nasal passages, persisting for 12 weeks or longer.[1] Symptoms may include nasal discharge, nasal obstruction, facial pain, and decreased sense of smell.[2] The recent expert consensus guidelines, the European Position Paper on rhinosinusitis and nasal polyps 2012 (EPOS 2012), gives the clinical definition of CRS in adults as:

- "inflammation of the nose and the paranasal sinuses characterised by two or more symptoms, one of which should be either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip):
    - ± facial pain/pressure
    - ± reduction or loss of smell

and either

- endoscopic signs of:
    - nasal polyps, and/or
    - mucopurulent discharge primarily from middle meatus and/or
    - oedema/mucosal obstruction primarily in middle meatus

and/or

- CT changes:
    - mucosal changes within the ostiomeatal complex and/or sinuses" [3]

An example of sinus mucosal changes seen in CRS is displayed in Figure 1.

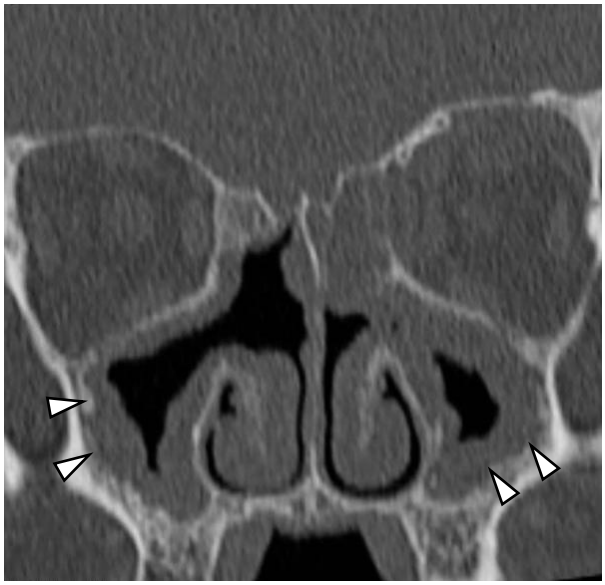


*Figure 1. Computed tomography of paranasal sinuses in the coronal plane, with arrowheads showing mucoperiosteal thickening consistent with CRS – case courtesy of A/Prof. Frank Gaillard, Radiopaedia.org, reproduced under Creative Commons BY-NC-SA 3.0 Unported licence*

### 1.1.2 Prevalence and impact of chronic rhinosinusitis

CRS affects over 2 million Australians, with an estimated prevalence of 10.5% in the general population. In 2002-2003, CRS was responsible for over 10 000 hospital admissions in Australia.[4] Multiple clinical and population-based studies have concluded that CRS sufferers have reduced quality of life (QoL).[5-7] In a population-based study, Fu and colleagues found that persons with self-reported CRS had lower QoL scores than non-sufferers on the Medical Outcomes Study

36-item Short Form Health Survey (SF-36) scale, with the largest differences in role emotional function, general health and role physical function domains.[8] Lange and colleagues assessed QoL in patients diagnosed with CRS by an otolaryngologist in an outpatient setting. CRS sufferers reported lower QoL compared to non-sufferers on both general health-related QoL and CRS-specific scales (European Quality of Life – 5 Dimensions (EQ-5D) and Sino-Nasal Outcome Test (SNOT)-22).[9]

### 1.1.3 Classification of chronic rhinosinusitis

CRS may be divided into two categories: CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP). Nasal polyps consist of inflamed, oedematous sinus mucosa. CRSwNP is defined by EPOS 2012 as chronic rhinosinusitis with bilateral, endoscopically visualised polyps in the middle meatus, whereas CRSsNP is defined as chronic rhinosinusitis with no visible polyps in the middle meatus, following decongestion if necessary.[3] Whilst both subclasses of CRS have been observed to share similar pathological features, such as inflammatory cell infiltrates, Caucasian patients with CRSwNP are more likely to have local and peripheral eosinophilia, and oedema.[10-12] In contrast, patients with CRSsNP typically have neutrophilic infiltrates and deposition of thick collagen fibres in the extracellular matrix.[13, 14] The inflammatory process in each of the two subclasses is characterised by distinct cytokine profiles. Elevated levels of interferon gamma and transforming growth factor beta are found in CRSsNP, whilst interleukin 5, eotaxin, and eosinophilic cationic protein are elevated in CRSwNP.[15, 16]

### 1.1.4 Management of chronic rhinosinusitis

#### 1.1.4.1 Medical management – systemic therapies

Standard systemic medical therapies for CRS include antibiotics and corticosteroids.

Oral antibiotics are widely used to treat exacerbations, and following sinus surgery. Two double-blind placebo-controlled studies of oral antibiotics in CRSwNP suggest antibiotics may be beneficial in improving certain symptoms and endoscopic signs. Van Zele et al found treatment with a 20-day course of doxycycline significantly reduced postnasal drip symptoms and polyp size.[17] Schalek et al compared a 3-week postoperative course of anti-Staphylococcal antibiotic (quinolone, amoxycillin/clavulanic acid, or co-trimoxazole) with placebo, and found a slight but not statistically significant improvement in symptom and endoscopic severity scores.[18] The small number of participants (23 patients) suggests that the study may have been under-powered.

In patients with CRSsNP, investigation has largely focused on the potential anti-inflammatory and anti-biofilm effects of long-term low dose oral macrolide antibiotics. *In vitro* studies show that clarithromycin produces a dose-dependent decrease in production of the pro-inflammatory cytokines interleukin 5 and 8, and granulocyte-macrophage colony stimulating factor, by sinus mucosal samples from CRS patients.[19] Erythromycin has also been shown to increase neutrophil apoptosis *in vitro*.[20] The results of clinical trials have been mixed. A double-blind randomised trial of three months of low dose roxithromycin found a statistically significant improvement in saccharine transit times, endoscopic

and symptom scores, compared to placebo.[21] Conversely, a multicentre randomised controlled trial (RCT) of 3 months low-dose azithromycin in recalcitrant CRS found no statistically significant differences in symptom severity, quality of life scores, or endoscopic scores between the azithromycin and placebo groups.[22]

Oral corticosteroids are typically prescribed in short courses of 2 to 3 weeks, in order to prevent the complications of long-term steroid use. Corticosteroids are postulated to have anti-inflammatory effects on the sinonasal mucosa, decrease vascular permeability, and decrease the glycoprotein content in mucous secretions.[23-27] Several RCTs in patients with CRSwNP show improved health-related quality of life and symptom severity compared to placebo, at the end of a 2 or 3 week course of treatment.[28-31] There is a paucity of evidence in the published literature regarding the efficacy of oral corticosteroids in CRSsNP.

#### 1.1.4.2 Medical management – topical therapies

Topical therapies for CRS consist of treatments applied directly to the sinonasal mucosa by various methods, including rinses (irrigations), sprays, drops, and nebulisers. It is important to note that these delivery methods are not equivalent in their penetration into the sinuses. Studies using radiographic contrast and radiolabelled nasal pump sprays and drops show poor penetration into the sinuses.[32, 33] Pulsating aerosols were found to have only 6.5% deposition in the sinuses.[32] Experimental modelling using nebulised gentamicin has shown the proportion deposited into the maxillary sinus varies from 0.25% to 10%, dependent of the size of the maxillary ostia and particle size.[34] In contrast, nasal

irrigations have improved penetration into the maxillary and frontal sinuses in patients who have undergone endoscopic sinus surgery.[35]

Sinonasal saline irrigations are a mainstay in the management of CRS. The application of hypertonic saline to the sinonasal mucosa has been shown to increase mucociliary clearance in patients with CRS, possibly by increasing ciliary beat frequency.[36, 37] Nasal hypertonic saline irrigations have been shown to improve quality of life and reduce CRS symptom severity.[37-39] Saline irrigations may also assist in removing intranasal crusts and inspissated secretions, and are commonly prescribed following endoscopic sinus surgery. Studies aiming to assess the benefit of saline irrigations during the post-operative period have reported mixed results. Liang et al found benefit from the addition of irrigations to post-operative care only in mild CRS.[40] Freeman et al's RCT reported a significant improvement in endoscopic scoring of discharge with saline irrigations compared to without at 3 weeks post-operatively, but this effect was not maintained at 3 month follow up.[41]

Topical corticosteroids are an important aspect of medical management of CRS. Intranasal corticosteroids (INCS) may be applied to the sinonasal mucosa by numerous delivery methods, including sprays, drops, and sinonasal irrigations. A recent meta-analysis of eighteen randomised controlled trials found that in comparison to placebo, INCS had a moderate effect size in reducing symptom severity.[42] In patients with CRSwNP, the INCS group had smaller polyp size, and a higher chance of improvement in polyp size compared to placebo. The four studies assessing the intervention in patients with CRSsNP did not find any

statistically significant difference in endoscopy scores between INCS and placebo. However, the meta-analysis included only studies using INCS sprays or drops, and did not include high volume nasal irrigations containing steroids. Whilst there is a lack of double-blind placebo-controlled trials of corticosteroid-containing irrigations in CRS, there is evidence that these irrigations may lead to a reduction in symptoms, as well as endoscopic and radiographic severity.[43]

The use of topical antimicrobials in CRSsNP has been explored in several RCTs. However, there is significant heterogeneity between trials in the antimicrobial agents used (neomycin, tobramycin, bacitracin/colimycin, mupirocin) and methods of intranasal administration (spray, nebuliser, sinus rinse). Three RCTs using intranasal spray or nebuliser showed no additional benefit from topical antibiotics.[44-46] Jervis-Bardy et al undertook a double-blind RCT of twice daily mupirocin in saline sinus rinses compared to saline rinses with oral antibiotics.[47] Following the course of treatment, 89% of participants who received topical mupirocin had sinus cultures negative for *S. aureus*, compared to 0% of the control group. A significant improvement in endoscopic scores compared to control was noted in the mupirocin group, but there was no significant difference between groups in symptom severity scores. Given the small number of RCTs, all with small numbers of participants, topical antimicrobials are not recommended by consensus guidelines including EPOS 2012 and the American Academy of Otolaryngology – Head and Neck Surgery (AAOHNS) 2015.[3, 48]

### 1.1.4.3 Surgical therapy

Surgical treatments for CRS are generally reserved for patients who do not respond to medical therapies. Conventional surgical approaches include nasal polypectomy, inferior meatal antrostomy, and Caldwell-Luc antrostomy, however endoscopic sinus surgery (ESS) is the most commonly performed surgical intervention for CRS. ESS is a collective term for a range of endoscopic procedures, which may be performed individually or in combination. These procedures include uncinectomy, maxillary antrostomy, anterior ethmoidectomy, posterior ethmoidectomy, sphenoidotomy, and frontal sinus drill-out. The primary aims of ESS are to improve patency of sinus ostia and facilitate mucociliary function, remove diseased tissue, and improve penetration of topical therapies to sinus mucosa.[49] As for many surgical treatments, there is a lack of placebo-controlled trials. However, large prospective studies have reported improvements in symptom severity, as well as in objective measures such as ciliary beat frequency and olfactory function.[50-54]

Two RCTs have compared ESS to medical treatment of CRS, with conflicting results. Hartog et al reported increased resolution of purulent rhinitis and loss of smell with saline irrigations followed by ESS in comparison to saline irrigations alone, in patients with chronic maxillary sinusitis.[55] It should be noted that neither treatment group received INCS during the study, contrary to current consensus guideline recommendations for medical management of CRS. Ragab et al found no differences in symptom severity, saccharine clearance time, or endoscopic scores between medical and surgical treatment groups, in a cohort of

patients with CRS or recurrent acute rhinosinusitis who had failed to improve with initial medical management.[56] Whilst both groups received alkaline nasal irrigations and INCS, the medical treatment group received a longer course of erythromycin, and a small number of medically-treated patients received a 9-day course of oral corticosteroids. It is unclear whether any patients in the surgery group received similar "rescue" therapy. In summary, the small number of studies and heterogeneity of study populations and treatments means the relative efficacy of surgery compared to medical therapies is still unclear.

### 1.1.5 Aetiology and pathogenesis of chronic rhinosinusitis

The aetiology of CRS is likely to be multifactorial, and has not been fully elucidated. However, the sinonasal mucosa may be seen as the interface between the environment, and the immune system. Thus the aetiology of CRS may be broken down into host factors, such as innate immunity, macro- and microstructure of the sinuses, and extrinsic factors, such as bacterial, viral and fungal infection.[57]

#### 1.1.5.1 Host factors

##### *1.1.5.1.1 Innate immunity*

The immune system has been implicated in CRS due to its inflammatory features, as well as the recurrent or persistent infections that appear to plague some sufferers. Many aspects of innate immunity are exhibited in sinus mucosa. The pseudostratified ciliated columnar epithelium, along with its mucociliary apparatus, forms a mechanical barrier to foreign material and invasion by microbes.

Epithelial, glandular and immune cells also secrete numerous host defence molecules, including cathelicidins, defensins, lysozyme, lactoferrin, and secretory leukoprotease inhibitor (SLPI). Defensins are found in nasal epithelium and neutrophils, as well as other epithelial surfaces. Defensins increase the membrane permeability of their targets (bacteria, fungi, viruses) by insertion into the cell membrane.[58] This appears to result in inhibition of bacterial DNA, RNA, and protein synthesis.[58] Cathelicidins have been identified in many mammals, and share a common "cathelin" domain as well as a variable C-terminal antimicrobial domain. The only human cathelicidin identified, human cationic antimicrobial peptide 18kDa (hCAP18), has a C-terminal peptide named LL-37. hCAP18 is found in neutrophil granules, nasal mucosa, and submucosal glands.[59-61] LL-37 binds to and disrupts the bacterial cell membrane, and also induces neutrophil, monocyte, and T-cell chemotaxis, as well as mast cell degranulation.[62-64]

Lysozyme and lactoferrin are both found in neutrophil granules and epithelial cells. Lysozyme hydrolyses peptidoglycan in the gram positive bacterial cell wall, but also has activity against gram negative bacteria.[65] Lactoferrin binds and sequesters iron, which is essential for many microbial cellular processes, leading to cellular starvation. Lactoferrin has also been shown to prevent biofilm formation by *Pseudomonas aeruginosa*.[66] SLPI is produced by neutrophils, macrophages, and respiratory epithelium. It has anti-bacterial and anti-fungal activity, and also appears to regulate opsonin degradation and processing of antimicrobial peptides.[67-69]

Aside from secreted host defence molecules, several pattern recognition receptors are expressed by respiratory epithelium, macrophages, dendritic cells, and B lymphocytes. These include toll-like receptors (TLRs) and palate, lung, and nasal epithelial clone (PLUNC) proteins. The TLR family is a family of membrane glycoproteins that are homologues of Toll, a receptor that is responsible for establishing dorsoventral polarity in insect embryogenesis. TLR ligands range from molecules found on bacteria and fungi, to viruses and endogenous (host) stimuli. One example is lipopolysaccharide (LPS), a component of the outer membrane of gram negative bacteria. In general, the end result of TLR activation is the expression of inflammatory cytokines, although negative modulation is also possible.[70] PLUNC proteins share a similar structure with the LPS binding proteins lipopolysaccharide binding protein (LBP) and bactericidal/permeability-increasing protein (BPI). Two family members, SPLUNC1 and LPLUNC2 are expressed in sinonasal tissue.[71]

Various derangements of the host defence mechanisms described above have been found in CRS sufferers. Delayed mucociliary clearance and impaired ciliary beat frequency have been measured in patients with CRS and their sinus tissue samples, compared to healthy controls.[54, 72-75] Air-liquid interface (ALI) cultures, where sinonasal epithelial cells are grown to confluence on a porous membrane and the apical surface exposed to air, have been used to explore the integrity of the epithelial barrier in CRS. Transepithelial resistance (TER) is measured between the apical and basal compartments, with lower resistance suggesting increased epithelial permeability. TER of cells from polypoid mucosa of CRSwNP

patients has been found to be lower than that of healthy controls.[76] Sinus epithelium from CRSwNP patients also appeared to have irregular expression of tight junction proteins.[76] Soyka et al also noted accentuation of these changes on exposure of the ALI cultures to IFNγ and IL-4.[76]

β-defensin 2 expression, as well as neutrophils containing α-defensin 1, 2, and 3, have been detected in nasal polyps and turbinate tissue from CRS patients, but not in healthy controls.[77] Increased LL-37 expression has been measured in nasal polyps and inferior turbinate tissue from CRS patients.[78] This has also been experimentally induced in nasal tissue explants from CRS patients in a dose-dependent response to fungal extracts.[79] Immunohistochemical studies by Fukami et al showed an apparent increase in lysozyme in the goblet cells of CRS patients, with both lysozyme and lactoferrin also localised to atypical mucosal glands.[80] Conversely, Psaltis et al found a decrease in lactoferrin expression in the sinus mucosa of CRS patients, including those with and without eosinophilic mucus, fungal culture, and fungal allergy.[81] Similarly, a decrease in SPLUNC1 and LPLUNC2 expression correlated with fewer submucosal glands has been found in nasal polyps in CRSwNP.[82] TLR-2, 5, 6, 7, 8, and 9 expression is increased in CRSwNP nasal polyp tissue, whilst only TLR-2 appears to be upregulated in CRSsNP sinus mucosa.[83, 84] Given that the majority of the aforementioned studies are cross-sectional in design, it is unclear whether the changes detected in CRS patients are the cause or consequence of the disease process. However, it can be concluded that an altered inflammatory response is a key feature of CRS phenotypes.

*1.1.5.1.2 Adaptive immunity*

Differences in the adaptive immune response in various CRS subtypes have been identified. The histopathology of nasal polyps in Caucasian populations is typified by infiltration of eosinophils, neutrophils, mast cells, and B lymphocytes. The inflammatory infiltrate in Asian populations is more variable, with a lower proportion of patients having a predominantly eosinophilic infiltrate.[85] Analysis of T cells and cytokines in CRSwNP sinus tissue has shown a dominance of CD4+ (helper) T cells and a $T_H2$ cytokine profile (IL-4 and IL-5).[86] The same study showed a predominance of CD8+ (cytotoxic) T cells over CD4+ T cells in CRSsNP tissue. Thymic stromal lymphopoietin (TSLP) expression and IL-33 receptor positive cells have both been found to be increased in CRSwNP sinus tissue, and could potentially drive the $T_H2$-polarised response.[87, 88] Interestingly, Shaw et al found IL-33 expression was similar in CRSwNP, CRSsNP, and healthy controls, suggesting that it may be the population of IL-33 responsive cells that plays a pathogenic role in CRSwNP. The postulated role of $T_H2$ cytokines in the CRSwNP inflammatory cascade is illustrated in Figure 2.

Increased levels of B cell activating factor (BAFF), as well as autoreactive antibodies have been identified in CRSwNP, implicating a role for B cells in the pathogenesis of CRSwNP.[89, 90] Feldman et al found that the frequency of IgG, IgA, and IgE-secreting B cells was significantly higher in nasal polyps than in tonsils – a secondary lymphoid organ. Nasal polyps also contained more plasmablasts expressing Epstein-Barr virus-induced protein 2 (EBI2), a marker of extrafollicular plasmablasts. This suggests that B cell activation in CRSwNP may be

extrafollicular, as opposed to occurring within germinal centres.[91] Conversely, tertiary lymphoid organs (TLOs) have been identified in the sinus tissue of patients with CRSwNP and CRSsNP.[92] Whilst the function of TLOs in CRS is yet to be explored, the presence of TLOs is associated with tissue eosinophilia and number of prior surgeries, suggesting a potential role in surgically-recalcitrant CRS.

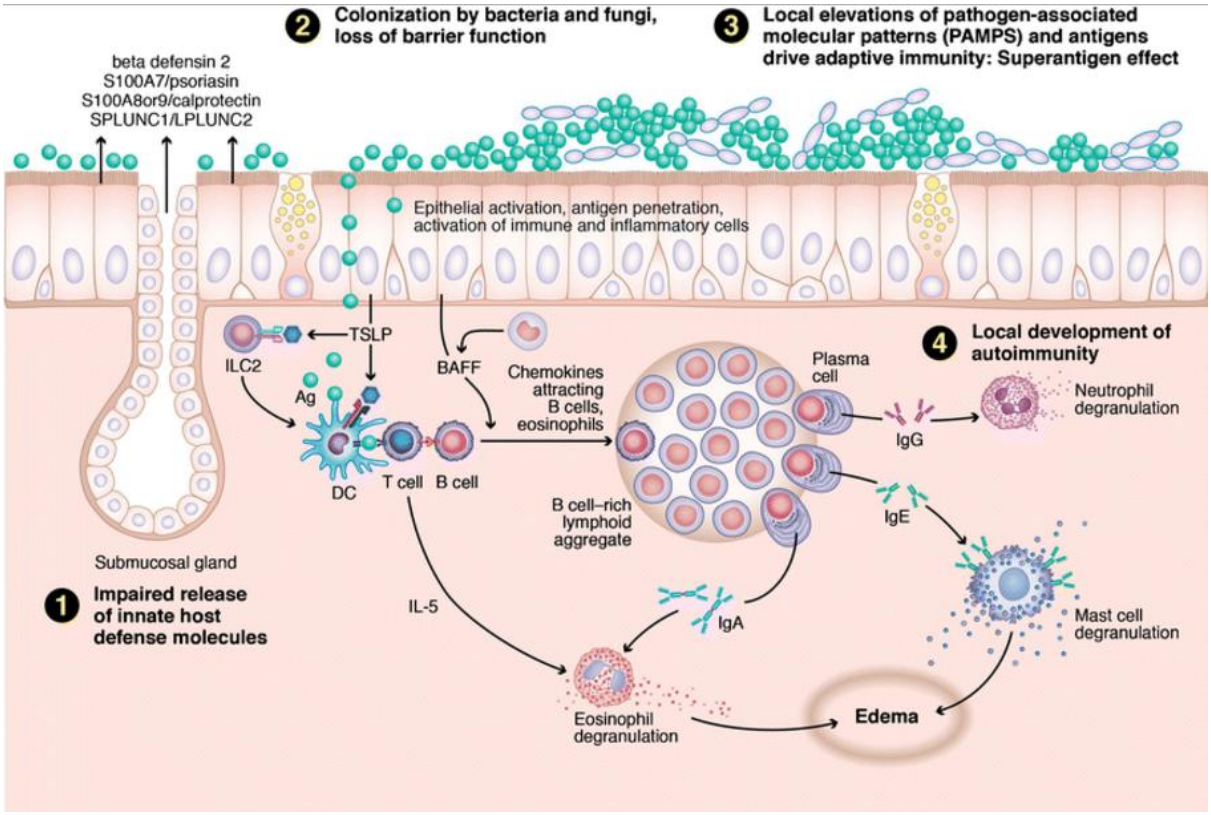



*Figure 2. Diagrammatic illustration of the immune barrier hypothesis in CRS and postulated derangements in innate and adaptive immunity – reproduced from Lam et al, with permission of Springer* [93]

*1.1.5.1.3 Allergy and atopy*

Atopy is the genetic tendency to develop allergic diseases such as allergic rhinitis, eczema, and asthma. Exposure to common aeroallergens may lead to the development of specific immunoglobulin E (IgE) antibodies to those allergens, which may be detected by serum or skin testing. Allergic rhinitis (AR) can be defined as an IgE-mediated inflammatory response of the nasal mucous membranes, after exposure to inhaled allergens.[94] Typical symptoms include sneezing, rhinorrhoea, nasal congestion, and nasal pruritis. Whilst some symptoms may overlap with those found in CRS, definitive diagnosis of AR relies on the detection of an IgE-mediated response to a specific allergen.[94]

Several observational studies have described the prevalence of atopy in various cohorts of CRS patients, ranging from 38% to 84%.[95-101] Unfortunately, these studies did not attempt to compare their prevalence estimates in CRS patients with prevalence in comparable groups of people without a diagnosis of CRS. Moreover, several population studies of atopy and allergy have noted large variations in prevalence dependent on geography and over time, making the need for a truly comparable control group imperative.[102-104] Contrary to assertions made in some studies that atopy is more prevalent in those suffering CRS, Leo et al found the prevalence of atopy in children with CRS to be comparable to that of the general paediatric population in Italy.[105] The prevalence of allergic sensitisation in the United States general population estimated by the National Health and Nutrition Examination Survey (NHANES) 2005-2006 was 44%, falling well within the range of values observed in the

groups of CRS patients studied.[106] Thus an epidemiological association between allergy or atopy and CRS is not consistently observed.

Whilst no causal relationship between allergic diseases and CRS has been proven, postulated mechanisms include:

- Obstruction of the sinus ostia by inflamed mucosa or secretions
- Delayed mucociliary clearance [72, 107]
- Penetration of allergens into the paranasal sinuses, causing local inflammation [108, 109]
- Altered inflammatory response and priming of immune cells [110-112]
- Neurogenic inflammation secondary to sensitisation of sensory afferent nerves [97]

Numerous studies have examined the relationship between atopy and CRS severity, with mixed results. Ramadan and colleagues found that amongst CRS patients who had never undergone sinus surgery, those with allergic rhinitis had higher (more severe) CT scores, compared to those with no allergies.[113] Another cross-sectional study of 225 patients undergoing revision endoscopic sinus surgery (ESS) found that patients with inhalant allergy had significantly higher Lund-Kennedy endoscopic and Lund-Mackay CT scores.[99] However, the same study found that patients with CRSwNP were significantly more likely to have inhalant allergy, and also that CRSwNP patients had significantly higher endoscopic and CT scores than CRSsNP patients. A similar pattern was noted by Robinson and colleagues in a study of 193 CRS patients.[114] Whilst Lund-Mackay scores were more severe in atopic patients, when patients were grouped

according to the presence or absence of nasal polyposis (CRSsNP or CRSwNP) there was no significant difference in Lund-Mackay scores between atopic and non-atopic patients within each group. In concurrence with Batra et al's study, CRSwNP patients had higher CT scores than CRSsNP patients. Atopic and non-atopic patients did not differ in Lund symptom scores, although atopic patients reported significantly more sneezing, reduced productivity, and reduced concentration on SNOT-20. Pearlman and colleagues performed a prospective study of 165 patients, finding that Lund-Mackay scores were similar between atopic and non-atopic patients.[115] The authors also noted that Lund-Mackay scores were higher in CRSwNP and asthmatic patients, but that atopy was not a significant factor.

Some investigators have attempted to identify a specific allergen, or class of allergens, relevant to CRS. Pearlman and colleagues identified that patients with cockroach allergy were significantly more likely to have a Lund-Mackay score greater than 10, and higher maxillary sinus scores.[115] Other studies have also noted a predominance of perennial allergies, such as to house dust mite, cockroach, animals, and moulds, over seasonal allergies in CRS patients.[96, 97]

*1.1.5.1.4 Hypersensitivity*

Aspirin-exacerbated respiratory disease (AERD), or non-steroidal anti-inflammatory drugs (NSAIDs)-exacerbated respiratory disease (NERD), is a clinical syndrome typified by aspirin or NSAID hypersensitivity, asthma, and CRSwNP with mucosal eosinophilic infiltrates.[116] A recent meta-analysis has estimated the prevalence of AERD in CRS patients to be 8.7%.[117] Several studies have

found increased severity of symptom, endoscopic, and radiographic scores in patients with AERD compared to CRSwNP patients without AERD.[118-120] Moreover, patients with AERD are more likely to undergo revision ESS.[118]

The NSAID hypersensitivity in NERD is not immunologically mediated, and therefore does not represent a true allergy. Cyclooxygenase (COX)-1 inhibition by NSAIDs is thought to lead to reduced prostaglandin $E_2$ ($PGE_2$) synthesis, over-production of cysteinyl-leukotrienes (cys-LT) via the 5-lipoxygenase (5-LO) pathway, and subsequent activation of mast cells and eosinophils.[121] 5-LO expression has also been found to be higher in NERD nasal polyps, as well as eosinophils expressing leukotriene $C_4$ ($LTC_4$) synthase, which is involved in cys-LT production.[122] Nasal epithelial cells from AERD patients have been shown to produce less $PGE_2$ when unstimulated *in vitro*, and more 15-hydroxyeicosatetraenoic acid (15-HETE), a pro-inflammatory arachidonic acid metabolite, upon stimulation with aspirin.[123] Additionally, nasal polyp histology from patients with NERD shows more eosinophils compared to in NSAID-tolerant patients with CRS.[122] Several mechanisms have been proposed to explain the characteristic eosinophilic infiltrate in NERD. These include increased systemic eotaxin-2 levels, platelet adherence to leukocytes enabling migration into inflamed tissues and cys-LT production, and IL-5 and granulocyte-macrophage colony stimulating factor expression.[124-126] Sanak et al identified a polymorphic variant of the $LTC_4$ synthase (*LTC4S*) locus as conferring increased risk for aspirin-intolerant asthma, although this result has not been consistently replicated across studies.[127] A meta-analysis of 13 studies found no increased risk of

asthma with *LTC4S* variants overall, but that the variants were associated with increased risk of asthma in Caucasian and aspirin-tolerant populations.[128] Other candidate genes under investigation that may be involved in or differentiate NERD and non-NERD CRS include periostin (*POSTN*), met proto-oncogene (*MET*), and protein phosphatase 1 regulatory subunit 9B (*PPP1R9B*).[129]

#### 1.1.5.2 Extrinsic & environmental factors

##### *1.1.5.2.1 Bacteria and bacterial communities*

Bacteria are frequently cultured from the sinus swabs and samples of mucosal tissue from CRS patients, giving rise to the hypothesis that bacteria are involved in the pathogenesis of CRS.

Although healthy paranasal sinuses were traditionally considered to be sterile, there is increasing evidence that this is not the case. Su et al attempted to compare the bacteria cultured from patients with chronic maxillary sinusitis undergoing Caldwell-Luc antrostomy, with those from the sinuses of control patients who did not have chronic sinusitis.[130] Bacteria were isolated from both nasal and sinus samples of all seven control patients. Klossek et al had comparable findings, with similar proportions of positive cultures from healthy control and CRS patients (81.3% and 83.1% respectively).[131]

More recently, molecular methods such as 16S ribosomal RNA (rRNA) genetic sequencing have been used to gain a more complete picture of the bacterial species present in healthy and diseased sinuses. These techniques allow identification of the various bacterial species present within a sample, as well as the relative abundance of each species. Compared to traditional culture-based

methods, there is also increased sensitivity for detection of species that are difficult to culture.[132] Several studies have identified *Staphylococcus aureus* as having increased relative abundance in CRS patients compared to healthy controls, whilst *Corynebacterium spp.*, *Staphylococcus epidermidis*, and *Propionibacterium acnes* have been found to have a higher relative abundance healthy sinuses.[132-135]

There have been many attempts to identify causal bacterial species in CRS, without consistent results. Some culture-based studies implicated anaerobic bacteria in the pathogenesis of chronic maxillary sinusitis, however other studies did not replicate this finding.[130, 136, 137] Doyle et al did not culture any anaerobes from endoscopically-guided ethmoid sinus biopsies in 59 patients with chronic ethmoid sinusitis.[138] Other similar studies found a predominance of aerobic species, namely *Staphylococcus spp.*, *P. aeruginosa*, *H. influenzae*, and *Streptococcus spp*.[131, 139-142] Given that molecular methods have displayed the microbial diversity of both healthy and diseased sinuses, it is unsurprising that the results of these culture-based studies show growth of a variety of species in different patient cohorts. Close examination of the methodologies used in these studies reveals numerous areas of heterogeneity, including criteria used to diagnose chronic sinusitis, methods of sample collection and transportation, and prior medical and surgical interventions in the study population.

Observational studies exploring associations between bacterial species and patient disease characteristics do identify *S. aureus* and *P. aeruginosa* as occurring more frequently in patients undergoing revision surgery.[140, 142, 143]

Given that the sinus cultures of asymptomatic individuals following endoscopic sinus surgery have been found to grow predominantly coagulase negative *Staphylococci*, with only 31% and 3% growing *S. aureus* and *P. aeruginosa* respectively, it is postulated that the latter organisms are associated with disease recalcitrance, rather than the surgery itself.[144] Higher relative abundance of *P. aeruginosa* has also been associated with lower disease-specific health related quality of life as measured by SNOT-22.[133]

Several pathophysiological mechanisms through which bacteria might contribute to the CRS disease process have been proposed. Certain bacteria might induce an aberrant immune response, leading to ongoing local inflammation in sinus tissues. Most investigation has focussed on the type 2 T-helper cell ($T_H2$) response that is characteristic of CRSwNP.[145] The presence of *S. aureus* and *P. aeruginosa* has been positively correlated with expression of the inflammatory cytokines interleukin-5 (IL-5), and tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ), on the sinus mucosa of patients with CRSwNP and CRSsNP respectively.[146]

One hypothesis is that the $T_H2$ inflammatory response is triggered by *S. aureus* superantigens. Specific IgE to toxic shock syndrome toxin 1, staphylococcal enterotoxins A (SEA) and B (SEB) has been detected in nasal polyp tissue, and found to be associated with higher tissue eosinophil counts.[147, 148] It has been demonstrated that T cells in nasal polyp tissue containing these toxins show oligoclonal expansions in the T cell receptor variable beta domain corresponding to the specific toxin present, suggesting the toxins result in lymphocyte

activation and proliferation.[148, 149] *In vitro* experiments have shown that stimulation of *ex vivo* nasal polyp tissue with SEB for 24 hours resulted in increased production of $T_H2$ proinflammatory cytokines IL-5 and IL-4, as well as decreased production of the anti-inflammatory cytokine IL-10.[150] In a similar study, Xu et al found a decrease in CD4+ CD25+ T-regulatory cells in both nasal polyp and turbinate tissue after stimulation with SEB for 24 hours.[151] IL-5, as well as IgE specific to *S. aureus* enterotoxins, have both been found to be markedly increased in patients with recurrent CRSwNP following ESS compared to those who did not experience recurrence.[152]

The apparent ability of sinonasal bacteria to evade antibiotic treatment has also been investigated, giving rise to the hypotheses that bacterial biofilms or intracellular bacteria may play a role in the pathogenesis of CRS.

A bacterial biofilm is a structured community of bacteria that is associated with a surface, and encased in a self-produced extracellular biopolymeric matrix, as seen in Figure 3. Bacteria in biofilms exhibit several differences from planktonic (free-floating) bacteria, including variation in metabolism throughout the biofilm, production of exopolysaccharide, and changes in gene expression.[153] These features can result in decreased sensitivity to antibiotics; bacteria in biofilms can be over 1000 times more resistant to antibiotics than their planktonic counterparts.[154] Thus biofilms may provide a niche in which bacteria can evade the host immune system and antimicrobial treatments, leading to ongoing disease. Scanning electron microscopy and confocal scanning laser microscopy have identified biofilms on the sinus mucosa of 44 to 55% of patients undergoing

ESS for CRS, compared to 0 to 8% of healthy control patients.[155-157] Several investigators have identified the presence of mucosal biofilms as a predictor of poor post-ESS outcomes, including more severe ongoing symptoms and worse Lund-Kennedy endoscopic scores, compared to patients without biofilms.[158, 159]

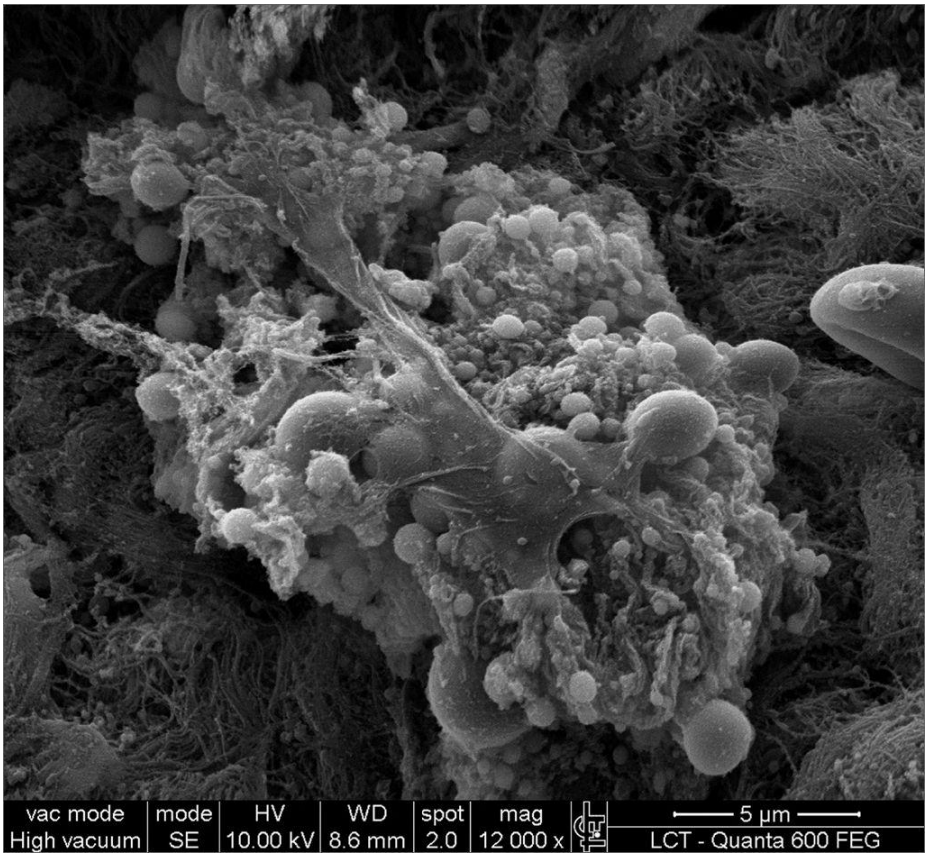



*Figure 3. Scanning electron micrograph of a bacterial biofilm on sinonasal mucosa from a patient with CRS – reproduced from Pinto Bezerra et al under Creative Commons BY 4.0 licence* [157]

In concordance with culture-based studies of patients requiring revision ESS, *S. aureus* and *P. aeruginosa* feature prominently in studies examining biofilm formation in CRS clinical isolates. Whether the microbial species comprising the biofilms alters the CRS disease process, and through which mechanisms, is not

yet fully elucidated. A prospective study of 150 CRS patients found that whilst *S. aureus* was the most frequently isolated species from endoscopically-guided swabs, samples from which *P. aeruginosa* was isolated were the most likely to form biofilms *in vitro*.[160] The authors also noted that patients with previous infections were more likely to harbour bacteria with biofilm-forming capacity. In a retrospective study of 24 patients, Foreman et al identified that patients with single-species *H. influenzae* biofilms at the time of ESS had a rapid resolution of CRS post-operatively, whilst the presence of either mono- or polymicrobial biofilms containing *S. aureus* was associated with more severe disease that took longer to resolve. Patients with polymicrobial biofilms containing *P. aeruginosa* also had increased symptomatic and radiographic severity pre-operatively.[161] Similarly, Bendouah et al found that the ability of *S. aureus* and *P. aeruginosa* isolates to form biofilm *in vitro* was associated with an unfavourable progression of disease following ESS.[162]

Although traditionally considered to be an extracellular pathogen, intracellular *S. aureus* has been identified in the sinonasal epithelium (Figure 4), mucous glands, myofibroblasts, and mast cells of CRS patients.[163-165] A prospective study of 51 patients undergoing ESS for CRS detected intracellular *S. aureus* in 39%, with all also having mucosal biofilms. Patients positive for intracellular *S. aureus* in their sinonasal mucosa had increased radiographic severity, and were more likely to have had previous surgery. These patients were also significantly more likely to have both early and late post-operative infections, compared to patients without biofilm or intracellular bacteria.[166]

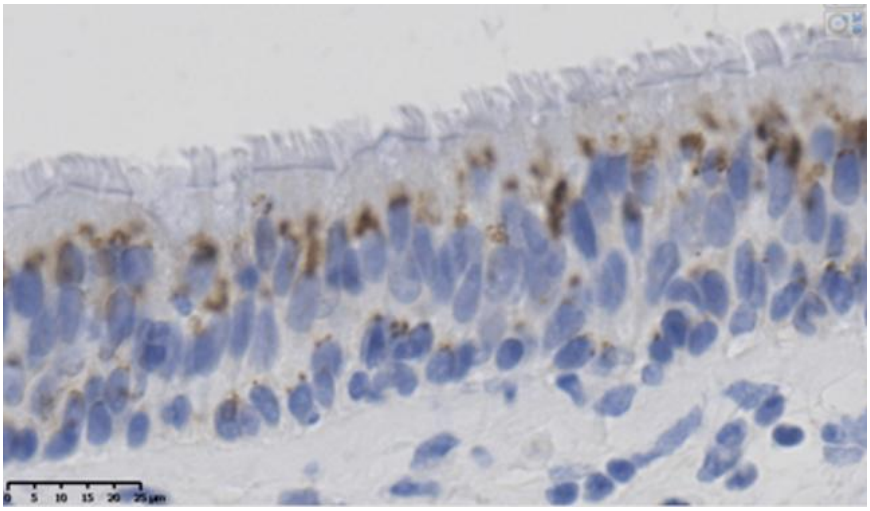


*Figure 4. Photomicrograph of immunohistochemistry staining of intracellular Staphylococcus aureus in sinonasal mucosal epithelium – reproduced from Ou et al under licence from John Wiley and Sons* [(167)]

The role that intracellular bacteria might play in the pathogenesis of CRS is unclear. Clement et al followed the clinical course of 3 patients with intracellular *S. aureus*, who all experienced recurrent infection with the same strain of *S. aureus* over time, despite multiple courses of antibiotics.[(163)] This suggests intracellular bacteria may provide a reservoir for recurrent infections, whilst being protected from antimicrobial treatments. It has also been postulated that epithelial invasion by *S. aureus* is an indicator of impaired epithelial barrier function.[(168)] As described above for extracellular *S. aureus*, intracellular *S. aureus* may have immunomodulatory effects. *In vitro* studies of nasal polyp epithelial cells with experimentally induced intracellular *S. aureus* infection measured an increase in IL-6 synthesis compared to uninfected controls.[(168)] Ou et al noted significantly higher lymphocyte counts in sinonasal mucosa samples with intracellular *S. aureus*, compared to those without.[(167)]

The role of the bacteria found in healthy sinuses, and whether these species contribute to the maintenance of the healthy state, is still being explored. It has been shown that in a polymicrobial community, as has been identified in the paranasal sinuses, the presence of one bacterial species may impact on the population of a co-existing species.[169, 170] *In vivo* experimental work by Abreu et al has shown that *Lactobacillus sakei* is able to exert a protective effect on murine sinuses infected with *Corynebacterium tuberculostearicum*.[171] *Corynebacterium* species have been implicated as determinants of nasal carriage of *S. aureus* in healthy humans. Yan et al found *C. accolens* to be associated with high relative abundance of *S. aureus*, whilst *C. pseudodiphtheriticum* was associated with absence or low abundance of *S. aureus*.[172] Rates of *S. aureus* nasal carriage are also significantly lower in the presence of serine protease-producing *S. epidermidis* strains.[173] This finding is supported by *in vitro* studies showing that the supernatant from these strains can inhibit *S. aureus* biofilm formation.[173]

Concurrently, the microbiome may also influence the host immune response. Murine studies suggest that the microbiome of the gastrointestinal tract influences the host adaptive immune response and promotes immune homeostasis.[174] Certain gut bacteria may have systemic anti-inflammatory effects. *Bacteroides fragilis* has been shown to influence development of T regulatory lymphocytes in the gastrointestinal tract, whilst other bacteria produce short chain fatty acids by metabolism of dietary fibre, which may also lead to differentiation of T regulatory cells.[175, 176]

In CRS, introduction of a potentially pathogenic bacterial species into the sinonasal tract might not only lead to acute or chronic infection, but also to permanent changes in the sinonasal microbiome. Cope et al used a murine model of acute *P. aeruginosa* sinusitis to assess both the acute inflammatory response and changes to the sinus microbiome in mice with depleted and intact microbiomes.[177] Mice treated with a course of antibiotics prior to inoculation with *P. aeruginosa* had a delayed resolution in pro-inflammatory IFNγ gene expression, compared to mice with intact microbiomes. Microbiome-depleted mice also had a longer duration of decreased microbiome richness in response to the *P. aeruginosa* infection. Persistent increased dominance of the *Pseudomonadaceae* family was noted in the microbiomes of both groups of mice, and interestingly the native *Pseudomonadaceae* remained dominant even after clearance of the infecting strain. Although the murine sinus microbiome differs significantly from that of humans, these results suggest that a bacterial infection may lead to persistent changes in the sinus microbiome, and subsequent changes in host immune response.

#### *1.1.5.2.2 Fungi*

A role for fungi in the pathogenesis of CRS has been hypothesised for both allergic fungal rhinosinusitis (AFRS) and CRS in general. The AFRS phenotype was first identified as a subset of patients with sinus histology sharing similarities with allergic bronchopulmonary aspergillosis, including eosinophilic mucin containing necrotic cellular debris, Charcot-Leyden crystals, and non-invasive fungal hyphae (Figure 5). These patients also had allergy to *Aspergillus* on skin

testing, and serum IgE and IgG specific to the fungi.[178] For this reason, it was initially postulated that type I immediate and type III delayed hypersensitivity to various fungal species caused AFRS.[179] However, sensitisation to the fungal species found in the sinuses is not always present. Moreover, disease severity appears to have a stronger correlation with total IgE than with fungal-specific IgG.[180]

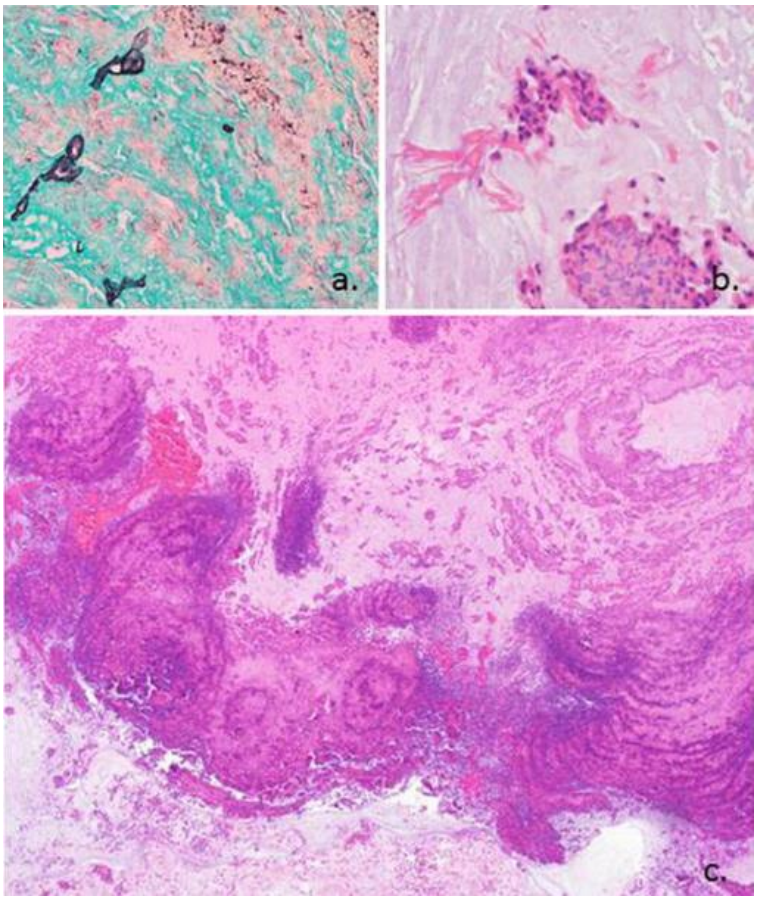



*Figure 5. Photomicrographs showing: a. Fungal elements stained with Grocott's methenamine silver; b. Eosinophilic mucin containing degenerated inflammatory cells and Charcot–Leyden crystals; c. Ripples of eosinophils and neutrophils creating a "tide line" or "tree ring" appearance typical of allergic fungal sinusitis – reproduced from Correll et al with permission from Springer* [181]

Other postulated immune reactions to fungal proliferation within the sinuses include the activation of pattern recognition receptors by fungus-derived complex carbohydrates, activation of protease-activating receptors, and resulting eosinophilic inflammation.[182, 183] Co-colonisation with bacteria and fungi is common, suggesting a role for bacterial superantigens in triggering the inflammatory response seen in AFRS.[184] Dutre et al found serum IgE specific to *S. aureus* superantigens in 16 out of 17 AFRS patients studied.[185] Evidence from animal models of AFRS also suggests that development of sinus fungal biofilms is dependent on the presence of bacterial biofilms or epithelial injury, and is not dependent on sensitisation to the fungus.[186, 187]

Using molecular techniques, fungi have been detected in 63 to 100% of patients with CRS who do not meet the diagnostic criteria for AFRS.[188, 189] Interestingly, whilst Gosepath et al also found fungal DNA in 66% of healthy control patients, no controls were positive for *Alternaria* DNA compared to all participants with CRS. The presence of fungus on staining or culture has been associated with ongoing symptoms and mucosal inflammation post-ESS.[158] It has thus been postulated that fungi may play a broader role in the pathogenesis of non-AFRS CRS.

Similar mechanisms of immune activation by fungi to those described for other micro-organisms have been proposed. Stimulation of ethmoid sinus mucosa from patients with CRSwNP with fungal extracts (*Aspergillus fumigatus, Alternaria alternata, Cladosporium herbarium*) resulted in increased IL-33 expression.[87] Peripheral blood mononuclear cells (PBMCs) from patients with

CRS have shown upregulation of IL-5 production following stimulation with *Alternaria*, *Aspergillus* and *Cladosporium* extracts, whereas this response was smaller in PBMCs from healthy controls, and even absent in the case of *Alternaria* stimulation of control PBMCs. A similar response in IL-13 production was seen.[190]

Unfortunately, several double-blind placebo-controlled trials have failed to show efficacy of topical and systemic antifungal agents in treating CRS.[189, 191-195] The ubiquitous nature of airborne fungi, its presence in the sinuses of control subjects, as well as the lack of efficacy of antifungal agents in clinical trials, makes it difficult to implicate fungi as a primary aetiological factor in CRS.

#### *1.1.5.2.3 Viruses*

Viral infection has been implicated in both the initiation and exacerbation of the CRS disease process. Many patients report that the onset of CRS was preceded with a viral upper respiratory tract infection (URTI), and acute rhinosinusitis is most often caused by viruses. However, no clear link between viral infections and the pathogenesis of CRS has been established.

Viruses have been detected in the nasal epithelium of up to 74% of CRS patients.[196] These viruses include rhinovirus, parainfluenza, influenza, respiratory syncytial virus, coronavirus, adenovirus, enterovirus, human metapneumovirus, and bocavirus. Case-control studies comparing the prevalence of viral infection in subjects with and without CRS show conflicting results. Cho et al detected virus more frequently in CRS patients compared to healthy controls, and also noted that co-infection with multiple viruses was more

common in CRS patients.[197] Liao et al found no difference in the frequency of virus detection between CRS and control groups.[196] However, no relationship between viral presence and CRS disease severity has been identified, with one study showing no difference in symptom, radiological or endoscopic scores between CRS subjects with and without virus identified in mucosal samples.[198]

Viral infections may result in remodelling of sinus mucosa, causing changes similar to those seen in CRS. Human rhinovirus infection of human bronchial epithelial cells and nasal polyp fibroblasts has been shown to result in upregulation of matrix metalloproteinase-9 (MMP-9) *in vitro*; clinical studies of subjects during rhinovirus infection also revealed an increase in nasal lavage MMP-9 concentration.[199, 200] MMPs enable inflammatory cell recruitment by regulating cytokines and chemokines, and disrupting cellular junctions and extracellular matrix. MMP-9 has been implicated in CRS-associated osteitis, and poor healing post-ESS.[201, 202] Infection of human nasal epithelial cells by rhinovirus has also been shown to increase bacterial adhesion, and reduce transepithelial resistance.[203, 204]

Polyinosinic-polycytidilic acid (poly(I:C)) is a synthetic viral (double-stranded RNA) analogue and TLR3 ligand, which has been used to explore the potential effects of viruses on the immune response in CRS. Golebski et al demonstrated increased TSLP expression in nasal polyp-derived epithelial cells that were stimulated with poly(I:C), compared to cells from healthy controls, suggesting viruses may also play a role in driving $T_H2$-biased inflammation.[205]

## 1.2 *Pseudomonas aeruginosa*

### 1.2.1 Classification and identification

*P. aeruginosa* is an obligate aerobic, motile, gram negative rod-shaped bacterium. It is frequently isolated from environmental habitats such as soil, water, plants, and animals, but is also found in various sources in the nosocomial setting, such as ventilators, mops, and even tap water. Data from the Human Microbiome Project suggests that *P. aeruginosa* is not a common constituent of the healthy human microbiota.[206] It is considered to be an opportunistic pathogen, and may cause life-threatening infections.[207]

It is distinguished from other bacterial species by growth from 37 up to 42 degrees Celsius, oxidase positivity, and the production of the green and blue pigments pyoverdin and pyocyanin. Some stains also produce pyorubin and pyomelanin, red and black pigments respectively. *P. aeruginosa* colonies have distinct morphologies when grown on bacteriological agar.[207] Wild-type environmental strains usually produce small, rough colonies, whilst clinical isolates can produce either large smooth colonies, slow-growing "small colony variants", or copious production of extracellular polysaccharide as seen in Figure 6. The latter are described as being mucoid, and are classically isolated from patients with cystic fibrosis or chronic infections.[207-209]

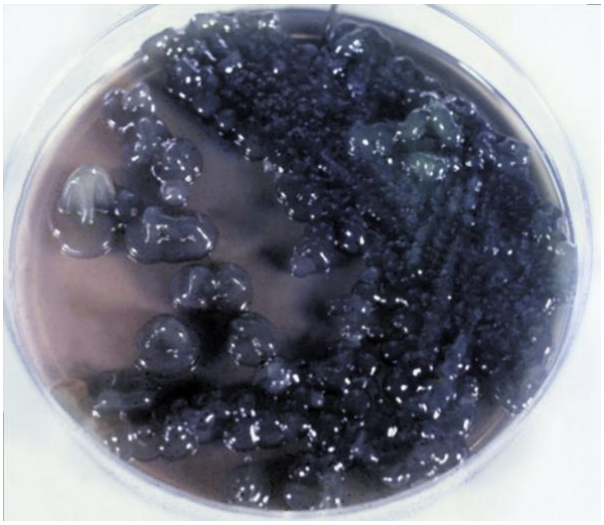


*Figure 6. Mucoid P. aeruginosa growing on MacConkey agar, demonstrating copious extracellular polysaccharide (alginate) production – reproduced from Pritt et al, by permission of the American Society for Clinical Pathology* [(210)]

### 1.2.2 Virulence factors

*P. aeruginosa* possesses a range of virulence factors, resistance genes, and metabolic adaptations, which contribute to its success as a pathogen. Virulence factors include pili, flagella, toxin secretion systems, quorum sensing, excreted pigments, and alginate (extracellular polysaccharide) production.

Common pili (or fimbriae) are hairlike appendages that enable bacteria to adhere to biotic and abiotic surfaces. Many *P. aeruginosa* strains possess type IV pili, which confer twitching motility and are crucial for biofilm formation.[(211)] The relationship between pili and pathogenicity is complex. In a murine model of corneal infection, Hazlett et al found both non-piliated and hyperpiliated *P. aeruginosa* mutants had decreased infectivity.[(212)] A study of *P. aeruginosa* attachment to injured murine trachea found that pili mediated attachment of

non-mucoid strains, but not mucoid strains.[213] The subunit of pili, pilin, is structurally encoded by the *pilA* gene, of which five alleles (groups I to V) have been identified in *P. aeruginosa*.[214, 215] Kus et al identified that a higher proportion of isolates from patients with cystic fibrosis possess group I pilins, which are able to be glycosylated.[215] It has been suggested that this glycosylation might strengthen binding to host surface receptors or other structures.[216]

Flagella are also important for bacterial adherence and motility. Flagella appear to be important in formation of a bacterial monolayer on a surface, the first step observed in *P. aeruginosa* biofilm formation.[211] Analogous to the role of pili, specific adaptations in the expression of flagella may be observed in isolates from cystic fibrosis patients. In a study of *ex vivo* isolates from patients with cystic fibrosis, Mahenthiralingam et al found non-motile isolates that lacked flagella were resistant to non-opsonic phagocytosis by murine macrophages.[217]

*P. aeruginosa* possesses type 1, 2, 3, 5 and 6 toxin secretion systems, illustrated in Figure 7. Type 1 secretion systems (T1SS) excrete toxins into the extracellular space, and consist of an outer membrane protein, an ATP-binding cassette transporter in the inner membrane, and an adaptor protein that connects the former two proteins. Proteins secreted by T1SS in *P. aeruginosa* include AprA, AprX, and HasAp. AprA is an alkaline protease; the function of AprX is unknown.[218, 219] HasAp binds haem and transports it to the outer membrane receptor, allowing haem internalisation.[220]

Type 2 secretion systems (T2SS) are formed by an inner membrane protein, a large outer membrane secretin channel, and a pseudopilus that is thought to push proteins through the channel. Numerous T2SS exoproteins have been discovered, and include proteolytic enzymes, alkaline phosphatase, phospholipases, exotoxin A, and chitin-binding protein.[221]

The type 3 secretion system (T3SS) is an intricate mechanism that utilises a supramolecular needle complex to transport exotoxins from the *P. aeruginosa* cytosol into a host eukaryotic cell. The needle complex consists of a multi-ring base and a needle-like filament. The filament is thought to act as a sensor for the host cell, as well as delivering the toxins through the bacteria cell wall. The needle complex also secretes proteins that form a membrane pore in the host cell membrane, known as the translocation apparatus. It is through this translocation apparatus that the toxins are able to move into the host cell cytoplasm.

The presence of this membrane pore alone may lead to cell death by increasing membrane permeability or activation of cellular defence mechanisms.[222-225] Four exotoxins that are secreted through the *P. aeruginosa* T3SS have been identified: ExoS, ExoT, ExoU, and ExoY. ExoS and ExoT have both N-terminal GTPase-activating protein and C-terminal ADP ribosyltransferase activity.[226-229] ExoS is transported to the Golgi apparatus and endoplasmic reticulum of the host cell, where it targets GTPases and causes actin cytoskeleton disruption.[228, 230] This results in rounding of epithelial cells and macrophages, and impairs phagocytosis *in vitro*.[231, 232] Rounding of epithelial cells may reduce epithelial

integrity.[233] ExoT has been noted to have similar effects to ExoS on host cells.[227, 234, 235] ExoU is a phospholipase, and ExoY is an adenylate cyclase.[236, 237] ExoU causes rapid host cell death.[238] The secretion of ExoY into the host cell cytoplasm results in raised intracellular cyclic AMP, altered gene expression, and disruption of the actin cytoskeleton.[237]

The presence of a functional T3SS has been shown to increase disease severity in animal models of infection, including pneumonia, keratitis, burn infection, and peritonitis.[224, 238-242] Associations between T3SS toxin secretion, disease severity, and treatment resistance have been observed in ventilator-associated pneumonia, and with mortality in non-cystic fibrosis acute pneumonia.[243-245]

In the type 5 secretion system (T5SS), proteins are transported across the inner membrane by the general secretory (Sec) pathway, and then through a β barrel protein in the outer membrane. The secreted protein either remains on the outer surface of the outer membrane, or is cleaved and released into the extracellular space. T5SS exoproteins found in *P. aeruginosa* include EstA, LepA, and CupB5. EstA appears to facilitate the production of rhamnolipid surfactant and subsequent swarming motility.[246] The protease LepA transactivates nuclear factor-kappa B (NF-κB)-driven promoter, and induces IL-8 production in human bronchiole epithelial cells *in vitro*.[247] CupB5 appears to be associated with *P. aeruginosa* fimbriae.[248]

Type 6 secretion systems (T6SS) are comprised of a puncturing device and sheath, enabling injection of proteins into targeted prokaryotic and eukaryotic cells.[249] Three T6SS have been identified in *P. aeruginosa*, termed H1-, H2-, and H3-T6SS.

These T6SS each secrete several different effector proteins, mostly targeted at other bacteria.[250]

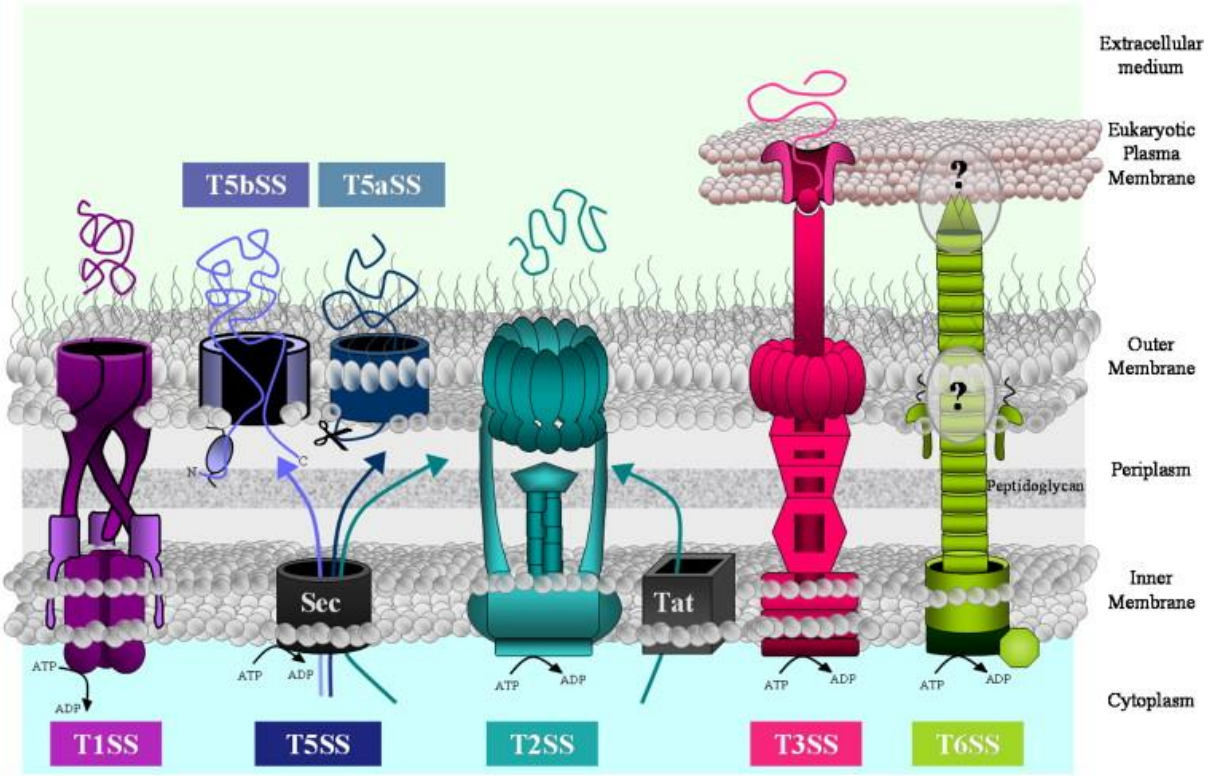



*Figure 7. Schematic diagram of protein secretion systems in P. aeruginosa – reproduced from Bleves et al, with permission from Elsevier* [221]

Quorum sensing is cell-cell communication within a bacterial species, via the production and release of signal molecules. A threshold concentration of the signal molecule sensed by the bacteria will trigger changes in gene expression. Lux-type quorum sensing systems are based on the production of N-acylhomoserine lactone (AHL), which can diffuse across cell membranes, and a transcriptional regulator receptor in the bacterial cytoplasm. Two lux-type systems, *las* and *rhl*, regulate virulence in *P. aeruginosa*, with strains lacking both *las* and *rhl* having greatly decreased virulence in animal models of pneumonia

and burn infection.[251-253] A quinolone quorum sensing system is also found in *P. aeruginosa*.[254]

The excreted pigments pyocyanin and pyoverdin may also be considered virulence factors. Pyocyanin is able to increase intracellular levels of reactive oxygen species, leading to cell lysis and release of extracellular DNA.[255, 256] Pyocyanin accelerates neutrophil apoptosis and impairs macrophage phagocytosis *in vitro* and in murine models of acute pneumonia.[257-259] Aside from helping the bacteria evade host immune cells, this may also lead to increased viscosity of mucus due to the release of high molecular weight DNA, impairing mucociliary clearance.[260] Pyocyanin has also been found to cause a concentration-dependent decrease in IL-2 secretion, IL-2 receptor expression on T lymphocytes, and B lymphocyte differentiation into plasma cells.[261, 262]

Pyoverdin is a siderophore, assisting iron uptake by chelating extracellular iron and enabling uptake by an outer membrane transporter.[263] It has also been shown to regulate production of exotoxin A and an extracellular endoproteinase, PrpL.[264]

Alginate production by mucoid strains of *P. aeruginosa* is generally an adaptation that occurs in chronic infection, typically in cystic fibrosis lung colonisation.[265, 266] Alginate not only impairs phagocytosis and opsonisation, but may also alter the host immune response.[267-273] *In vitro* studies show alginate-induced impairment in neutrophil chemotaxis and lymphocyte transformation.[274] The potential for alginate to enhance adhesion of *P. aeruginosa* to epithelial surfaces has been

explored, with conflicting results. Doig et al demonstrated a wide variation in the adherence of mucoid strains to buccal and tracheal epithelium.[275]

### 1.2.3 Antimicrobial resistance

#### 1.2.3.1 Mechanisms of intrinsic antimicrobial resistance

Many factors contribute to the ability of *P. aeruginosa* to evade or resist antimicrobial treatments. These factors include biofilm formation, innate antibiotic resistance, and acquired antibiotic resistance.

Intrinsic resistance refers to the mechanisms limiting the activity of antibiotics in wild-type isolates. The outer membrane of gram negative bacteria is a barrier to the penetration of antibiotics, particularly to small hydrophilic antibiotics such as beta-lactams and quinolones, which must pass through water-filled porin channels. *P. aeruginosa* isolates with greatly decreased outer membrane permeability compared to other gram negative bacteria have been described, although this cannot fully explain the species' intrinsic resistance to many antibiotics.[276, 277] Low outer membrane permeability works in tandem with other mechanisms of intrinsic resistance, such as efflux pumps and beta-lactamase.[278] Intrinsic drug efflux pumps found in *P. aeruginosa* include MexAB-OprM and MexXY-OprM.[279, 280] Both are able to extrude tetracycline and erythromycin, whilst the former can also extrude quinolones, other macrolides, chloramphenicol and most beta-lactams, and the latter can extrude aminoglycosides.[279, 281, 282] AmpC is a chromosomally-encoded inducible beta-lactamase that is able to hydrolyse most beta-lactams, with the exception of fourth generation cephalosporins and carbapenems.[207, 283]

Biofilm formation comprises four main stages, illustrated in Figure 8. Firstly, bacteria adhere to a biotic or abiotic surface. Secondly, the bacteria begin to secrete an extracellular matrix, usually consisting of exopolysaccharides, proteins, and extracellular DNA. Thirdly, the biofilm matures in a coordinated fashion, often utilising quorum sensing systems. Subpopulations of cells in different regions of the biofilm may appear. Lastly, planktonic cells are dispersed from the biofilm.

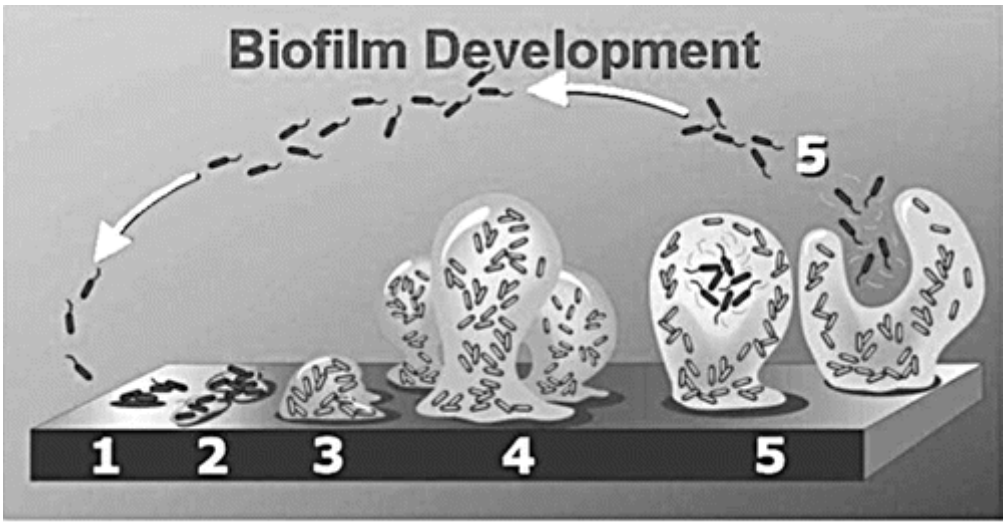



*Figure 8. Diagram showing the stages of biofilm formation – reproduced with permission from Stoodley et al [(153)]: 1. Attachment of bacteria to surface, 2. Secretion of exopolysaccharides, 3 & 4. Development and maturation of biofilm architecture, 5. Dispersion of single bacteria from the biofilm.*

The ability of biofilms to tolerate antimicrobial treatments occurs via numerous mechanisms. These involve both the extracellular matrix, as well as changes in the embedded bacteria as they adopt the biofilm lifestyle. Extracellular DNA and mucin found in the sputum of cystic fibrosis patients has been shown to

electrostatically bind positively-charged antibiotics and antimicrobial peptides, such as aminoglycosides and polymyxins.[284-286] Similarly, Chiang et al found that the addition of exogenous DNA to flow-cell biofilms of a DNA release-deficient *P. aeruginosa* mutant increased tolerance to aminoglycoside treatment.[287] Extracellular DNA has also been shown to induce expression of *pmr* genes *PA3552-PA3559* in *P. aeruginosa* biofilms, resulting in decreased susceptibility to cationic antimicrobial peptides.[288] The same investigators also noted decreased susceptibility to aminoglycosides. Alginate production by biofilms of mucoid *P. aeruginosa* has been demonstrated to decrease susceptibility to tobramycin, compared to a non-alginate producing isogenic strain.[289]

Differential metabolism between bacteria in different regions of a biofilm may also contribute to increased tolerance to antimicrobials. Several studies have demonstrated that metabolic activity is highest in parts of the biofilm that are closest to the bulk medium, and lowest deep within the biofilm.[290-294] It has been theorised that bacteria with low growth rates are less susceptible to antibiotics that target cellular growth processes such as cell wall synthesis, transcription and translation. However, the interaction between metabolic activity and antimicrobial tolerance varies depending on the antimicrobial agent in question. Whilst some antibiotics, such as tobramycin, ciprofloxacin and tetracycline, are less effective in killing the less metabolically active bacteria within a biofilm, colistin and chlorhexidine appear to have greater activity against these bacteria.[290, 294, 295] *In vitro* studies of *P. aeruginosa* biofilms have shown that a subpopulation of metabolically active cells develops colistin tolerance by

induced expression of *pmr* genes, which mediate modification of cell membrane lipopolysaccharide.[294, 296]

Persister cells are bacteria that have differentiated into a dormant non-dividing or slowly-dividing state, as opposed to simply having slower growth due to limitation of oxygen or nutrients. Evidence of persister cells has been described in numerous *in vitro* studies of antimicrobial tolerance in *P. aeruginosa* biofilms.[295, 297, 298] Several genes that appear to relate to the development of the persister phenotype in *P. aeruginosa* have been identified, including genes related to mucoid conversion, twitching motility, phenylalanine synthesis, and type IV pili.[299] Genes regulating bacterial stress response, such as *spoT*, *relA*, *dksA*, and *rpoS*, have also been implicated as persister genes in *P. aeruginosa*.[300, 301]

Several other genes that may contribute to antimicrobial tolerance have been found to be upregulated in *P. aeruginosa* biofilms. Increased expression of drug efflux pumps including MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MuxABC-OpmB, has been identified in *P. aeruginosa* biofilms. Increased expression of *mexAB-oprM*, *mexCD-oprJ* and *muxABC-opmB* genes in the upper layer of *P. aeruginosa* PA01 strain biofilms appears to be necessary in the development of colistin tolerance, in conjunction with the aforementioned *pmr* genes.[294, 302] *MexAB-oprM* and *mexCD-oprJ* are also required for biofilm formation in the presence of sub-inhibitory concentrations of azithromycin, with an increase in *mexC* expression within these biofilms.[303] Interestingly, Gillis et al noted that the expression of both *mexAB-oprM* and *mexCD-oprJ* did not increase tolerance to

other substrate antibiotics, as might be expected.[303] Liao et al found that MexAB-OprM and MexEF-OprN contribute to *P. aeruginosa* biofilm tolerance to tobramycin, trimethoprim, tetracycline, and kanamycin.[304]

The *ndvB* gene appears to be expressed only in *P. aeruginosa* PA14 strain biofilms, and not in planktonic cells of the same strain. This gene encodes for an enzyme involved in the synthesis of periplasmic glucans that are able to bind and sequester tobramycin. A PA14 strain carrying a *ndvB* mutation was found to have decreased biofilm tolerance to tobramycin, gentamicin, ciprofloxacin, ofloxacin, and chloramphenicol.[305]

#### 1.2.3.2 Mechanisms of acquired antimicrobial resistance

Mechanisms of acquired antimicrobial resistance in *P. aeruginosa* predominantly involve antimicrobial-modifying enzymes, usually acquired via plasmids, transposons or integrons. These include narrow- and extended-spectrum beta-lactamases (ESBL), oxacillinases, and aminoglycoside-modifying enzymes. Narrow-spectrum beta-lactamases confer resistance to penicillins and narrow-spectrum cephalosporins, with some enzymes in this category able to be inhibited by clavulanic acid.[306, 307] ESBL are also able to hydrolyse third and fourth generation cephalosporins, and in some cases, aztreonam or carbapenems.[308] Oxacillinases, also known as OXA-type beta-lactamases, may have narrow- or broad-spectrum activity against beta-lactams.[309, 310] OXA-type beta-lactamases that are able to hydrolyse carbapenems have been identified, although reports of *P. aeruginosa* isolates expressing these enzymes are sporadic.[311-313] Carbapenem resistance may also be conferred by

metallobetalactamases, including VIM, IMP and NDM enzymes, and *Klebsiella pneumoniae*-type carbapenemase (KPC).[314-316] Metallobetalactamases have been identified in isolates responsible for single-hospital outbreaks of nosocomial *P. aeruginosa*, as well as in the widespread multidrug-resistant *P. aeruginosa* serotype O12.[317, 318] Aminoglycoside-modifying enzymes include aminoglycoside nucleotidyl-transferase (2')-I and aminoglycoside acetyltransferase (6')-II, which confer resistance to gentamicin, tobramycin, and in the case of the latter, netilmicin.[319]

Another mechanism of acquired aminoglycoside resistance in *P. aeruginosa* is methylation of 16S rRNA, preventing the antibiotic from binding to the rRNA. RmtA and RmtD are 16S rRNA methylases that have been identified in *P. aeruginosa*, likely acquired through intergeneric lateral gene transfer from aminoglycoside-producing bacteria.[320, 321] The former was identified in an isolate with high-level resistance to all parenteral aminoglycosides, whilst the latter confers resistance to amikacin, tobramycin, and gentamicin.[320, 321]

Drug efflux pumps MexCD-OprJ and MexEF-OprN have been identified in isolates with acquired resistance to fluoroquinolones.[322, 323] Point mutations in the genes encoding for DNA gyrase may also decrease the binding affinity of quinolones for the enzyme, and result in resistance.[324-326]

#### 1.2.3.3 Epidemiology of antimicrobial resistance

Antibiotic classes traditionally used to treat *P. aeruginosa* infections include some penicillins, cephalosporins, fluoroquinolones, aminoglycosides and

carbapenems. Unfortunately, the prevalence of antibiotic resistance and multi-drug resistant strains is increasing. The SENTRY Antimicrobial Surveillance Program is a global antimicrobial surveillance study that collects clinical isolates and performs centralised antimicrobial susceptibility testing, with the aim of collecting prevalence-based data. SENTRY data from patients hospitalised with *P. aeruginosa* pneumonia in the United States and the Europe and Mediterranean region between 2009 and 2012 showed meropenem resistance rates of 25% in Europe and 16% in the USA. Levofloxacin resistance was found in 29% and 21% in Europe and USA respectively.[327] SENTRY data from the Latin America region for 2008 to 2010 for all *P. aeruginosa* isolates showed resistance rates of 25%, 41%, and 34% to piperacillin/tazobactam, ciprofloxacin, and meropenem respectively.[328] Rates of carbapenem resistance in *P. aeruginosa* clinical isolates from around the world are shown in Figure 9. In Australia, rates of antibiotic resistance in *P. aeruginosa* clinical isolates remain comparatively low, ranging from 4.0% resistance to meropenem, to 6.7% to ciprofloxacin and 10.3% to piperacillin/tazobactam.[329] However, emerging concerns include the appearance of carbapenemase-expressing gram negative bacteria throughout the country, albeit at low rates.[329]

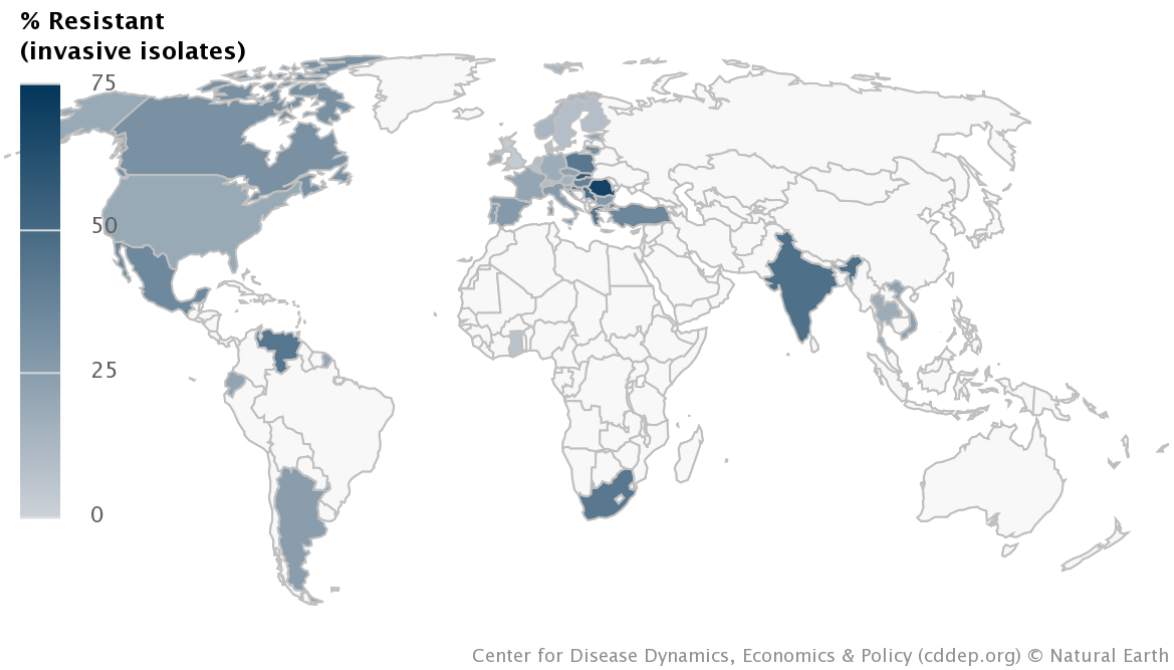



*Figure 9. Map showing rates of carbapenem-resistant P. aeruginosa by country – from ResistanceMap, Center for Disease Dynamics, Economics & Policy* [(330)]

Multidrug resistance (MDR) is defined as acquired non-susceptibility to at least one agent in each of three or more antimicrobial categories, whilst extended drug resistance (XDR) is non-susceptibility to at least one agent in all but two or fewer antimicrobial categories.[(331)] Data from the International Network for Optimal Resistance Monitoring program (INFORM) collected between 2012 and 2015 found that 15.4% and 9.4% of USA *P. aeruginosa* clinical isolates were MDR and XDR respectively.[(332)] Data from the US Centers for Disease Control and Prevention (CDC) shows that *P. aeruginosa* is responsible for 51000 healthcare-associated infections per year in the USA, with 13% of these being caused by multi-drug resistant strains.[(333)]

Recent global data on the prevalence of antibiotic resistance in *P. aeruginosa* isolates from CRS patients is scarce. However, single-centre studies performed in North America provide some insights. Kingdom and Swain (USA) reported 26% aminoglycoside (gentamicin and tobramycin) and 17% ciprofloxacin resistance in *P. aeruginosa* isolates from CRS patients undergoing ESS between 1997 and 2001.[334] Guss et al (USA) examined the prevalence of fluoroquinolone-resistant *P. aeruginosa* in bacterial sinus cultures between 2002 and 2007. The authors reported 13% and 5% of isolates were resistant to levofloxacin and ciprofloxacin respectively.[335] Genoway et al (Canada) found resistance rates in *P. aeruginosa* isolates from CRS patients over a 13-month period of 0% to gentamicin, 30% to ciprofloxacin, and 50% to meropenem.[336] The results of these studies must be interpreted with caution, as with the exception of Guss et al's study, the number of *P. aeruginosa* isolates examined in each study was small.

### 1.2.4 Significance of *Pseudomonas aeruginosa* in respiratory diseases

#### 1.2.4.1 Role of *Pseudomonas aeruginosa* in chronic rhinosinusitis

As described previously, *P. aeruginosa* has been identified in a significant proportion of CRS sufferers' sinuses through both culture-based and molecular methods. The prevalence of *P. aeruginosa* colonisation in the sinonasal mucosa of healthy humans varies from 0% to 4%, although estimates as high as 9% have been derived from 16S rRNA sequencing studies.[133, 337-340] The reported proportion of CRS patients with cultures identifying *P. aeruginosa* varies from 7% to 15%, but prevalence of *P. aeruginosa* in CRS microbiome studies using molecular detection methods is as high as 21%.[133, 140, 143, 334, 336, 341-343]

Importantly, Cleland et al's microbiome study noted that *P. aeruginosa* had a high mean relative abundance in CRS patients compared to healthy control patients.[133]

In a cross-sectional study of 265 CRS patients, Nadel and colleagues found prior sinus surgery, use of systemic steroids, and nasal irrigations were associated with higher rates of cultures positive for *P. aeruginosa*.[142] These associations may either imply that these treatments introduce or increase the pathogenicity of *P. aeruginosa* in CRS, or conversely suggest that patients with cultures positive for *P. aeruginosa* have more severe disease, and are thus more likely to receive the aforementioned interventions. Cleland et al's finding that higher relative abundance of *P. aeruginosa* is associated with lower disease-specific health related quality of life supports the latter conjecture.[133]

The association between *P. aeruginosa* and the need for revision sinus surgery is unclear. Numerous studies have also noted an increased frequency of *P. aeruginosa*-positive cultures from patients undergoing revision sinus surgery.[140, 142, 143, 344] Conversely, Kingdom and Swain's study of 101 CRS patients did not find any significant difference in the proportion of patients or cultures that grew *P. aeruginosa* between primary and revision surgery groups.[334] Studies attempting to define the sinus microbial flora of CRS patients post-ESS have identified a variable proportion of patients with *P. aeruginosa* positive cultures. Al-Shemari and colleagues reported *P. aeruginosa* in 3% and 24% respectively of asymptomatic and symptomatic groups of such patients.[144] A similar study by Bhattacharyya and Kepnes found *P. aeruginosa* in 7.2% of cultures from

symptomatic post-ESS CRS patients.[341] None of these studies were able to compare post-operative with pre-operative or intra-operative culture results, and thus the significance of these findings is unclear. Further longitudinal studies are required to elucidate whether the presence of *P. aeruginosa* in CRS is predicative of a subgroup of patients who will have recurrent or recalcitrant disease following ESS.

#### 1.2.4.2 Role of *Pseudomonas aeruginosa* in cystic fibrosis

Cystic fibrosis is an inherited disorder, resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The CFTR protein functions as a chloride ion channel, and also regulates other cellular ion channels. The altered function of these channels causes decreased water content, and thus increased viscosity, of the mucus that is found in the respiratory, gastrointestinal, and reproductive systems. The resulting changes in the lung microenvironment predispose CF patients to recurrent lung infections, with *P. aeruginosa*, *Burkholderia cepacia*, and *Achromobacter xylosoxidans* being common Gram-negative pathogens. CRSwNP is considered to be one of the disease characteristics of CF. One study found that 100% of paediatric and adult patients with CF had CRS symptoms, with 96% of patients having nasal polyps visualised on endoscopic examination.[345] Patients with CF are likely to have more severe CRS compared to those without CF, as measured by changes on nasendoscopy, computed tomography, and increased olfactory thresholds. [345, 346]

*P. aeruginosa* is frequently isolated from the sinuses of patients with cystic fibrosis (CF), with reported rates of 18.5% to 49% in cultures taken during

ESS.[347-352] *P. aeruginosa* colonisation of the paranasal sinuses has been shown to be a reservoir of recurrent pulmonary infections in CF patients. [350, 353-355] There is also evidence supporting the hypothesis that *P. aeruginosa* infections are acquired from environmental sources. Genotyping studies of isolates from CF patients have shown that initial pulmonary infections are caused by a diversity of genotypes, reflecting the variation present in the environment. Subsequent infections have been caused by a clonal (isogenic) isolate in some cases, whilst in other cases a different genotype was identified. This suggests both environmental sources, as well as a reservoir of persistent bacterial colonisation such as the sinuses, as a source for recurrent pulmonary infections.[356-358]

Chronic pulmonary infection by *P. aeruginosa* in CF is of particular concern, as this has been associated with deterioration in lung function.[359] Interestingly, chronic pulmonary infections are often caused by the same strain, meaning that serial isolates from a chronically infected patient are isogenic.[356, 360] However, phenotypic changes may occur over time, which can affect the strain's expression of virulence factors.[360] More specifically, the conversion of the colonising strain to a mucoid phenotype is correlated with a significant increase in morbidity and mortality.[361] This phenotypic progression may not be limited to the lower respiratory tract. Hansen and colleagues discovered diversification of strains in the sinuses of paediatric CF patients, including the acquisition of microbiological traits that are known to correlate with poor lung disease prognosis.[362]

# 1.3 Bacteriophages

## 1.3.1 Definition and taxonomy

Bacteriophages, or phages, are viruses that infect bacteria. They are considered to be the natural predators of bacteria, and evidence of these naturally-occurring viruses was first documented in the early 1900s. Twort identified microorganisms that were able to pass through a filter, and appeared to "dissolve" or prevent the growth of bacteria under certain conditions.[363] D'Herelle made similar observations of a microorganism isolated from the faecal samples of patients with *Shigella* dysentery, and termed these microorganisms "bacteriophages".[364] Phages may have either single-stranded or double-stranded DNA or RNA genomes. Electron microscopy has provided evidence of diverse phage morphology; phages may be enveloped or non-enveloped, with tailed, polyhedral, filamentous, or pleomorphic-shaped particles.[365] The current International Committee on Taxonomy of Viruses (ICTV) taxonomy classifies phages into one order, 14 families, and 37 genera, with several other families under consideration (Table 1).[365, 366] The majority of characterised phages are tailed, and fall under three families in the Caudovirales order: Myoviridae, Siphoviridae, and Podoviridae.[367]

| Shape | Nucleic acid | Family | Genera | Particulars | Example | Members |
|---|---|---|---|---|---|---|
| Tailed | dsDNA (L) | *Myoviridae* | 6 | Tail contractile | T4 | 1,320 |
| | | *Siphoviridae* | 7 | Tail long, noncontractile | λ | 3,229 |
| | | *Podoviridae* | 4 | Tail short | T7 | 771 |
| Polyhedral | ssDNA (C) | *Microviridae* | 4 | Conspicuous capsomers | $\phi$X174 | 40 |
| | dsDNA (C, S) | *Corticoviridae* | 1 | Complex capsid, lipids | PM2 | 3? |
| | dsDNA (L) | *Tectiviridae* | 1 | Double capsid, lipids, pseudo-tail | PRD1 | 19 |
| | dsDNA (L) | SH1* | | Double capsid, lipids | SH1 | 1 |
| | dsDNA (C) | STIV* | | Turret-shaped protrusions | STIV | 1 |
| | ssRNA (L) | *Leviviridae* | 2 | Poliovirus-like | MS2 | 39 |
| | dsRNA (L, M) | *Cystoviridae* | 1 | Envelope, lipids | $\phi$6 | 3 |
| Filamentous | ssDNA (C) | *Inoviridae* | 2 | Long filaments, short rods | M13 | 67 |
| | dsDNA (L) | *Lipothrixviridae* | 4 | Envelope, lipids | TTV1 | 7 |
| | dsDNA (L) | *Rudiviridae* | 1 | Stiff rods, TMV-like | SIRV-1 | 3 |
| Pleomorphic | dsDNA (C, S) | *Plasmaviridae* | 1 | Envelope, no capsid, lipids | L2 | 5 |
| | dsDNA (C, S) | *Fuselloviridae* | 1 | Lemon-shaped, envelope, lipids? | SSV1 | 11 |
| | dsDNA (L, S) | — | 1** | Lemon-shaped, envelope | His1 | 1 |
| | dsDNA (C, S) | *Guttaviridae* | 1 | Droplet-shaped | SNDV | 1 |
| | dsDNA (L) | Ampullaviridae* | | Bottle-shaped, helical NC | ABV | 1 |
| | dsDNA (C) | Bicaudaviridae* | | Two-tailed, development cycle, helical NC | ATV | 1 |
| | dsDNA (L) | Globuloviridae* | | Envelope, spherical, lipids, helical NC | PSV | 1 |

C, circular; L, linear; M, multipartite; NC, nucleocapsid; S, supercoiled; —, no name; *, nonclassified; **, genus *Salterprovirus*. Members indicate numbers of phages examined by electron microscopy, excluding phage-like bacteriocins and known defective phages (based on computations from January 2006; from reference 13).

*Table 1. Bacteriophage phage families and associated characteristics – reproduced with permission from Ackermann [(365)]*

### 1.3.2 Structure of viral particles

The structures of the tailed *Escherichia coli* "T-even" phages (T2, T4, and T6) are some of the most studied. The typical structure of *Myoviridae* tailed phage particles is illustrated in Figure 10. Tailed phages have a quasi-icosahedral capsid or head, comprised of multiple capsid proteins.[368] The head contains the phage double-stranded DNA genome. The tail of the phage is attached to the head by the connector, comprised of additional proteins.[369] T-even and other *Myoviridae* phages have complex tail structures that are able to contract prior to DNA injection into the bacterial host. The tail is formed by a contractile sheath, a base plate wedge and hub, whiskers and long tail fibres, the latter two of which are attached to the base plate.[370] Other tailed phages have either short or long non-contractile tails.[367]

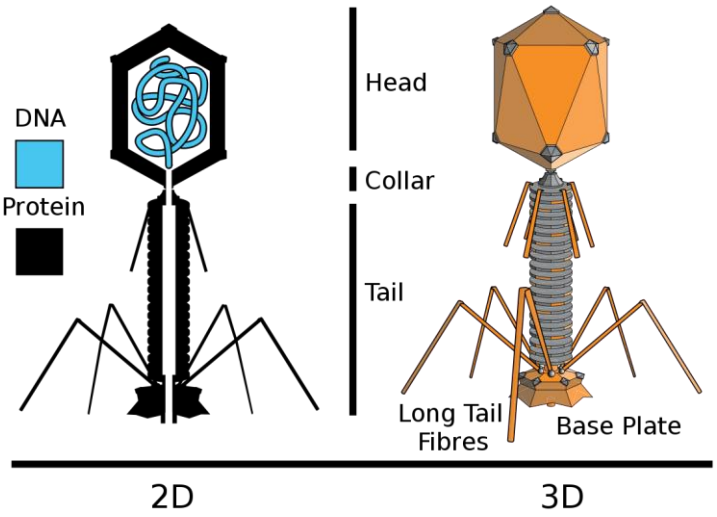



*Figure 10. Myoviridae T4 phage structure - By Adenosine (original); en:User:Pbroks13 (redraw), reproduced under Creative Commons BY-SA 2.5 licence, https://commons.wikimedia.org/w/index.php?curid=4128278*

### 1.3.3 Bacteriophage life cycles

Whilst phage genomes contain all the genetic information necessary for self-replication within a host bacterium, phages themselves have no internal method of energy generation or protein production. Thus bacteriophage replication is dependent on their ability to infect host bacteria.[371] Similar to other viruses, both lytic and lysogenic replication cycles have been identified in phages. Obligate lytic phages are only able to replicate via the lytic pathway, whereas temperate phages may undergo either lytic or lysogenic cycles. The lytic phage life cycle is illustrated in Figure 11, and the various possible outcomes of temperate phage infection are shown in Figure 12. In both cycles, the initial step is adsorption of the phage to a surface structure on a potential host cell. This is a two-step process, in which the first stage is a reversible attachment, whereas the second stage is irreversible binding. The host cell membrane is then breached, and phage nucleic acids are transported into the host cell. In tailed phages, adsorption occurs by attachment of tail fibres to pili, flagella, or other host cell surface structures. The bacterial cell wall is then digested by enzymes located in the tail tip, allowing injection of the phage DNA into the host.[372] Gene expression, formation of capsid and tail components, phage genome replication, and packaging of the phage genome into capsids, may commence at this stage, by redirecting the host cell replication "machinery" and metabolism.[373]

In lytic phages, the mature progeny phages are released into the extracellular space by lysis of the host bacterium, killing the bacterium in the process. Tailed phages produce holins and endolysins (peptidoglycan hydrolases), which

penetrate the plasma membrane and damage cell wall peptidoglycan respectively.[372]

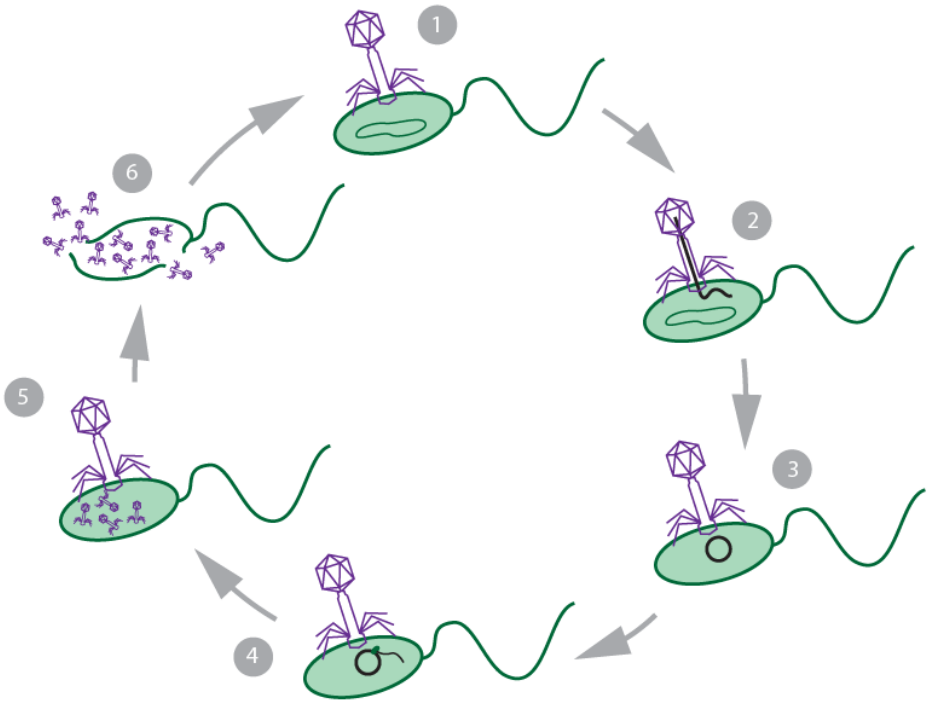



*Figure 11. Lytic phage life cycle: 1. Adsorption of phage to host, 2 & 3. Injection of phage genome into host, 4. Transcription and translation of phage genome using host cell machinery, 5. Formation of phage particles within host, 6. Release of phage particles by host cell lysis.*

In a lysogenic cycle, the phage genome remains within the host cell in a dormant state, typically integrated into the host cell genome, and is replicated when the host cell replicates. The dormant phage genome may also persist within the bacterial cell as a plasmid. Temperate phages have the ability to choose either a lysogenic or lytic cycle; they may initially choose a lysogenic cycle, but conversion to a lytic cycle can occur later.[372, 373] A state of chronic infection may also occur,

where mature phages are released from the host cell by budding or extrusion, without lysing the host cell.[373]

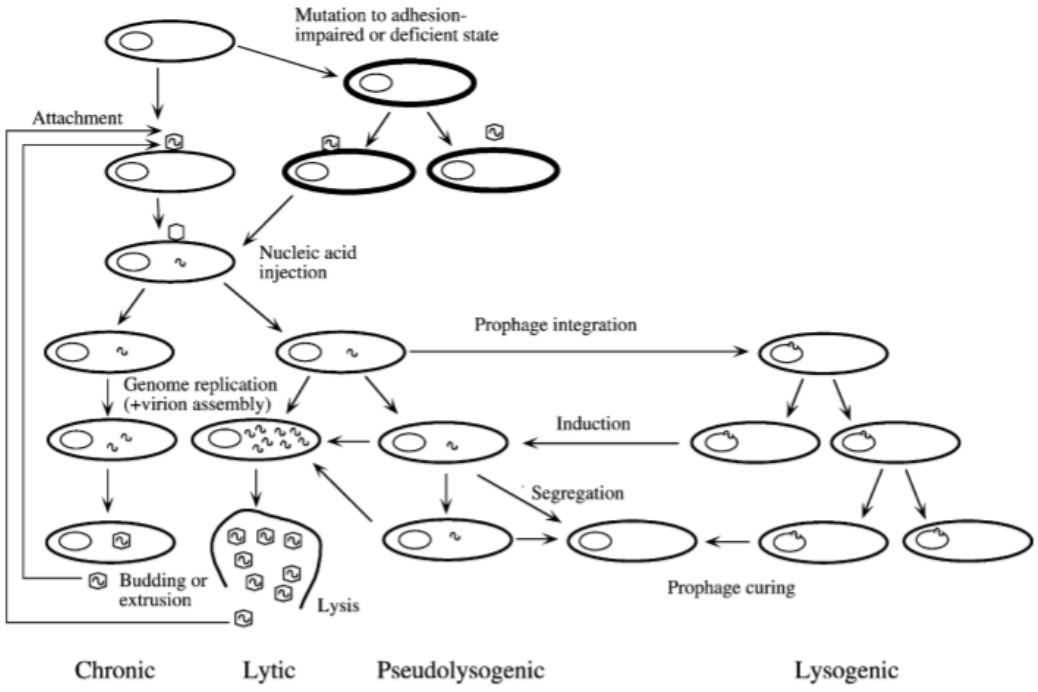



*Figure 12. Bacteriophage life cycles: chronic infection, lytic, lysogenic, and pseudolysogenic. Adapted from Paul et al[374] by Weinbauer[373]. Reproduced with permission from Federation of European Microbiological Societies and Elsevier.*

## 1.3.4 Considerations in the therapeutic use of bacteriophages

### 1.3.4.1 Lytic versus lysogenic phages

Lytic bacteriophages have obvious therapeutic potential in treating infections with their ability to lyse and kill bacteria. Whilst the majority of therapeutic research and use of phages has been focussed on lytic phages, non-lytic filamentous phages have been used to introduce antibacterial agents into bacteria or to induce increased sensitivity to antibiotics. Several studies have

shown the bacterial killing ability of filamentous phages genetically engineered to encode lethal proteins such as restriction endonuclease, holin, modified catabolite gene activator protein, and toxins Gef and ChpBK.[375-378] The suggested advantages of this approach include minimising endotoxin release.[375, 376] Lu and Collins demonstrated the use of a filamentous phage genetically modified to suppress a mechanism of *E. coli* defence against bactericidal antibiotics, subsequently increasing the sensitivity of *E. coli* to ofloxacin, gentamicin, and ampicillin.[379]

#### 1.3.4.2 Host range

The group of bacterial strains or species that a phage is able to infect is termed its "host range". The majority of characterised phages have a host range limited to a subset of strains within a single or closely-related bacterial species; however polyvalent phages that can infect multiple bacterial species have been identified.[380, 381] The limited host range of many phages may be beneficial for preserving commensal bacterial flora when targeting a single pathogenic strain. Conversely, the host range may restrict the application of phage therapy when multiple strains or species may cause an infection or disease. Proposed solutions to this problem include the use of a cocktail of different phages, isolation of polyvalent phages, and screening of bacterial clinical isolates against panels of phages to select the most appropriate phage for treatment.[382]

### 1.3.4.3 Predicting the effect of bacteriophage therapy on a bacterial population

Whilst traditional pharmacokinetic and pharmacodynamic parameters can be used to predict the concentration- or time-dependent bacterial killing of a dose of a conventional antibiotic, modelling lytic phage infection and replication in a population of bacteria is significantly more complex. Approaches that have been used include empirical methods such as quantifying net changes in phage abundance, measuring phage nucleic acid synthesis by radiolabelling, or measuring the rate of decay in the phage population under steady state conditions, as well as mathematical modelling based on estimates of rate of phage adsorption, time between infection and lysis, and burst size.[383-386]

Regarding the bacterial killing effect of a single dose of lytic phages on a bacterial population, active and passive modes of treatment have been defined. Passive treatment is bacterial killing mediated by the phages present in the dose, whilst active treatment refers to ongoing bacterial infection and killing by the progeny phage that are released following phage replication within the initially infected bacteria in the sample. Multiplicity of infection (MOI) is often defined as the number of virions (or phages) that are added per potential host cell during infection, although the literal definition is the number of adsorbed phages per potential host cell.[387] In practice, passive treatment implies a high MOI, and active treatment a lower MOI.

Abedon has proposed phage therapy equivalents of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), parameters which are commonly used in dosing metrics for conventional antibiotics.[388]

Abedon argues that MBC is a function of initial bacterial density, volume of the treatment environment, and time elapsed since treatment. The proposed model allows for stochastic phage-bacteria interactions, and adsorption of multiple phages to a bacterium. Calculations for dosing intervals and time until bacterial eradication are also proposed. Whilst Abedon takes into account decay (loss) of phages and limitations to phage proliferation, he does not address the development of, or selection for, bacteria that are resistant to the phage.

It is evident that a plethora of highly complex models can be derived in order to predict optimal phage dosing. However, the practical application of these models is limited by the variable characteristics of phage-bacteria interactions when using different phages or bacterial strains, as well as the fact that the quantity of bacteria present in a clinical infection is rarely known.[382, 389] Despite this, mathematical models may be useful in providing estimates of effective doses in order to prevent or explain treatment failure.[388]

#### 1.3.4.4 Bacterial resistance to bacteriophage infection

Mechanisms of bacterial resistance to phage infection can be found at all stages of phage infection, from adsorption to translation of the phage genome.

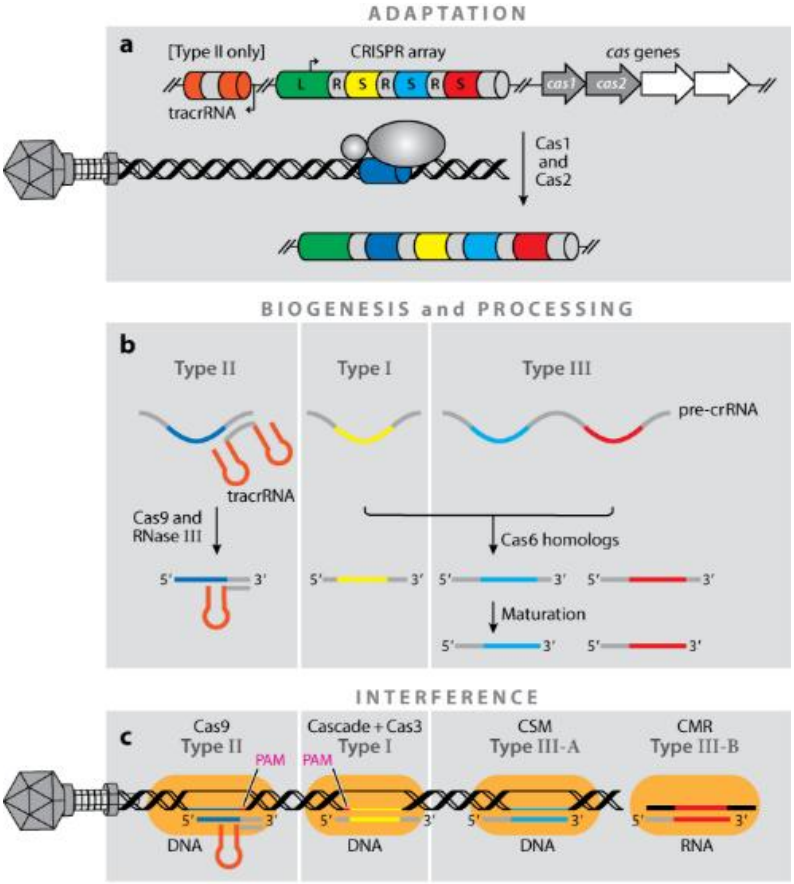
Resistance may occur due to lack of receptor expression on the bacterial cell surface.[390] This may be mediated by prophages; for example, *Salmonella* ε-prophages encode for enzymes that modify cell membrane polysaccharides, preventing infection by other phages.[391] Another approach is to mask the receptor; *E. coli* outer membrane lipoprotein TraT interacts with the T-even

phage receptor outer membrane protein A (OmpA) to inhibit phage adsorption.[392] Small molecules that exhibit competitive antagonist activity for cell surface receptors may also be produced by bacteria. *E. coli* produces microcin J25 (MccJ25) under nutrient limitation, which blocks FhuA, an iron transporter and receptor for T5 phage.[393]

Superinfection exclusion (Sie) systems are able to block phage DNA injection or translocation, and have been identified in *E. coli* and *Lactococcus lactis*.[394-396] Sie proteins are often encoded for by phages or prophages. The *E. coli* Sie protein Imm blocks DNA injection, whilst another Sie protein, Sp, inhibits phage base plate lysozymes.[394, 395]

Bacteria have a number of defence systems against mobile genetic elements (MGEs), such as phages. Restriction-modification (RM) systems are considered to be the bacterial innate immune system against MGEs. Restriction endonucleases are enzymes that recognise foreign DNA, and cleave within, near, or far from the recognition site. They work in conjunction with methyltransferases, which methylate and thus protect native DNA from cleavage. The earliest discovered restriction endonucleases, EcoB and EcoK, were identified in *E. coli* strains that were able to resist bacteriophage infection.[397, 398] Since these discoveries, more the 800 different restriction enzymes have been isolated from bacteria. CRISPR-Cas systems are a mechanism of adaptive immunity to MGEs. Clustered regularly interspaced short palindromic repeats (CRISPR) arrays consist of spacer sequences derived from the MGE, inserted between short repeats. Transcription of CRISPRs produces CRISPR RNAs, whilst expression of *cas* genes produces Cas

endonucleases. Together, these components recognise and degrade MGEs as illustrated in Figure 13.[399]




Dy RL, et al. 2014.
Annu. Rev. Virol. 1:307–31

*Figure 13. The three different types of CRISPR-Cas systems and their mechanisms for phage interference. CRISPR-based interference is divided into three sequential steps: a. adaptation or the acquisition of phage-derived spacers; b. CRISPR and cas expression, CRISPR RNA (crRNA) biogenesis, and processing; and c. crRNA-directed interference. a. The CRISPR array is composed of repeats (R) interspaced by spacers (S) and a leader (L) sequence. Typically, near the CRISPR array is the cas operon. Part of the phage DNA is incorporated as a spacer at the leader-proximal end of the CRISPR through the action of Cas proteins. Type II CRISPR-Cas systems also possess a trans-activating crRNA (tracrRNA) that aids crRNA processing and interference. b. Once transcribed, the long precursor crRNA (pre-crRNA) is processed into crRNA by Cas proteins and host RNase III (in the Type II*

*systems). c. The crRNA guides the Cas complex to complementary sequences (protospacers) in the phage genome and elicits their degradation. In Type I systems, Cas3 is required for degradation. Next to each protospacer is a protospacer-adjacent motif (PAM; pink) important for spacer acquisition and interference. Figure and caption reproduced with permission from Dy et al.*[400]

Finally, abortive infection (Abi) systems trigger the death of phage-infected bacteria, whilst often also blocking phage replication. They are usually dormant proteins that are activated by phage proteins. *E. coli* Abi protein RexAB causes cell membrane depolarisation, whilst similar Abi protein PifA causes the cell to leak ATP by disturbing membrane integrity.[401, 402] Other *E. coli* Abi systems, Lit and PrrC, block translation of phage and host proteins by cleaving an elongation factor and $tRNA^{Lys}$ respectively.[403, 404]

### 3.4.5 Bacteriophage immunogenicity and pharmacokinetics *in vivo*

The first demonstration of the immunogenic properties of phage in mammals was the identification of phage-neutralising antibodies in the serum of rabbits injected with phage lysates.[405] A high degree of inter-individual and inter-strain variability in antibody formation has been noted in rabbits and mice immunised with phage.[406, 407] The magnitude of the murine humoral immune response to fd phage appears to have genetic determinants and be dependent on thymus-derived cells.[406] In addition, some phages appear to have greater immunogenicity than others.[408, 409]

The specific phage antigen that the anti-phage antibody is directed towards has implications for its impact on the phage-bacteria interaction. Antibodies generated by phage antigens may have a range of functions other than neutralisation of infectivity. In T-even phages, both tail and head proteins have been identified as antigens.[410-412] Antibodies to the former are able to neutralise phage infectivity, and also induce phage aggregation and complement fixation. Conversely, antibodies to the head proteins do not appear to have infectivity neutralisation properties.[410-412] In studies of *Staphylococcus* phage serology in rabbits, temporal differences were noted in the development of infectivity neutralising and complement fixing antibodies following an initial intravenous injection of phage. In the response to repeated phage injections, the two different types of antibody developed in parallel to each other as displayed by Figure 14, consistent with a secondary humoral immune response.[413]

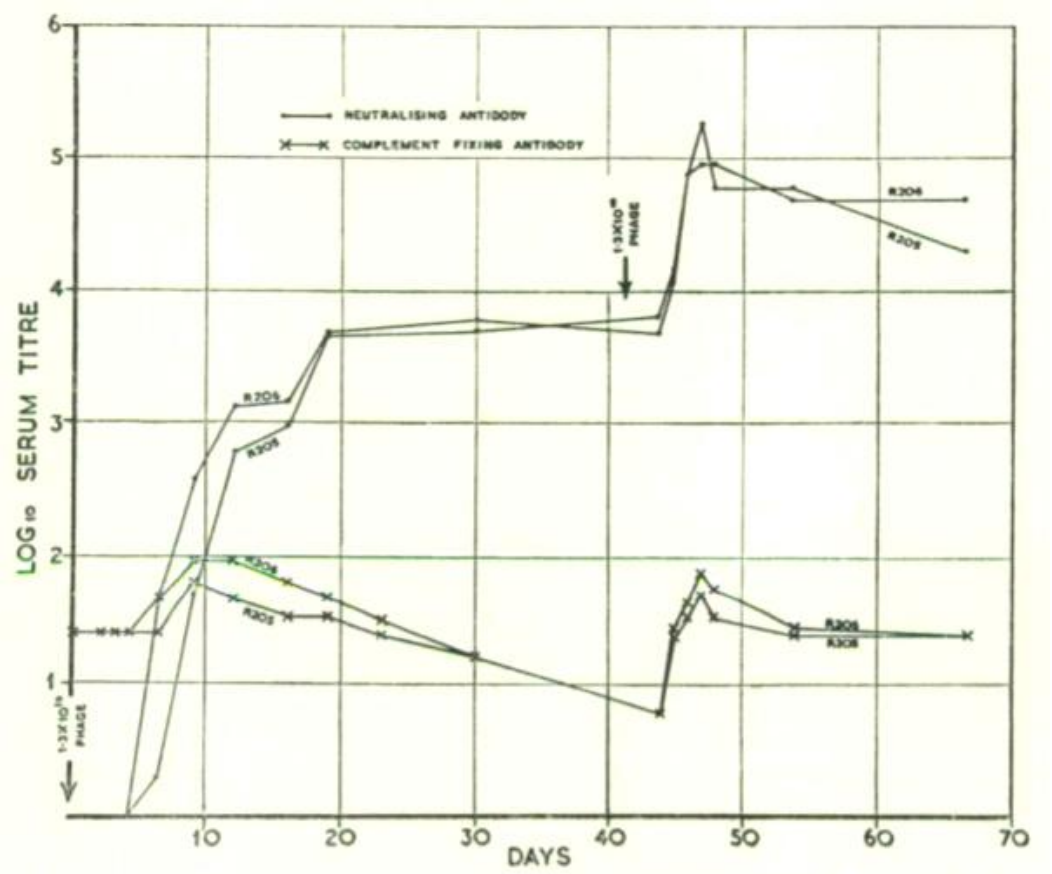



**Fig. 2.** Production of complement fixing and phage neutralizing antibody in rabbits R205 and R206, immunized with phage 3A.

*Figure 14. Reprinted by permission from Macmillan Publishers Ltd: Immunology and Cell Biology, Rountree*[413]*, © 1952*

The *in vitro* dynamics of antibody neutralisation of phage infectivity have been studied for various different phages. Studies by Andrewes and Elford of *Staphylococcus*, "*B. coli*" and "*B. dysenteriae*" phages showed that for a range of concentrations of phage particles exposed to a given dilution of anti-phage serum, a constant proportion of phage was neutralised.[408] Burnet et al examined the neutralisation of *Streptococcus* and *Staphylococcus* phages, and enterophage particles exposed to very high concentrations of anti-phage antibodies.[409] The authors describe a logarithmic inactivation of up to 99% of

phage particles; the remaining active phages appeared to be more resistant to neutralisation. Andrewes and Burnet and their colleagues, as well as d'Herelle, hypothesised that this proportional resistance to serological inactivation of phage infectivity was due to heterogeneity within the phage population.

*In vivo* studies comparing phage neutralising antibody production and phage elimination in mice following intravenous injection of *E. coli* phage ΦX174 noted increasing antibody titres from day 3 to day 14 post-injection.[414] An accelerated decrease in viable phage counts in the blood was observed at 32 to 35 hours post-injection, and coincided with the production of phage neutralising antibody. Whilst this pattern was observed in both germ-free and conventionally reared mice, a more rapid rate of phage elimination was seen in germ-free mice following injection of higher titres of phage ($10^8$ and $10^9$ PFU/mL). Only conventionally reared mice exhibited signs of endotoxaemia following injection of these titres, suggesting possible lytic effects of the phage on gut flora.

More recently, Łusiak-Szelachowska et al examined serum from 122 patients treated with phage therapy, as well as 30 healthy volunteers, for the presence of anti-phage antibodies.[415] Participants received at least 6 weeks of specific phage therapy for genitourinary, soft tissue, bone, or respiratory infection. Low levels of anti-phage activity were noted in the sera of healthy volunteers, and patients who received phage therapy via the oral route. 23% and 9% of patients who received locally-applied phage therapy had high and medium levels of anti-phage activity respectively in their sera during therapy. 7 of 15 patients with high levels of serum anti-phage activity experienced clinical improvement, indicating that

the development of anti-phage antibodies does not preclude successful treatment.

### 1.3.5 *Pseudomonas aeruginosa* bacteriophages

#### 1.3.5.1 Bacteriophage activity against *Pseudomonas aeruginosa in vitro*

Several investigators have isolated, characterised, and tested *P. aeruginosa* phages against various laboratory reference strains and clinical isolates, using a variety of methods. Given that different phages have been tested against highly variable panels of *P. aeruginosa* strains, it is difficult to generalise results from a single study. However, a number of studies have demonstrated phage activity against multidrug-resistant strains and strains from cystic fibrosis patients, in both planktonic and biofilm models.[416-421]

Whilst the minimum biofilm eradication concentration (MBEC) assay (Calgary biofilm device) or microtitre plate assays are the most commonly used methods to assess biofilm removal by phages, it is of note that some studies attempted to replicate *in vivo* conditions more closely. Fu et al found that pre- or post-treatment of hydrogel-coated urinary catheters with phage M4 resulted in a significant decrease in *P. aeruginosa* biofilm on the catheters.[422] Morales et al tested the activity of a phage cocktail in treating biofilms of *P. aeruginosa* CF isolates grown in an artificial sputum medium, in order to simulate CF respiratory tract conditions. Inhibition and reduction in biofilm up to 99% and 98% respectively were noted.[421]

Experiments by Barr et al observing the movement of fluorescent-labelled *E. coli* T4 phage in physiological mucin showed slower diffusion with increasing mucin concentration.[423] Closer examination of the diffusive movement revealed a subdiffusive pattern that actually increased adsorption rates of T4, compared to a T4 mutant (T4Δ*hoc*) that does not adhere to mucin. Barr et al's study did not examine whether this increased adsorption translated to increased bacterial killing, but does illustrate the ability of tailed phages to move through mucus, as would be found on a mucosal surface.

Several studies provide evidence that at least some *P. aeruginosa* phages are able to move through biofilms and exopolysaccharides. Hanlon et al examined the effect of phages on the viscosity of *P. aeruginosa* exopolysaccharide, with findings of up to 40% reduction in viscosity.[424] Phages were able to diffuse through the exopolysaccharides and resulted in a 1-log decrease in viable cells within the biofilm. Vilas-Boas et al have shown phage penetration of *P. aeruginosa* biofilms using locked nucleic acid (LNA) fluorescence in situ hydridisation (FISH).[425]

*In vitro* studies assessing the effect of potential *in vivo* limiting or inhibiting factors of bacteriophage therapy are relatively scarce. Knezevic et al examined the effect of environmental conditions, such as temperature, pH, carbohydrates, amino acids, exopolysaccharides, and lipopolysaccharide, on the activity of one *Podoviridae* and three *Siphoviridae* phages.[426] Findings for temperature and pH were highly variable dependent on the phage tested, although a very low pH of 1.5 significantly affected the viability of all phages. Maximum viability for all

phages was at approximately pH 7. At pH 3, viability varied from 15% to 70%, with viability of 50% to 75% at pH 5 (Figure 15). Adsorption decreased at lower temperatures for all phages, but the decrease was minimal in *Podoviridae* compared to other phages. All phages maintained activity after incubation in rat serum. Some of the phages were neutralised to varying degrees by carbohydrates, amino acids, lipopolysaccharides, and exopolysaccharides. These results have implications for the selection of appropriate routes of administration in clinical trials, and emphasise the importance of assessing these characteristics in phages being investigated for clinical use.

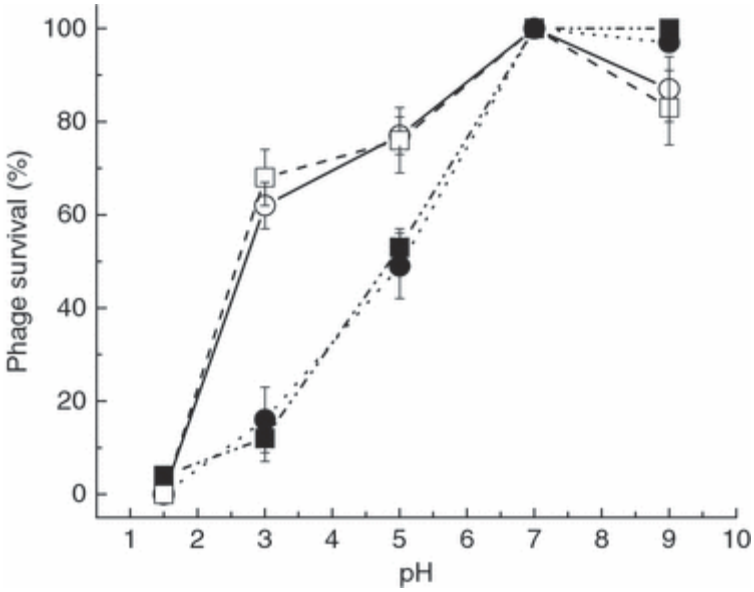



*Figure 15. Effect of pH on phage viability after 30 min at 37°C: δ (○); J-1 (•); σ-1 (□); and 001A (▪). Values are the mean ± SD of three determinations. Figure reproduced from Knezevic et al with permission from The Society for Applied Microbiology.*[426]

Krylov et al examined commercially available *P. aeruginosa* phage preparations (all produced in Russia) and identified five frequently isolated constituent phage

"species": PB1-, phiKMV-, PaP1-, KPP10-, and PaP3/LUZ24-like phages.[427] PB1-like phages are considered good candidates for phage therapy due mutations conferring extended lytic activity, absence of lysogenic effects, and a low rate of bacterial resistance.[428, 429] Whilst phiKMV-like phages exhibit high growth rates and a broad range of lytic activity in some cases, pseudolysogenic activity and incompatibility with other phages was detected, casting doubt over the appropriateness of these phages for therapy.[427, 430] PaP3 is a temperate phage, and closely-related phages isolated by Krylov et al appeared to have a high degree of genetic instability.[427, 431] Although a broad range of lytic activity is desirable when selecting phages for therapy, it is evident that several other characteristics must be examined to select a theoretically safe and effective phage for clinical use. There is a lack of published efficacy data on the commercial preparations that were examined by Krylov et al, and so at this stage these characteristics cannot be correlated with clinical outcomes.

#### 1.3.5.2 Efficacy of bacteriophages in the treatment of *Pseudomonas aeruginosa* infections *in vivo*

Numerous animal studies have been performed using *P. aeruginosa* phages to treat a variety of infections, including corneal, lung and gastrointestinal infections in mice, infected ulcers in catfish, and chronic otitis in dogs.[432-439] These studies showed promising results, with either improved recovery (murine corneal infection) and survival (murine lung and gastrointestinal infections, catfish ulcers) of phage-treated animals compared to untreated animals, or

improved clinical scores (canine chronic otitis) or decreased bacterial counts in phage-treated animals (murine lung infection).

Clinical studies involving the use of *P. aeruginosa* phages to treat infections in humans were performed as early as 1959, when Bertoye and colleagues used phages to treat ear infections and meningitis.[440, 441] Numerous clinical studies and case series were published in the following years, including on the treatment of infected wounds and burns, and urogenital inflammation with phages. Unfortunately, many of these studies did not include controls or only reported limited data.[442]

In more recent years, Wright et al performed a randomised, placebo-controlled clinical trial using phage therapy to treat chronic antibiotic-resistant *P. aeruginosa* otitis.[443] Ear swabs were taken to confirm *P. aeruginosa* infection, and sensitivity of the bacterial isolate to at least one of six bacteriophages in the treatment cocktail, prior to treatment. Only a single dose of treatment or placebo was administered intra-aurally. The investigators found a statistically significant decrease in both patient self-reported symptom severity and clinician-assessed signs of infection. A statistically significant decrease in *P. aeruginosa* counts compared to baseline was noted at 3 and 6 weeks post-treatment, for the phage treatment group. This decrease was not seen in the placebo group. Importantly, phage titres were increased at post-treatment visits in the treatment group, suggesting phage replication.

Preliminary data from a clinical trial of a cocktail of *E. coli*, *Enterococcus*, *Proteus*, *Staphylococcus* and *Streptococcus* phages for treating symptomatic urinary tract

infection has shown a significant reduction in bacterial concentrations in the urine of treated patients.[444] The cocktail used was adapted from a commercially available phage cocktail. Urine cultures were screened for sensitivity to the phage cocktail prior to treatment, albeit with only 39% being sensitive and 15% displaying intermediate sensitivity, with presumably only sensitive patients receiving the phage cocktail.

Conversely, a trial using a mixture of PA and *Staphylococcus aureus* bacteriophages to treat infected burns showed no change in bacterial load following treatment.[445] Similarly, Rhoads et al conducted a double-blind phase 1 trial of a phage cocktail comprising of *P. aeruginosa*, *E. coli* and *S. aureus* phages, for treatment of venous leg ulcers.[446] 39 participants completed 12 weeks of therapy, where sterile saline or the phage cocktail respectively were applied to the wound using an ultrasonic debridement device. Concomitant standard wound dressings were used. They reported no difference between treatment and control groups in healing frequency or rate.

A number of factors may explain the apparent treatment failure in these trials. Both trials had small participant numbers and may have been insufficiently powered to assess efficacy. Rose et al employed a within-subject study design, meaning that the phage cocktail and standard antimicrobial dressings were applied to different areas of the same burn. The post-treatment biopsies for each area were taken two to five hours following treatment application. Only a very small bacterial load was measured in 8 out of 10 patients prior to treatment, giving rise to the possibility that there were insufficient bacteria in the wound to

allow the phages to replicate to any measurable degree in this short timeframe. Unfortunately, post-treatment phage titres were not reported. The clinical trial conducted by Rhoads et al was a phase 1 safety trial, and thus not designed to assess efficacy. No data regarding the presence of bacterial infection in the ulcers prior to treatment is reported, and inclusion criteria included patients with non-infected ulcers. The outcomes assessed were healing frequency and rate, both of which may be influenced by many factors other than infection. Again, post-treatment phage titres were not reported.

Clinical use of bacteriophage therapy has occurred in some former Soviet Union and eastern European countries since the 1920s, in particular Georgia, Russia, and Poland. Research into and application of phage therapy has been driven by several institutions, most prominently the Eliava Institute in Tbilisi, Georgia, and the Institute of Immunology and Experimental Therapy in Wroclaw, Poland.[447, 448] Whilst both institutes have begun to publish this work in Western scientific publications, it is likely that some significant preclinical research and clinical trials have been omitted from literature published in English, or published with great brevity. For example, a double-blind randomised controlled trial of prevention of dysentery in Red Army soldiers in four different regions reported a 10-fold reduction in the incidence of dysentery in the treatment group compared to the control group, but did not report the number of participants in each group.[448, 449] In contrast, a randomised placebo-controlled trial demonstrating efficacy of phage therapy for the prevention of diarrhoeal diseases in 30000 children,

performed in Georgia in 1963 and 1964, has now been summarised in a review by Sulakvelidze et al.[448, 450]

More recently, several case reports describing the successful use of bacteriophage therapy to treat multidrug resistant *P. aeruginosa* infections have been published[451-455]. These cases include pneumonia in a CF patient and in lung transplant recipients, bone and joint infection, and sepsis. Another case report describes the injection of phage OMKO1 and ceftazidime into a phlegmonous collection adjacent to an infected aortic graft, in a patient requiring continuous intravenous ceftazidime to suppress the *P. aeruginosa* infection and prevent sepsis[456]. Antibiotics were successfully ceased 4 weeks later, following exploratory surgery for bleeding (involving graft excision and replacement) and wound cultures growing *Candida* only. It is unclear in the latter case whether the resolution of infection can be attributed to the phage, given antibiotics were also administered and the infected graft was removed.

#### 1.3.5.3 Safety of *Pseudomonas aeruginosa* bacteriophage therapy *in vivo*

Animal studies performed in mice and dogs have not reported any safety concerns relating to the administration of *P. aeruginosa* phages via a number of routes (oral, intra-muscular, intra-peritoneal, intra-aural, intra-nasal, and intra-ocular).[432, 433, 436-439, 457, 458] However, these studies were not specifically designed to assess safety, and thus lack a non-infected treatment arm. Holguin et al reported 100% survival in control group mice that were only inoculated with phage treatment, but no other safety outcomes such as tissue histology were reported.[416]

Studies designed to assess the safety of preparations containing phages of other bacterial species have been performed in mice (oral *E. coli* T7 phage) and sheep (intra-sinus *S. aureus* phages).[459-461] Park et al examined clinical condition, gastrointestinal histopathology, and inflammatory cytokine responses in mice fed $10^9$ plaque forming units (PFU) of *E. coli* T7 phage daily for 10 days.[460] There was no mortality, and no differences were noted in clinical signs between phage-fed and control groups. Histopathology of the stomach, small intestine, and colon was normal in all groups. The only significant difference in blood inflammatory cytokine levels between the phage-fed and control group was an increase in IL-17A in phage-fed mice of less than 10%. The other cytokines measured were IL-1α, IL-1β, IL-2, IL-10, IL-12, IFN-γ, TNF-α, G-CSF, and GM-CSF. The studies of *S. aureus* phage treatment of rhinosinusitis in a sheep model are discussed below.

Where reported, safety outcomes of the human clinical trials already discussed have been favourable. Wright et al did not report any serious or treatment-related adverse events, with treatment-emergent adverse events being reported in similar proportions of participants in the test and control arms (50% and 42% respectively).[443] The trial performed by Rose et al did not report any adverse events, clinical or laboratory test abnormalities.[445] Rhoads et al found no significant difference in the number or quality of adverse events between treatment and control arms.[446] Sulakvelidze et al note that there have been no reports of serious complications associated with the use of phage therapy in the published literature.[448]

Other safety trials have focussed on oral administration of phage therapy. McCallin et al conducted a detailed safety analysis of a Russian commercially-available phage cocktail targeted at *E. coli* and *Proteus* infections.[462] Metagenomic analysis revealed the presence of two potentially "undesirable" genes – one conferring acid resistance in *E. coli*, and the other encoding for a serum resistance factor in an *Escherichia fergusonii* (non-pathogenic) prophage. The phage cocktail and placebo were given, in random order, to 15 adults and children. No severe adverse events were reported. Neither phage nor antibodies to phage were detected in serum samples. Faecal microbiome analysis of four adult participants showed variability within both phage and placebo treatment periods. Another safety trial designed to assess the safety of orally-administered *E. coli* T4 phage in 15 healthy human volunteers reported no serious or treatment-related adverse events.[463] No significant rise in liver enzymes or serum antibody formation to the phage was detected. Whilst *E. coli* phage was detected in the stool samples of all participants receiving the higher phage dose, no phage was detected in serum samples.

Międzybrodzki et al have reported safety outcomes of 153 patients treated for a variety of infections at the Phage Therapy Unit of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Wrocław.[464] Statistical analysis of blood and biochemical parameters measured at baseline and during up to 12 weeks of therapy showed only a clinically insignificant decrease in platelet count and a small increase in aspartate transaminase (AST) at 3 to 6 weeks, and reductions in γ-glutamyl transpeptidase (GGT) at 1 to 6 weeks. Adverse events

included gastrointestinal symptoms in patients administered oral phage therapy, and local discomfort or reactions at the site of topical administration in 18% of patients who received therapy via this route. However, only 1.4% of the latter patients discontinued therapy due to the adverse reaction. Superinfection and fever occurred in 4.6% and 3.3% of patients respectively. No serious treatment-related adverse events were reported.

### 1.3.6 Potential role for bacteriophage therapy in the management of chronic rhinosinusitis

A thorough literature review has not revealed any published clinical trials or *in vivo* animal studies using bacteriophages to treat *P. aeruginosa* infections in the paranasal sinuses. Drilling et al used the sheep rhinosinusitis model to assess the safety and efficacy of using a *S. aureus* bacteriophage cocktail (CT-SA) to treat *S. aureus* sinus infections.[461] The investigators found a significant decrease in *S. aureus* biofilm in sinuses treated with CT-SA, compared to untreated controls. Safety parameters assessed included integrity of cilia on the sinus mucosa, and the presence of phage in other organs such as kidney, spleen, liver and brain. Although no safety concerns were noted, the safety arm only received 3 days of CT-SA treatment, limiting the applicability of results to short-term treatment.

Drilling et al performed a further study using the sheep rhinosinusitis model to assess the safety of long-term intra-sinus administration of two other *S. aureus* phages, P68 and K710.[459] Sheep had both sinuses treated with a cocktail of the two phages twice daily, for 20 days. Analysis of sinus histopathology for inflammation, oedema, fibrosis, and goblet cell hyperplasia showed no

significant differences between phage-treated and control animals. Similarly, no significant difference was noted in SEM assessment of sinus mucosa ciliary integrity between groups. Phage was not detected in any of the serum samples taken throughout the treatment period.

A recent phase 1 clinical trial of CT-SA (also known as AB-SA01) sinus flushes for the treatment of *S. aureus* infections in CRS patients has reported an excellent safety profile[465]. Patients received twice-daily sinus flushes containing up to $3 \times 10^9$ PFU/mL AB-SA01 for up to 14 days. Only mild treatment-emergent adverse effects were noted, including diarrhoea, epistaxis, cough, nose and throat pain, and an asymptomatic transient decrease in serum bicarbonate level.

# Chapter 2: Activity of bacteriophages in removing biofilms of *Pseudomonas aeruginosa* isolates from chronic rhinosinusitis patients

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## Contextual statement

This study was an initial screening step in assessing the *in vitro* efficacy of a bacteriophage cocktail for treatment of *Pseudomonas aeruginosa* infection in chronic rhinosinusitis. The aims of this study were to analyse the *P. aeruginosa* strains found in the sinuses of patients with chronic rhinosinusitis and/or cystic fibrosis, and to assess the ability of the anti-*P. aeruginosa* bacteriophage cocktail to remove biofilms of these clinical isolates *in vitro*.

# Statement of authorship

| | |
|---|---|
| Title of Paper | Activity of Bacteriophages in Removing Biofilms of *Pseudomonas aeruginosa* Isolates from Chronic Rhinosinusitis Patients |
| Publication Status | ☑ Published<br>☐ Accepted for Publication<br>☐ Submitted for Publication<br>☐ Unpublished and Unsubmitted work written in manuscript style |
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## Principal Author

| | | | |
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| Name of Principal Author (Candidate) | Dr. Stephanie Anne Fong | | |
| Contribution to the Paper | Study design, execution of experiments, data analysis, and writing of manuscript | | |
| Overall percentage (%) | 75% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
| Signature | | Date | 18/1/2020 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

the candidate's stated contribution to the publication is accurate (as detailed above);

permission is granted for the candidate in include the publication in the thesis; and

the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| | | | |
|---|---|---|---|
| Name of Co-Author | Dr. Amanda Drilling | | |
| Contribution to the Paper | Study design, execution of experiments, data analysis, and review of manuscript | | |
| Signature | | Date | 22/11/2019 |

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| Name of Co-Author | Dr. Sandra Morales | | |
| Contribution to the Paper | Study design, and review of manuscript | | |
| Signature | | Date | 13/1/2020 |

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| Name of Co-Author | Dr. Marjolein Eline Cornet | | |
| Contribution to the Paper | Collection of clinical samples, data collection, and review of manuscript | | |
| Signature | | Date | 17/12/2019 |

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## 2.1 Abstract

**Introduction**: *Pseudomonas aeruginosa* infections are prevalent amongst chronic rhinosinusitis (CRS) sufferers. Many *P. aeruginosa* strains form biofilms, leading to treatment failure. Lytic bacteriophages (phages) are viruses that infect, replicate within, and lyse bacteria, causing bacterial death.

**Aim:** To assess the activity of a phage cocktail in eradicating biofilms of *ex vivo P. aeruginosa* isolates from CRS patients.

**Methods**: *P. aeruginosa* isolates from CRS patients with and without cystic fibrosis (CF) across three continents were multi-locus sequence typed and tested for antibiotic resistance. Biofilms grown *in vitro* were treated with a cocktail of four phages (CT-PA). Biofilm biomass was measured after 24 and 48 hours, using a crystal violet assay. Phage titrations were performed to confirm replication of the phages. A linear mixed effects model was applied to assess the effects of treatment, time, CF status, and multidrug resistance on the biomass of the biofilm.

**Results:** The isolates included 44 strain types. CT-PA treatment significantly reduced biofilm biomass at both 24 and 48 hours post-treatment ($p<0.0001$), regardless of CF status or antibiotic resistance. Biomass was decreased by a median of 76% at 48 hours. Decrease in biofilm was accompanied by a rise in phage titres for all except one strain.

**Conclusion:** A single dose of phages is able to significantly reduce biofilms formed *in vitro* by a range of *P. aeruginosa* isolates from CRS patients. This represents an exciting potential and novel targeted treatment for *P. aeruginosa* biofilm infections and multidrug resistant bacteria.

## 2.2 Introduction

Chronic rhinosinusitis (CRS) is an inflammatory condition of the nose and paranasal sinuses, persisting for 12 weeks or longer. Bacterial biofilms have been implicated in recalcitrant CRS and increase the tolerance of bacteria to antibiotics through numerous mechanisms. These include metabolic heterogeneity of the bacteria within the biofilm, enzymatic deactivation, anionic charges due to extracellular DNA within the biofilm matrix, and changes in gene expression[466]. Biofilms have been found on the sinonasal mucosa of up to 54% of CRS sufferers, compared to 8% of control patients[155]. Furthermore, multiple studies have noted a higher prevalence of biofilms in patients who are undergoing revision surgery[155, 156]. In particular, the presence of biofilm-forming *Pseudomonas aeruginosa* strains has been associated with poor resolution of symptoms and signs of CRS following endoscopic sinus surgery[162].

*Pseudomonas aeruginosa* has been identified in the sinuses of 9% of CRS patients, and is associated with poorer quality of life measured by disease severity scores such as the Visual Analogue Scale (VAS) and Sinonasal Outcome Test-22 (SNOT-22)[133, 140]. *P. aeruginosa* sinus infections also commonly afflict patients with cystic fibrosis (CF), with the species being identified in sinus cultures of up to 49% of CF patients with CRS[349]. Moreover, *P. aeruginosa* is intrinsically resistant to many classes of antibiotics, and acquired antibiotic resistance is increasing worldwide. New therapeutic strategies are therefore required to combat these difficult to treat bacterial infections in the context of CRS.

Bacteriophages are viruses that infect bacteria. Lytic bacteriophages are able to lyse their host bacterium, after replicating themselves within the host bacterium. Lysis of the host not only kills the bacterium, but also releases the progeny copies of the phage for re-infection of other bacteria[371]. Bacteriophages are species specific, and so can be used to target pathogenic bacteria, without disturbing non-harmful commensal bacteria[467]. Bacteriophages are also able to penetrate bacterial biofilms[425].

These characteristics make bacteriophages an attractive non-antibiotic therapy for treating bacterial biofilms in CRS. This study aims to assess the activity of a cocktail (mixture) of four *P. aeruginosa* bacteriophages (CT-PA) in removing *ex vivo* biofilms formed by PA isolates from CRS patients, both with and without CF. The prevalence of antibiotic resistance in these clinical isolates was also assessed.

## 2.3 Methods

### Bacterial strains and growth conditions

This study was approved by the Human Research Ethics Committee of The Queen Elizabeth Hospital, Adelaide, South Australia.

*P. aeruginosa* strains were isolated by an independent pathology laboratory (Adelaide Pathology Partners, Adelaide, South Australia) from endoscopically-guided sinus swabs, from patients who met the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012 criteria for chronic rhinosinusitis[3]. Clinical *P. aeruginosa* isolates of patients with CF were kindly donated by the Department of Otorhinolaryngology, Academic Medical Centre (Amsterdam, Netherlands) and *P. aeruginosa* sinus isolates from CRS patients with and without CF were kindly donated by the Department of Otolaryngology-Head and Neck Surgery, University of Alabama at Birmingham (Birmingham, AL). *P. aeruginosa* isolates were stored in 25% glycerol in nutrient broth at -80°C. *P. aeruginosa* laboratory reference strain ATCC 15692 (PAO1) was obtained from American Type Culture Collection (Manassas, VA, USA) as a control for phage sensitivity and biofilm assays. Isolates were plated from frozen glycerol stocks onto 1.5% nutrient agar, and broth cultures were grown in nutrient broth. Agar plates and broth cultures were incubated at 37°C.

### Bacteriophage cocktail

Stocks of 4 anti-*P. aeruginosa* bacteriophages (Pa 193, Pa 204, Pa 222, Pa 223), as well as heat-inactivated stocks, were supplied by AmpliPhi Biosciences

(Brookvale, New South Wales, Australia). Pa 193 and Pa 204 are of the Myoviridae family, and Pa 222 and Pa 223 are of the Podoviridae family. All 4 phages have been characterised as strictly lytic by genome sequencing (unpublished data). Prior to each assay, the stock suspension of each bacteriophage was titrated against a selected *P. aeruginosa* bacterial strain using the soft agar overlay small drop assay, as described below. Equal concentrations of each bacteriophage were combined to form the bacteriophage cocktail (CT-PA).

**Multi-locus sequence typing**

The protocol for multi-locus sequence typing (MLST) of *P. aeruginosa* isolates has been described by Curran et al[468]. For each strain, a single colony was used to inoculate 5mL of nutrient broth, and the culture was grown overnight on a shaker at 37°C. DNA extraction from a 1mL aliquot of the overnight culture was performed using the DNeasy Blood and Tissue DNA extraction kit (Qiagen, Hilden, Germany), following the protocol for Gram negative bacteria recommended by the manufacturer. PCR of the 7 MLST loci was performed using the Taq PCR kit (New England Biolabs, Ipswich MA, USA) using the protocol and primers described by Curran et al (GeneWorks, Adelaide, South Australia). PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Sanger sequencing was performed on the purified PCR products by two external laboratories (SA Pathology and Australian Genome Research Facility, Adelaide, South Australia). Sequences were checked for base miscalls, and contigs were assembled using GeneStudio Professional, version 2.2.0.0 (GeneStudio Inc.). The sequence of each locus was matched to the allele sequences in the *Pseudomonas aeruginosa* MLST database (pubmlst.org/paeruginosa/) and assigned the corresponding allele number. The profile of alleles for each isolate was then matched to the sequence type profiles in the MLST database.

**Phylogenetic and BURST analysis**

Phylogenetic analysis of the clinical isolates and laboratory reference strain PAO1 were conducted using MEGA version 6.06[(469)]. MLST sequence alleles for each of the 7 loci were aligned using the MUSCLE programme in MEGA[(470)]. The concatenated alignments were used to generate dendrograms using the Neighbour-Joining method with bootstrapping analysis (2000 replicates)[(471)]. Evolutionary distances were computed using the Maximum Composite Likelihood method, with ambiguous positions removed for each sequence pair[(472)]. Outgroup sequences were identified through BLAST searching for sequences of sufficient homology from other *Pseudomonas* species. eBURST version 3 (eburst.mlst.net) was used to identify clonal complexes and BURST groups[(473)]. Bootstrapping analysis with 2000 replicates was used for all BURST analyses.

**Minimum inhibitory concentration assays**

Resistance to commonly used antibiotics was determined using broth microdilution minimum inhibitory concentration (MIC) assays, as described by Wiegand et al[(474)]. Antibiotics tested were: gentamicin, ciprofloxacin, ceftazidime, piperacillin, and amikacin, obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Isolates were designated as being sensitive, resistant, or having intermediate sensitivity to the antibiotics based on Clinical and Laboratory Standards Institute (CLSI) cut-offs.

**Sensitivity to CT-PA phages & enumeration of phage**

The ability of the 4 phages to lyse each bacterial isolate was tested using the spot test described by Mazzocco et al, with a drop size of 5µL[(475)]. All plaque assays were performed in duplicate. Enumeration of phage in stocks using the small drop plaque assay system was performed prior to each assay, with a concentration of $10^8$ PFU/mL used for isolate sensitivity assays. A selected reference bacterial strain was used for titration of each of the 4 different phage stocks. The small drop plaque assay was also used to assess the phage

concentration in the liquid contents of the biofilm assay wells, at 48 hours after treatments were applied.

**Biofilm assay**

The microtitre dish biofilm formation assay as described by O'Toole was used to assess the ability of CT-PA to eradicate *P. aeruginosa* biofilms *in vitro*[476]. A 1.0 McFarland unit suspension in 0.45% saline of the isolate was diluted into 10 times the volume of nutrient broth (Oxoid, Hants, UK), and gently mixed by inversion. 150µL/well of the resulting suspension was plated into a clear polystyrene 96-well plate (Greiner Bio-One, Kremsmünster, Austria). Wells adjacent to the edge of the plate were filled with 180µL sterile PBS as a sterility control. The plate was then incubated for 48 hours on a gyratory mixer at 37°C. After 48 hours, the liquid contents of the bacterial wells were gently aspirated, followed by washing twice with sterile PBS to remove any remaining planktonic cells. Treatments included each of the 4 phages and the phage cocktail in nutrient broth at concentrations of $10^7$ and $10^8$ PFU/mL, as well as equivalent volumes of heat-inactivated stocks of the 4 phages, with PBS as negative control and 2.5% cetylpyridinium chloride (Sigma-Aldrich, St Louis, MO, USA) as a positive control. 180µL of each treatment was plated in quadruplicate and biofilms were assessed at 24 and 48 hours after treatment. 48 hour treatment plates had 50µL/well nutrient broth replenished at 24 hours. At the designated time point (either 24 or 48 hours after treatment), liquid contents of the wells were transferred into a fresh plate for post-treatment phage titration. The biofilm plates were gently washed twice with sterile PBS, and then stained with 190µL/well 0.5% crystal violet (Sigma, St Louis, MO, USA) for 30 minutes. The stained plates were rinsed by two rounds of gentle immersion into distilled water, and left to dry overnight. The crystal violet stain was eluted by application of 200µL/well 30% acetic acid (Chem-Supply, Adelaide, South Australia) and the plate was incubated at room temperature for 30 minutes. Absorbance at 595 nm was measured for each well using the Fluostar Optima microplate reader (BMG Labtech, Ortenberg, Germany), with 200µL 30% acetic acid in unstained wells used as blanks.

**Statistics**

Linear mixed effects models were applied to assess the effects of group, time point, CF status and multidrug resistance on absorbance (A595) data from the microtitre plate biofilm formation assay. A595 values were log transformed prior to analysis due to violations of the distributional assumptions of linear regression. Pairwise, post hoc comparisons for group were assessed at $p<0.010$ due to the large number of comparisons made. The data were analysed using SAS v9.4 (SAS Institute Inc., Cary, NC, USA).

## 2.4 Results

**Multiple MLST sequence types identified amongst CRS clinical isolates**

In total, 47 *P. aeruginosa* isolates were collected from the upper and lower airways from 44 patients suffering from CRS and/or CF in 3 continents. These included 19 isolates from the upper and lower respiratory tracts of patients with CF (Amsterdam, Netherlands), 7 sinus isolates from CRS patients with and without CF (Birmingham, AL, USA), and 21 sinus isolates from CRS patients with and without CF (Adelaide, Australia). Isolate characteristics are displayed in Table 1. MLST revealed 44 distinct sequence types. Isolate MLST profiles and burst groups are detailed in Supplementary Table 1. One clinical isolate from a CF patient in the Netherlands was of the same sequence type as PA01. Two Australian non-CF isolates shared an identical sequence type (348), and another sequence type (274) was shared by one isolate from each of Australia, the United States, and the Netherlands.

BURST analysis identified only one clonal complex, using the most stringent definition of sequence types sharing six or more alleles in common. The clonal complex consisted of two single locus variants isolated from the same CF patient. The phylogeny inferred by the Neighbour-Joining method is displayed in Figure 1.

**Table 1: Characteristics of *P. aeruginosa* clinical isolates studied**

| | | CF isolates | Non-CF isolates | Total |
|---|---|---|---|---|
| **Country of origin** | **Australia** | 2 | 19 | 21 |
| | **Netherlands** | 19 | 0 | 19 |
| | **USA** | 3 | 4 | 7 |
| **Total** | | **24** | **23** | **47** |

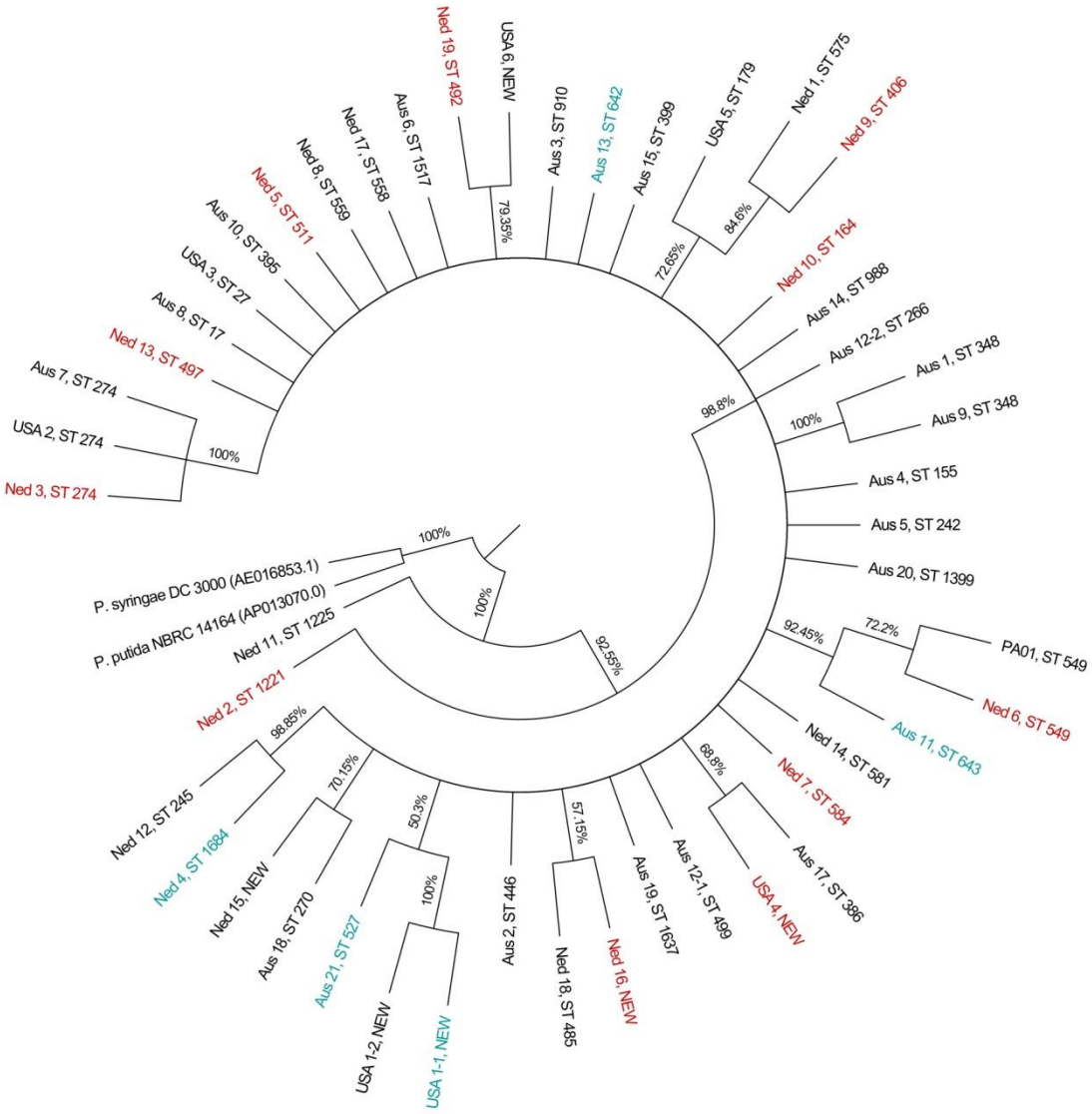


**Figure 1: Condensed phylogenetic tree obtained using the Neighbour-Joining method**

Phylogeny was inferred using the Neighbor-Joining method. The condensed topology after branches with bootstrap values of less than 50% have been collapsed into polytomies is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. There were a total of 2882 positions in the final dataset. Multidrug resistant isolates are labelled in red, and CT-PA phage cocktail resistant isolates are labelled in teal.

**CT-PA host range in CRS clinical isolates**

40/45 isolates (89%) were lysed by CT-PA, with partial or full activity spots seen on the small drop plaque assay. Examples of full and partial activity spots are shown in Figure 2. Two isolates could not be tested; one due to interference from plaques throughout the bacterial lawn including areas where the phage had not been applied (thought to be due to induction of a prophage), and the other due to poor growth in culture. When tested individually, 73%, 53%, 73%, and 71% of isolates were sensitive to Pa 193, Pa 204, Pa 222, and Pa 223 respectively, as displayed in Table 2. 39/45 isolates (87%) were susceptible to 2 or more of the 4 phages.

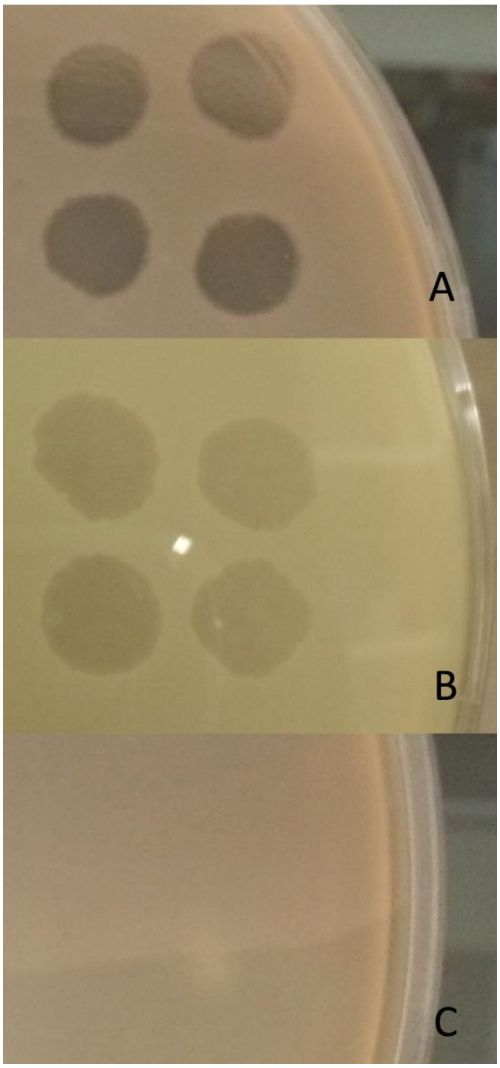


**Figure 2: Spot test assay**

Phage activity on the spot test assay was graded as: (A) Full activity – a completely clear (non-turbid) spot with no bacterial growth observed within the spot, (B) Partial activity – a turbid spot easily seen with the naked eye, or a clear spot containing isolated bacterial colonies, or (C) No activity – no easily discernible spot seen with the naked eye.

**Table 2: Host range of phages and phage cocktail in CRS clinical isolates**

| *P. aeruginosa* isolate | Phage treatment | | | | |
|---|---|---|---|---|---|
| | **Pa 193** | **Pa 204** | **Pa 222** | **Pa 223** | **CT-PA** |
| **PAO1** (reference strain) | ++ | ++ | ++ | 0 | ++ |
| **Aus 1** | 0 | 0 | ++ | ++ | ++ |
| **Aus 2** | Unable to test | | | | |
| **Aus 3** | ++ | ++ | + | ++ | ++ |
| **Aus 4** | ++ | 0 | ++ | ++ | ++ |
| **Aus 5** | ++ | ++ | ++ | ++ | ++ |
| **Aus 6** | + | 0 | + | + | + |
| **Aus 7** | + | 0 | ++ | ++ | ++ |
| **Aus 8** | 0 | 0 | ++ | ++ | ++ |
| **Aus 9** | 0 | 0 | + | + | + |
| **Aus 10** | ++ | ++ | 0 | 0 | ++ |
| **Aus 11** | 0 | 0 | 0 | 0 | 0 |
| **Aus 12** | ++ | ++ | 0 | 0 | ++ |
| **Aus 13** | 0 | 0 | 0 | 0 | 0 |
| **Aus 14** | ++ | ++ | + | 0 | ++ |
| **Aus 15** | ++ | ++ | + | + | ++ |
| **Aus 16** | ++ | ++ | ++ | ++ | ++ |
| **Aus 17** | ++ | ++ | 0 | 0 | ++ |
| **Aus 18** | + | + | + | + | ++ |
| **Aus 19** | ++ | ++ | + | + | ++ |
| **Aus 20** | ++ | ++ | ++ | ++ | ++ |
| **Aus 21** | 0 | 0 | 0 | 0 | 0 |
| **Ned 1** | + | 0 | 0 | ++ | ++ |
| **Ned 2** | ++ | ++ | ++ | ++ | ++ |
| **Ned 3** | 0 | 0 | ++ | ++ | ++ |
| **Ned 4** | 0 | 0 | 0 | 0 | 0 |
| **Ned 5** | ++ | ++ | ++ | ++ | ++ |
| **Ned 6** | + | ++ | + | + | ++ |
| **Ned 7** | + | + | ++ | ++ | ++ |
| **Ned 8** | ++ | 0 | 0 | ++ | ++ |
| **Ned 9** | ++ | ++ | 0 | 0 | ++ |
| **Ned 10** | ++ | ++ | + | ++ | ++ |
| **Ned 11** | ++ | ++ | ++ | ++ | ++ |
| **Ned 12** | + | 0 | + | 0 | + |
| **Ned 13** | ++ | 0 | + | + | ++ |
| **Ned 14** | 0 | 0 | + | + | + |
| **Ned 15** | ++ | ++ | ++ | + | ++ |
| **Ned 16** | + | 0 | + | 0 | + |
| **Ned 17** | Unable to test | | | | |
| **Ned 18** | 0 | 0 | ++ | + | ++ |
| **Ned 19** | + | 0 | 0 | 0 | + |

| | | | | | |
|---|---|---|---|---|---|
| **USA 1-1** | 0 | 0 | 0 | 0 | 0 |
| **USA 1-2** | 0 | 0 | + | ++ | ++ |
| **USA 2** | ++ | ++ | ++ | ++ | ++ |
| **USA 3** | ++ | ++ | ++ | ++ | ++ |
| **USA 4-2** | ++ | ++ | ++ | ++ | ++ |
| **USA 5** | ++ | ++ | + | + | ++ |
| **USA 6** | + | + | ++ | + | ++ |
| **Total CI sensitive** (%) | **33** (73) | **24** (53) | **33** (73) | **32** (71) | **40** (89) |
| **Total CI resistant** (%) | **12** (27) | **21** (47) | **12** (27) | **13** (29) | **5** (11) |

++ : Full activity spots on small drop plaque assay

+ : Partial activity spots on small drop plaque assay

0 : No activity on small drop plaque assay

CI: clinical isolates

**Multidrug resistance in CF clinical isolates**

Isolates that displayed sensitivity to CT-PA were tested for antibiotic resistance, using CLSI cut-offs for intermediate resistance and resistance. The number of isolates displaying resistance or intermediate resistance to the five antibiotics tested is shown in Table 3. 11 isolates, all from CF patients, were multidrug resistant according to the definition proposed by Magiorakos et al[(331)].

**Table 3: Antibiotic resistance of clinical isolates**

| | **No. of resistant isolates** (%) | | |
|---|---|---|---|
| | **CF isolates** | **Non-CF isolates** | **All isolates** |
| **Gentamicin** | **9** (43) | **1** (5) | **10** (25) |
| **Amikacin** | **10** (48) | **1** (5) | **11** (28) |
| **Ciprofloxacin** | **13** (62) | **2** (11) | **15** (38) |
| **Ceftazidime** | **12** (57) | **1** (5) | **13** (33) |
| **Piperacillin** | **3** (14) | **0** (0) | **3** (8) |
| **Multidrug resistant** | **11** (52) | **0** (0) | **11** (28) |
| **Total no. of isolates tested** | **21** | **19** | **40** |

**Biofilm reduction by CT-PA**

All 40 isolates that displayed sensitivity to CT-PA on the spot test assay, as well as reference strain ATCC 15692, were tested using the microtitre dish biofilm assay. Absorbance readings from the crystal violet biofilm assays of all isolates at 24 and 48 hours after treatment are displayed in Figure 3. Statistically significant reductions in biofilm biomass compared to the negative treatment control (nutrient broth) were seen with both $10^7$ and $10^8$ PFU/mL of CT-PA, Pa 222 and Pa 223 at both 24 and 48 hours ($p < 0.001$). Significant reductions were also seen with $10^7$ PFU/mL Pa 193 and $10^8$ PFU/mL Pa 204 at 48 hours ($p < 0.01$).

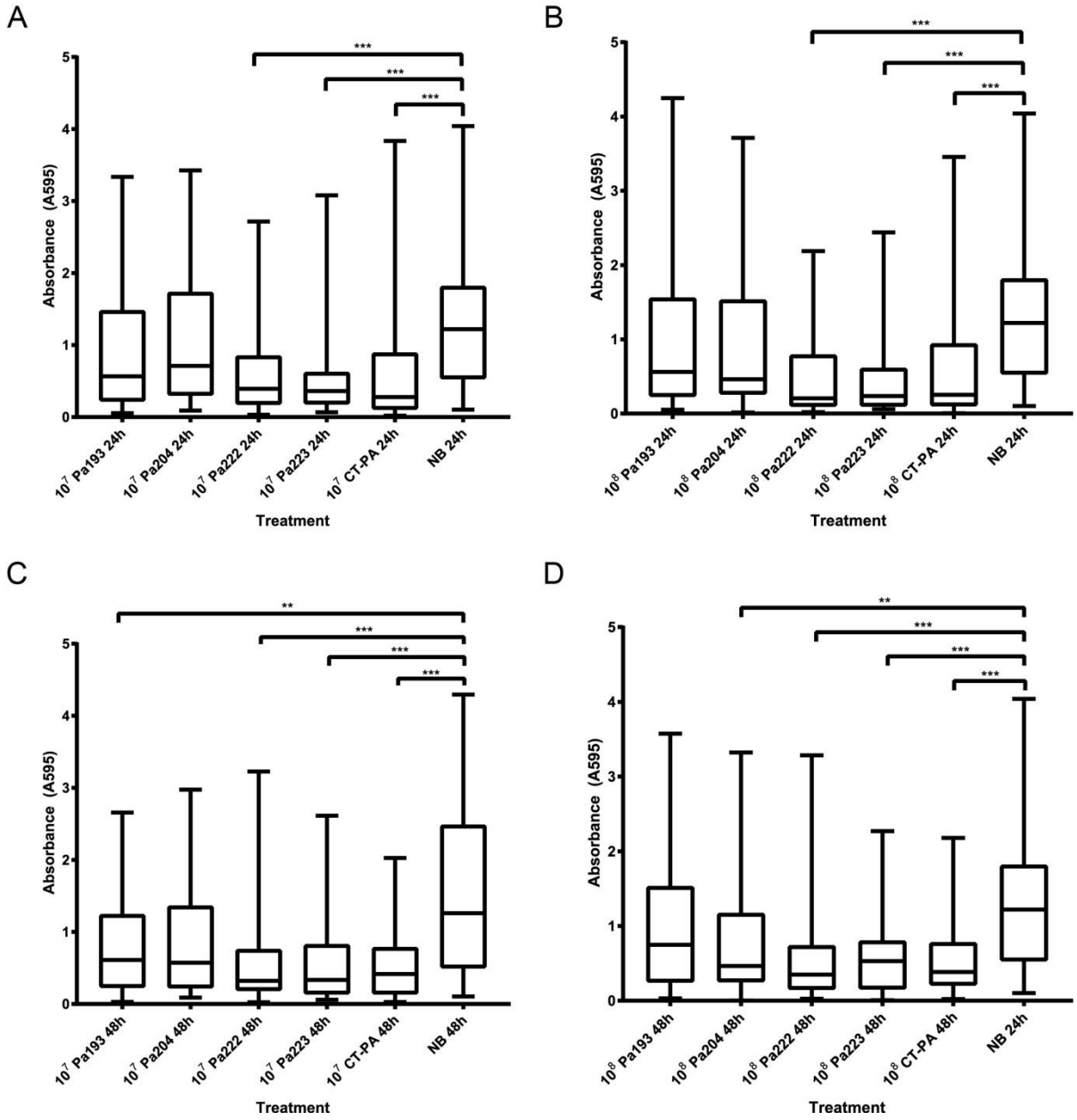


**Figure 3: Absorbance at 595 nm of crystal violet-stained biofilms**

Biomass of biofilms (of all 40 isolates and ATCC 15692) as measured by absorbance at 595 nm of crystal violet-stained biofilms after treatment with: **(A)** $10^7$ PFU/mL phage treatments or growth media control for 24 hours, **(B)** $10^8$ PFU/mL phage treatments or growth media control for 24 hours, **(C)** $10^7$ PFU/mL phage treatments or growth media control for 48 hours, **(D)** $10^8$ PFU/mL phage treatments or growth media control for 48 hours. Boxplot whiskers represent range (minimum to maximum values). (NB: nutrient broth; ** $p < 0.01$; *** $p < 0.001$)

For all isolates tested, the median biofilm biomass reduction was 70% and 64% after 48 hours treatment with the lower and higher concentrations of CT-PA respectively. 34 (85%) and 32 (80%) of the clinical isolates respectively showed a reduction in biomass compared to the negative treatment control at 48 hours, as displayed in Figure 4. The median biomass reductions at the 24-hour time point were similar for both concentrations of CT-PA at 67%. The maximum biomass reductions were 98.6% and 99.6% at 24 hours, and 99.4% and 98.8% at 48 hours, for the lower and higher concentrations respectively.

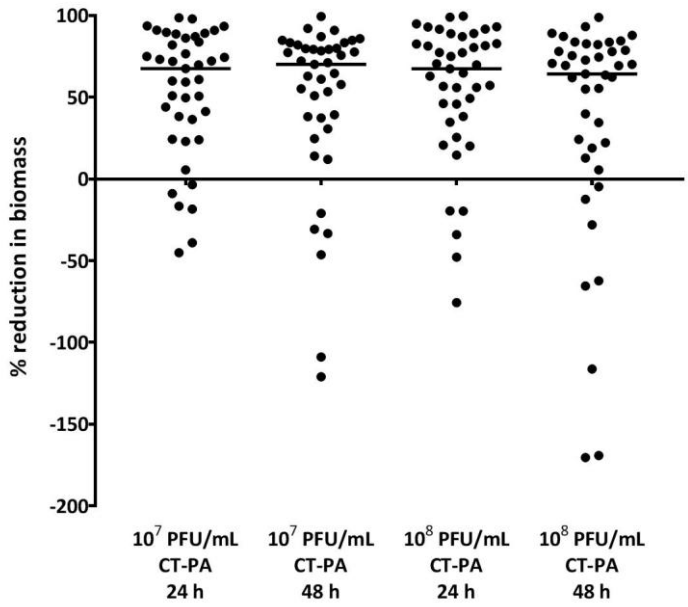


**Figure 4: Percentage reduction in biofilm biomass with CT-PA treatment**

Percentage reduction in biofilm biomass compared to growth media control after 24 and 48 hours treatment with lower and higher concentrations of CT-PA. Dots represent the average of quadruplicate treatments, and horizontal lines indicate median values.

Analysis of two-way interactions showed no statistically significant interactions between CF status or multidrug resistance and treatment effect.

**Post-treatment phage titres**

For all except three of the 40 isolates tested, titres of two or more of the four phages had increased after 48 hours treatment in the biofilm assay. This indicates successful phage infection and replication, and good complementation between the four phages comprising the CT-PA cocktail. Of the remaining three isolates, two showed an increase in titres of one phage, and one did not show an increase in any phage titres. Post-treatment phage titres for PAO1 and the 40 clinical isolates are displayed in Table 4.

**Table 4: Post-treatment phage titres**

| Strain | Post-treatment phage titres (PFU/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $10^8$ PFU/mL treatment | | | | $10^7$ PFU/mL treatment | | | |
| | Pa 193 | Pa 204 | Pa 222 | Pa 223 | Pa 193 | Pa 204 | Pa 222 | Pa 223 |
| **PAO1** | 2.0E+10 | 3.0E+09 | 4.0E+10 | 1.0E+08 | 1.0E+10 | 2.0E+09 | 2.0E+10 | 1.0E+09 |
| **Aus 1** | 1.1E+08 | 5.7E+07 | 4.9E+07 | 1.0E+02 | 4.3E+06 | 4.0E+06 | 1.8E+08 | 2.0E+02 |
| **Aus 3** | 1.8E+10 | 9.2E+09 | 4.3E+09 | 3.9E+09 | 9.0E+09 | 2.9E+09 | 8.5E+08 | 6.5E+02 |
| **Aus 4** | 3.0E+08 | 9.0E+08 | 3.0E+08 | 1.0E+07 | 7.0E+08 | 4.0E+09 | 5.0E+06 | 3.0E+05 |
| **Aus 5** | 8.0E+10 | 2.4E+09 | 6.4E+08 | 2.0E+03 | 5.0E+09 | 1.3E+10 | 1.3E+09 | 1.2E+09 |
| **Aus 6** | 7.0E+07 | 2.0E+09 | 3.2E+09 | 2.1E+05 | 7.8E+06 | 7.6E+10 | 8.3E+09 | 2.1E+06 |
| **Aus 7** | 2.2E+09 | 1.0E+08 | 5.0E+08 | 4.0E+08 | 9.0E+09 | 1.0E+07 | 2.0E+08 | 1.0E+09 |
| **Aus 8** | 2.0E+03 | 8.0E+08 | 2.0E+03 | 1.0E+07 | 3.0E+09 | 2.0E+09 | 1.0E+08 | 5.0E+05 |
| **Aus 9** | 2.0E+07 | 4.8E+03 | 2.4E+09 | 1.3E+03 | 4.1E+04 | 6.0E+02 | 7.8E+08 | 2.1E+03 |
| **Aus 10** | 5.0E+07 | 1.0E+11 | 9.3E+08 | 1.2E+06 | 3.0E+06 | 7.0E+09 | 2.0E+09 | 8.0E+05 |
| **Aus 12** | 2.3E+09 | 2.6E+10 | 5.1E+09 | 3.8E+03 | 7.3E+08 | 8.2E+10 | 4.5E+09 | 2.7E+04 |
| **Aus 14** | 2.7E+08 | 5.3E+10 | 9.0E+07 | 4.8E+06 | 1.7E+08 | 2.4E+09 | 2.0E+06 | 3.9E+05 |
| **Aus 15** | 1.2E+11 | 3.0E+10 | 1.2E+10 | 5.1E+10 | 2.0E+11 | 3.0E+11 | 4.0E+10 | 2.0E+05 |
| **Aus 16** | 2.0E+10 | 1.0E+11 | 3.0E+09 | 2.0E+08 | 1.0E+10 | 1.0E+10 | 2.0E+07 | 2.0E+07 |
| **Aus 17** | 1.9E+10 | 5.2E+11 | 1.9E+10 | 9.5E+06 | 5.9E+10 | 1.5E+11 | 1.5E+10 | 5.2E+06 |
| **Aus 18** | 9.0E+08 | 1.0E+08 | 2.0E+10 | 2.5E+08 | 3.0E+07 | 2.0E+07 | 7.0E+10 | 1.0E+08 |
| **Aus 19** | 5.0E+08 | 2.0E+03 | 3.0E+10 | 2.0E+03 | 2.0E+03 | 2.0E+03 | 1.0E+09 | 2.0E+03 |
| **Aus 20** | 1.0E+10 | 4.0E+10 | 9.0E+07 | 1.0E+10 | 2.0E+10 | 2.0E+03 | 1.7E+07 | 5.0E+05 |
| **Ned 1** | 4.0E+07 | 5.3E+06 | 7.0E+05 | 5.9E+03 | 2.2E+06 | 7.1E+06 | 9.5E+03 | 6.4E+03 |
| **Ned 2** | 7.2E+09 | 1.1E+10 | 5.7E+07 | 1.5E+08 | 8.0E+09 | 2.0E+08 | 2.1E+07 | 8.0E+07 |
| **Ned 3** | 8.0E+07 | 1.9E+07 | 3.6E+08 | 1.5E+08 | 9.8E+05 | 2.0E+06 | 3.5E+07 | 5.7E+08 |
| **Ned 5** | 2.5E+10 | 2.1E+11 | 4.0E+07 | 7.7E+07 | 1.8E+10 | 1.1E+11 | 4.0E+07 | 9.0E+05 |
| **Ned 6** | 8.6E+09 | 2.4E+10 | 7.1E+08 | 1.8E+09 | 5.1E+09 | 1.7E+10 | 1.2E+08 | 5.2E+08 |
| **Ned 7** | 6.8E+10 | 1.5E+09 | 7.4E+07 | 8.4E+07 | 5.5E+10 | 1.6E+09 | 1.2E+07 | 2.6E+08 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Ned 8** | 4.3E+10 | 2.2E+12 | 2.2E+09 | 2.1E+06 | 7.1E+10 | 2.4E+05 | 3.7E+13 | 1.5E+11 |
| **Ned 9** | 2.2E+10 | 2.3E+10 | 1.2E+07 | 5.5E+10 | 4.4E+10 | 1.0E+11 | 2.3E+06 | 1.6E+04 |
| **Ned 10** | 1.7E+08 | 1.1E+10 | 5.5E+06 | 7.0E+04 | 7.0E+07 | 1.7E+10 | 3.1E+05 | 2.0E+06 |
| **Ned 11** | 1.9E+09 | 3.9E+09 | 1.2E+09 | 8.5E+08 | 9.0E+09 | 6.9E+09 | 1.5E+09 | 2.4E+07 |
| **Ned 12** | 2.0E+08 | 1.0E+08 | 5.0E+09 | 1.7E+05 | 2.5E+06 | 1.0E+08 | 8.0E+09 | 4.0E+05 |
| **Ned 13** | 4.0E+09 | 3.2E+07 | 4.6E+08 | 1.0E+03 | 5.4E+09 | 4.6E+09 | 6.2E+08 | 0.0E+00 |
| **Ned 14** | 1.1E+09 | 5.3E+09 | 1.5E+08 | 1.8E+07 | 1.5E+09 | 4.5E+09 | 6.9E+07 | 4.3E+07 |
| **Ned 15** | 2.4E+11 | 1.9E+10 | 5.3E+09 | 4.9E+06 | 6.3E+10 | 1.5E+10 | 7.3E+09 | 5.6E+06 |
| **Ned 16** | 2.0E+11 | 3.4E+08 | 1.7E+08 | 5.1E+07 | 6.8E+10 | 3.0E+08 | 4.8E+08 | 8.0E+06 |
| **Ned 18** | 3.2E+07 | 1.4E+07 | 3.7E+09 | 1.8E+06 | 2.3E+06 | 1.4E+06 | 5.4E+09 | 2.7E+06 |
| **Ned 19** | 4.2E+08 | 1.2E+09 | 7.0E+07 | 2.2E+07 | 2.3E+06 | 6.2E+09 | 0.0E+00 | 6.8E+05 |
| **USA 1-2** | 3.0E+07 | 1.3E+07 | 3.0E+09 | 1.0E+06 | 6.0E+05 | 1.0E+07 | 3.0E+03 | 2.0E+06 |
| **USA 2** | 1.0E+09 | 2.0E+10 | 1.0E+09 | 1.0E+05 | 2.0E+10 | 3.0E+10 | 1.0E+11 | 6.0E+04 |
| **USA 3** | 1.0E+08 | 1.0E+12 | 1.0E+10 | 1.0E+01 | 1.0E+07 | 2.0E+06 | 8.0E+09 | 8.0E+03 |
| **USA 4-2** | 1.4E+09 | 9.0E+09 | 1.6E+09 | 1.0E+08 | 1.0E+10 | 2.0E+10 | 2.0E+09 | 1.0E+07 |
| **USA 5** | 1.0E+09 | 9.0E+09 | 2.0E+10 | 1.0E+05 | 1.0E+11 | 1.0E+09 | 5.0E+10 | 3.0E+04 |
| **USA 6** | 2.6E+10 | 2.0E+11 | 1.2E+10 | 1.2E+06 | 3.8E+11 | 4.0E+11 | 8.0E+09 | 5.0E+08 |

## 2.5 Discussion

CT-PA bacteriophage cocktail displayed suitable anti-biofilm activity *in vitro*. It had a broad host range in the 45 isolates tested, with 89% of isolates susceptible. The use of a cocktail as opposed to individual phages increased the host range significantly, with only 53% to 73% of isolates being susceptible to each of the four phages individually. This is consistent with previous observations that the use of a cocktail of phages rather than individual phages improves activity by expanding the host range and by preventing the development of bacteriophage-insensitive mutant bacteria[477-479].

The lytic effect of CT-PA on planktonic bacteria translated well to a reduction in biofilm, consistent with previous reports showing efficacy of bacteriophage to reduce biofilm *in vitro* and *in vivo*[461, 477]. Moreover, the increase in phage titres following treatment for almost all isolates implies successful phage infection, replication, and lysis of host bacteria, given the strictly lytic nature of all phages comprising the CT-PA phage cocktail.

Our results suggested slightly more biofilm removal by $10^7$ PFU/mL compared to $10^8$ PFU/mL CT-PA after 48 hours treatment, despite similar anti-biofilm activity of the two treatment concentrations at 24 hours. Studies of other *P. aeruginosa* phages have demonstrated that a higher concentration of phage treatment does not always result in greater magnitude of biofilm removal or bacterial killing[426, 480]. A non-linear relationship may be expected for some phage combinations over time due to the self-replicating nature of phage therapy[388].

The isolates in our study sample consisted of 44 MLST sequence types from both the sinuses of non-CF CRS sufferers, and the upper and lower respiratory tracts of CF patients. BURST and phylogenetic analysis was consistent with the predominantly non-clonal population structure that has been described by *P. aeruginosa* strain typing studies[468, 481]. Clonal groups (identical ST) and clonal complexes were uncommon within our collection of isolates. The clonal and BURST groups identified contain isolates from both CF and non-CF CRS patients, suggesting that *P. aeruginosa* sinus infections in both groups may initially be caused by similar strains. This is consistent with the observation made by Cramer et al that the dominant clones amongst CF patients are also common in the environment and other human disease habitats[482].

However, frequent antibiotic resistance was displayed by CF isolates, with 52% of the CF strains tested meeting the criteria for multidrug-resistance. Rates of antibiotic resistance in non-CF CRS isolates were much lower, and comparable to those observed in similar studies of CRS patients and in data from national antimicrobial surveillance programs[327, 329, 332, 334, 483]. Higher rates of antibiotic resistance in *P. aeruginosa* isolates from CF patients compared to non-CF patients have been well-documented[484, 485]. Although some of the isolates in our study were from the lower respiratory tract of CF patients, a high degree of similarity in the adaptive mutations and gene expression of CF sinus and lung *P. aeruginosa* isolates has been found[486]. Furthermore, the paranasal sinuses have been found to be a reservoir of recurrent pulmonary infections in CF patients, with identical strains often identified in the upper and lower respiratory tracts of patients[350, 353-355]. Thus the selective pressures in the upper respiratory tract of

CF patients, including antibiotics administered for management of bacterial sinus infections, have potential implications for the development of chronic lung infections[362]. The progression to chronic *P. aeruginosa* lung infection in CF has been shown to lead to deterioration in lung function and decreased life expectancy[359, 361].

The proportion of clinical isolates that were resistant to lysis by the CT-PA phage cocktail was small (11%), and did not include any multidrug resistant isolates. Importantly, the anti-biofilm activity of CT-PA was not affected by multidrug resistance, or whether the isolate was from a CF patient. The CF isolates tested were largely comprised of isolates with a small colony or mucoid phenotype. These phenotypes are associated with chronic infection, and display increased tolerance to various antibiotics[335, 362, 487-489]. These findings suggest that phage therapy may have potential in treating CF patients with chronic *P. aeruginosa* infection, for whom clinicians are currently faced with a dilemma of how to treat respiratory infections without creating further antibiotic resistance. We have previously demonstrated the ability of high-volume nasal irrigations to penetrate into the paranasal sinuses following functional endoscopic sinus surgery (FESS).[490] The phage cocktail could be administered via nasal irrigations in order to treat recurrent sinus infections following FESS.

*In vivo* animal studies have demonstrated enhanced bacterial clearance and prolonged survival in phage-treated animals (in *Galleria mellonella*, canine otitis, murine lung, corneal and peritoneal *P. aeruginosa* infection models)[432, 433, 437, 438, 491, 492]. A limited number of early-stage clinical trials of phage therapy for treatment of *P. aeruginosa* infections have been undertaken. A double-blind randomised controlled trial of a topically-applied phage cocktail for antibiotic-resistant *P. aeruginosa* chronic otitis showed statistically significant improvement from baseline in both symptom and clinical severity scores in the phage treatment group[443]. No such improvement was observed in the placebo group. Conversely, two clinical trials of phage cocktails targeting multiple bacterial pathogens (*P. aeruginosa*, *S. aureus* and *E. coli*) for treatment of burns

and chronic leg ulcers respectively showed no significant differences between phage and control therapies[445, 446].

The aforementioned clinical trials all reported excellent safety profiles. A study of long-term safety of a *S. aureus* phage cocktail applied topically to sheep sinuses found no local or systemic safety concerns[459]. Although not widespread, clinical use of phage therapy has occurred through phage therapy and research institutes, such as the Eliava Institute in Tbilisi, Georgia, and the Institute of Immunology and Experimental Therapy in Wroclaw, Poland[447, 448]. An analysis of 153 patients treated for various infections with phage therapy at the latter institute revealed no major safety concerns[464].

Whilst further evaluation and optimisation of phage cocktail treatment of *P. aeruginosa* infections in CRS and CF are required, the safety profile of phage therapy and its activity against antibiotic-resistant isolates make it an attractive potential therapeutic option. Further pre-clinical and clinical studies will be of great benefit in determining whether phage therapy can fulfil the potential demonstrated in this study.

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# Chapter 3: Safety and efficacy of a bacteriophage cocktail in an *in vivo* model of *Pseudomonas aeruginosa* sinusitis

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## Contextual statement

This study follows on from the promising *in vitro* results, with the aim of assessing the *in vivo* safety and efficacy of the bacteriophage cocktail in treating *P. aeruginosa* sinusitis in a large animal model. Sheep have paranasal sinuses that are similar in size to humans, and so the sheep rhinosinusitis model was chosen as it allows treatment to be administered as a flush into the sheep's frontal sinuses. This is comparable to the use of frontal trephine flushes or transnasal sinus flushes in humans.

# Statement of authorship

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## Principal Author

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| Name of Principal Author (Candidate) | Dr. Stephanie Anne Fong | | |
| Contribution to the Paper | Study design, execution of experiments, data analysis, and writing of manuscript | | |
| Overall percentage (%) | 75% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
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## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

the candidate's stated contribution to the publication is accurate (as detailed above);

permission is granted for the candidate in include the publication in the thesis; and

the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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## 3.1 Abstract

*Pseudomonas aeruginosa* (PA) is a bacterial pathogen that frequently displays antibiotic resistance. Its presence within the sinuses of chronic rhinosinusitis sufferers is associated with poorer quality of life. Obligately lytic bacteriophages (phages) are viruses that infect, replicate within, and lyse bacteria, causing bacterial death. The aims of the current study were to assess the safety and efficacy of a PA phage cocktail (CT-PA) in a sheep model of rhinosinusitis.

The sheep rhinosinusitis model was adapted to simulate PA infection in sheep frontal sinuses. To assess efficacy, after a 7-day biofilm formation period, sheep received twice-daily frontal trephine flushes of CT-PA or saline for one week. Biofilm quantitation on the frontal sinus mucosa was performed using LIVE/DEAD BacLight staining. To assess safety, sheep received twice-daily frontal trephine flushes of CT-PA or vehicle control for 3 weeks. Blood and faecal samples were collected throughout treatment. Histopathology of frontal sinus, lung, heart, liver, spleen, and kidney tissue was performed. Sinus cilia were visualised using scanning electron microscopy (SEM).

The Efficacy arm showed a statistically significant reduction in biofilm biomass with all concentrations of CT-PA tested ($p<0.05$). Phage titration of sinuses showed phage presence was maintained for at least 16 hours after the final flush. All Safety arm sheep completed 3 weeks of treatment. Phage was detected consistently in faeces and sporadically in blood and organ samples. Histology and SEM of tissues revealed no treatment-related damage.

In conclusion, CT-PA was able to decrease frontal sinus PA biofilm at concentrations of $10^8$ to $10^{10}$ PFU/mL. No safety concerns were noted.

## 3.2 Introduction

Chronic rhinosinusitis (CRS) is an inflammatory condition of the nose and paranasal sinuses, persisting for 12 weeks or longer[3]. The prevalence of CRS amongst the general population ranges from 8% to 12% between different continents, and it carries a significant economic burden[493-497]. The aetiology of CRS is theorised to be multifactorial. Proposed aetiological factors include bacteria, viruses, fungi, and disruptions in the sinus microbiome, as well as an aberrant host immune response. Conventional treatments used in the management of CRS include sinus irrigations, topical and systemic corticosteroids, antibiotics, and endoscopic sinus surgery. From a public health perspective, CRS accounts for a significant burden of antibiotic use, with antibiotics being prescribed during 69% of 47.9 million outpatient visits for CRS, over a 5-year period in the United States[498].

*Pseudomonas aeruginosa* is a gram negative bacterial species that is present in the sinonasal tract of 9% of CRS patients, and up to 49% of patients with both cystic fibrosis (CF) and CRS[140, 349]. Increased abundance of *P. aeruginosa* in the sinus microbiome has been associated with poorer quality of life in CRS patients[133]. Additionally, the presence of *P. aeruginosa* on sinus cultures has been associated with CRS patients undergoing revision sinus surgery, suggesting that this species may play a role in disease recalcitrance[140].

One of the foremost concerns regarding the overuse of antibiotics is the emergence of antimicrobial resistance in bacteria[333]. Since the discovery of the antibacterial properties of penicillin in the 1940s, the introduction of new

antibiotics to widespread clinical use has been followed by the appearance of bacteria that are resistant to those antibiotics[499]. In particular, *P. aeruginosa* clinical isolates display significant rates of both intrinsic and acquired antibiotic resistance[207, 327].

In addition to antibiotic resistance, the formation of bacterial biofilms on the sinonasal mucosa of CRS patients plays a role in disease recalcitrance[155, 156, 162]. Bacterial biofilms consist of a community of bacteria living within a self-produced extracellular polymeric matrix, and attached to a biotic or abiotic surface[500]. Biofilm formation confers increased tolerance to antibiotics through various mechanisms, including impeded antibiotic penetration through the biofilm matrix, expression of resistance genes, accumulation of antibiotic degrading enzymes, and changes in cellular metabolism[466]. Prince et al demonstrated that more than 75% of both monomicrobial and polymicrobial sinus cultures containing *P. aeruginosa* from CRS patients were able to form biofilms *in vitro*[501]. The presence of *P. aeruginosa* in polymicrobial biofilms in the sinuses of CRS patients *in vivo* has also been associated with increased symptomatic and radiological severity[161].

Bacteriophages (phages) have been proposed as a novel treatment for bacterial infections, especially those caused by multidrug resistant bacteria[448, 502]. Obligately lytic bacteriophages are bacterial viruses that infect, replicate within, and then lyse bacteria to release copies of the phage, resulting in bacterial death[371]. Phages offer several advantages over conventional antibiotics in targeting bacterial infection in CRS. They are able to penetrate bacterial biofilms,

have activity against multidrug resistant isolates, and are species specific, preventing deleterious effects on commensal bacterial flora[425, 467, 503]. We have recently reported on the *in vitro* activity of an anti-*P. aeruginosa* phage cocktail (CT-PA) against *P. aeruginosa* CRS clinical isolates, including several multidrug resistant isolates[503].

We have previously developed the sheep sinusitis model, which simulates *S. aureus* bacterial biofilm infection in the frontal sinuses[504]. We adapted this model to simulate *P. aeruginosa* biofilm infection in CRS. The aims of this study were to assess the *in vivo* efficacy and safety of the anti-*P. aeruginosa* phage cocktail using this animal model, as well as to assess the local and systemic distribution of the bacteriophages when applied topically to the paranasal sinus mucosa.

## 3.3 Methods

This study was approved by the Animal Ethics Committees of the University of Adelaide and the South Australian Health and Medical Research Institute, Adelaide, South Australia. The use of previously collected *P. aeruginosa* clinical isolates in this study was approved the Human Research Ethics Committee of The Queen Elizabeth Hospital, Adelaide, South Australia.

**Bacterial strains and growth conditions**

A clinical CRS *P. aeruginosa* strain was isolated from an endoscopically-guided sinus swab and multilocus sequence typed (MLST), as previously described[503]. The chosen isolate was MLST sequence type 1399 (isolate Aus 20), which displays

significant biofilm formation. This isolate is sensitive *in vitro* to all four individual phages that comprise the phage cocktail, as well as to the four phages combined into a cocktail, as described below[503]. The isolate was stored in 25% glycerol in nutrient broth at -80°C. Isolates were plated from frozen glycerol stocks onto 1.5% nutrient agar plates, and broth cultures were grown in nutrient broth. Agar plates and broth cultures were incubated at 37°C.

**Bacteriophage cocktail**

1 mL aliquots of a bacteriophage cocktail (CT-PA) containing equal concentrations of each of 4 *P. aeruginosa* bacteriophages (Pa193, Pa204, Pa222, Pa223) in phosphate buffered saline supplemented with magnesium (PBS+Mg) were supplied by AmpliPhi Biosciences (Sydney, New South Wales, Australia). The cocktail was produced by combining equal concentrations of each individual phage; thus a cocktail with concentration 4 x $10^8$ PFU/mL was comprised of 1 x $10^8$ PFU/mL of each individual phage. Pa193 and Pa204 are of the Myoviridae family, and Pa222 and Pa223 are of the Podoviridae family. All 4 phages have been characterised as strictly lytic by genome sequencing (unpublished data).

**Adaptation of the Sheep Rhinosinusitis model**

The sheep rhinosinusitis model as described by Ha et al was adapted for use in this study[504].

A total of 32 Merino cross wethers sheep were used. 12 sheep were used in the model Optimisation arm, 14 sheep in the treatment Efficacy arm, and 6 sheep in the treatment Safety arm. Power calculations based on the results of a similar

study by Drilling et al indicated that 5 sinuses per group in the Efficacy arm would be sufficient to detect the estimated minimum effect size, at an alpha-level of 0.05 with 80% statistical power[461]. Two sheep in the Efficacy arm did not complete the study protocol, necessitating the use of 2 additional sheep. One sheep died from aspiration pneumonitis secondary to regurgitation during induction of anaesthesia, and another sheep had aberrant sinus anatomy that prevented effective occlusion of the frontal sinus ostia by nasal packing.

*Optimisation arm protocol*

The aims of the Optimisation arm were to assess the amount of *P. aeruginosa* bacterial culture required to achieve consistent biofilm formation in the frontal sinuses, and to assess any negative impact on animal welfare in the adapted model. Three groups of 4 sheep each were used. Each group consisted of 2 sheep with *P. aeruginosa* culture inoculated into the frontal sinuses bilaterally, and 2 contemporaneous control sheep that had only sterile 0.9% saline inoculated into their frontal sinuses. Infection sheep in the 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ groups were inoculated with 1 mL x $10^7$ CFU/mL, 1 mL x $10^8$ CFU/mL, and 2 mL x $10^8$ CFU/mL respectively into each frontal sinus. Control sheep received equivalent volumes of sterile saline. Animal welfare was monitored a minimum of twice daily through assessment for signs of pain, anorexia, dehydration, respiratory distress, and systemic infection. After a 7 day biofilm formation period, sheep were humanely killed, and the frontal sinuses harvested for biofilm quantification and fluorescence in situ hybridisation (FISH) biofilm detection. Microbiology swabs (Σ-Transwab Amies, Medical Wire & Equipment, Corsham, England) were taken

from the inside of each harvested frontal sinus, and sent to a clinical laboratory (Clinpath Laboratories, Adelaide, South Australia) for culture.

*Efficacy arm protocol*

The treatment Efficacy arm protocol was adapted from the protocol previously described by Drilling et al[461]. Following the insertion of pre-sterilised frontal trephines and nasal packing, a gentle lavage of 2 mL sterile saline into the frontal sinuses was performed, and microbiology swabs (Σ-Transwab Amies, Medical Wire & Equipment, Corsham, England) of the lavage fluid were sent to a clinical laboratory (Clinpath Laboratories, Adelaide, South Australia) in order to assess the commensal flora of the sheep's sinuses. Each frontal sinus was then inoculated with 2 mL of $10^8$ CFU/mL *P. aeruginosa* culture, based on the results of the Optimisation arm. Frontal trephines were capped and nasal packing remained in situ for 7 days following inoculation, to allow time for biofilm formation before the treatment period commenced. Each treatment group consisted of 3 sheep, each receiving a flush of treatment agent diluted into 50 mL sterile 0.9% saline twice daily, into each frontal trephine. Sheep received the same treatment to their left and right frontal sinuses. The four different treatments were 1 mL of: 1) 0.9% saline, 2) 4 x $10^8$ PFU/mL CT-PA, 3) 4 x $10^9$ PFU/mL CT-PA, and 4) 4 x $10^{10}$ PFU/mL CT-PA. Sheep were housed in individual pens, with at least 1 metre between the pens of sheep that were receiving different treatments, to prevent cross-contamination. Efficacy arm treatment groups are listed in supplemental data (Supplementary Table 1). Following 7 days' treatment, sheep were humanely killed and frontal sinuses harvested for biofilm

quantification, histopathology, and phage enumeration. Microbiology swabs were also taken from the inside of each harvested sinus, and sent to the clinical laboratory for culture as described above.

Faecal samples were collected from all phage-treated sheep prior to the administration of the first flush, and on Day 7 of treatment. 9mL blood samples were collected into Vacuette lithium heparin tubes without gel (Greiner Bio-One, Kremsmünster, Austria) from sheep via a jugular vein catheter at 1, 2 and 4 hours following the first trephine flush, and again at 1, 2, 4 and 8 hours following the morning trephine flush on Day 7 of treatment. Phage enumeration was carried out on faecal and blood samples as described below.

*Safety arm protocol*

The treatment Safety arm protocol was adapted from the protocol described by Drilling et al[461]. Two treatment groups of 3 sheep each were used. Sheep had twice-daily flushes of treatment agent diluted in 50mL sterile 0.9% saline into each frontal trephine, for 21 days. The two treatments were 1 mL 4 x $10^{10}$ PFU/mL CT-PA and 1mL PBS+Mg (vehicle control). Safety arm treatment groups are listed in supplemental data (Supplementary Table 2). Following the treatment period, sheep were humanely killed, and frontal sinuses as well as brain, heart, lungs, liver, spleen and kidneys were harvested for histopathology and phage enumeration. Sinuses were also assessed for ciliary integrity, as described below.

Faecal samples were collected from sheep on Days 8 and 15 of treatment, and following humane killing. Blood samples were collected via jugular vein catheters into Vacuette lithium heparin tubes without gel from sheep on Days 1, 8, and 15 of treatment, and just prior to humane killing. Phage enumeration was carried out on faecal and blood samples as described below.

**Biofilm quantification on sinus mucosa**

A previously defined protocol was used to quantify biofilm present on the frontal sinus mucosa[505, 506]. Frontal sinuses were placed into Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad CA, USA), and transported to the laboratory on ice. Under the laminar flow hood, two 1 $cm^2$ pieces of mucosa were dissected at random from each frontal sinus, and stained with LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies Australia, Mulgrave VIC, Australia). The entire piece of mucosa was then examined at 20x magnification using a confocal microscope (Zeiss LSM700, Carl Zeiss AG, Oberkochen, Germany). Z-stack images (80 slices, interval 0.80) were taken of the three largest biofilms from each piece of mucosa. COMSTAT version 2.1 was used to measure the biomass of biofilms, with threshold set manually to minimise background staining[507-509].

**Fluorescence in situ hybridisation (FISH) biofilm detection**

A 1 $cm^2$ piece of mucosa from each sinus in the Optimisation arm was collected after humane killing, and transported to the laboratory in DMEM, on ice. Samples were stored at -80°C prior to FISH analysis (protocol previously validated

by Foreman et al)[510]. Samples were defrosted and processed following the protocol described by Foreman et al, using an *E. coli*/*P. aeruginosa* PNA FISH probe (AdvanDx, Woburn MA, USA)[510] that specifically detects *P. aeruginosa* and *E. coli* with Texas Red and fluorescein-labelled probes respectively. Following the FISH protocol, epithelial cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Sydney, Australia). The mucosa was then examined at 20x, 40x, and 80x magnification using a confocal microscope (Zeiss LSM700, Carl Zeiss AG, Oberkochen, Germany) to identify *P. aeruginosa* biofilms.

**Histopathology**

A 1 $cm^2$ piece of sinus mucosa was collected from a standardised location in each frontal sinus after humane killing and immersion-fixed in 10% neutral buffered formalin. Tissue samples were then paraffin-embedded, cut at 6 µm, and stained with haematoxylin and eosin (H&E). Based on a previously published protocol, the parameters in Supplementary Table 3 were graded by a veterinary pathologist (J.F.) who was blinded to the treatment groups[511].

**Phage sensitivity testing of post-treatment isolates**

*P. aeruginosa* isolates cultured from sinuses inoculated with saline in the Optimisation arm and from phage-treated sheep in the Efficacy arm post-treatment were tested for sensitivity to CT-PA using the spot test assay described by Mazzocco et al[475].

**Assessment of sinus ciliary integrity**

Assessment of mucosal ciliary integrity was performed using scanning electron microscopy, as described by Ha et al[504]. Following humane killing sheep in the Safety groups, a 1 $cm^2$ piece of mucosa was dissected from each frontal sinus and placed directly into sterile PBS. Samples were transported to the laboratory on ice, and sonicated (Soniclean 80T, Soniclean, Adelaide, Australia) in an ice bath for 30 minutes to remove the adherent mucus layer. Samples were then transferred into 1 mL of electron microscopy fixative (4% paraformaldehyde/1.25% glutaraldehyde in PBS with 4% sucrose). After 72 hours, samples were washed in buffer, post-fixed in osmium tetroxide, and dehydrated by placing in sequentially increasing concentrations of ethanol (70%, 90%, and 100%), and then hexamethyldisilazine. Samples were then mounted onto stubs, coated with platinum, and viewed using a Philips XL30 field emission scanning electron microscope (Philips, Amsterdam, Netherlands).

**Haematology and blood biochemistry**

Blood samples were collected from Safety sheep via a central venous catheter inserted into the jugular vein, prior to the first phage flush (Day 1) and prior to humane killing (Day 22). Vacuette K3EDTA and serum separator clot activator tubes (Greiner Bio-One, Kremsmünster, Austria) were used to collect blood for haematology and serum biochemistry panels respectively. Testing was performed by a clinical laboratory (SA Pathology, Adelaide, South Australia).

**Phage enumeration in tissues and faeces**

Following humane killing and harvesting of frontal sinus mucosa, a 1 $cm^2$ piece of mucosa from each sinus was dissected aseptically and placed directly into 5 mL sterile phosphate buffered saline (PBS). 1 $cm^3$ organ samples were collected from the brain, lungs, heart, liver, kidney, and spleen of all Safety arm sheep, as well as 4 Efficacy arm sheep (# 19, 21, 22 and 23). All samples were transported to the laboratory in separate 5mL aliquots of sterile PBS, on ice. Phage titre in faecal and organ samples was measured using previously published methodology[461]. 1 g of sheep faeces in 5 mL sterile PBS spiked with 20 µL of $10^8$ PFU/mL CT-PA was used as a positive control for faecal samples. 5 mL sterile PBS spiked with 20 µL $10^8$ PFU/mL CT-PA was used as a positive control for organ and sinus samples. Phage enrichment of samples was performed as described by Drilling et al, with the use of the *P. aeruginosa* isolate in place of *S. aureus*[461].

**Phage enumeration in blood**

Blood tubes were transported to the laboratory on ice, and centrifuged at 1500 rpm (Eppendorf 5810R centrifuge, Eppendorf AG, Hamburg, Germany) for 20 minutes at 4°C. 3 mL of sheep blood spiked with 200 µL of $10^8$ PFU/mL CT-PA was used as a positive control. The plasma was then filtered through a 0.22 µm syringe filter. The small drop plaque assay was used to titrate the filtered plasma for phage. Sample phage enrichment was performed as described above.

**Statistics**

Biofilm biomass data were analysed as repeated observations clustered within piece, sinus and sheep. A linear mixed effects model was applied to assess group differences with sinus and sheep included as random factors. A log transformation was applied to the data prior to analysis due to violations of the distributional assumptions of a linear regression model (normally distributed with constant variance). The data were transformed back to the original scale prior to reporting. As a result, post hoc comparisons represent the ratio of two geometric means. All analyses were completed using SAS v9.4 (SAS Institute Inc., Cary, NC, USA).

Kruskal-Wallis H test (SPSS version 24, IBM, Armonk NY, USA) was performed to compare histopathological parameters between groups.

## 3.4 Results

**Optimisation arm**

Sheep across all groups displayed mild to moderate appetite loss in the first 24 to 48 hours following insertion of trephines and nasal packing, which resolved after 48 hours. One Control sheep ("inoculated" with saline only) was excluded from analysis due to a large amount of purulent discharge that developed around its frontal trephines, with swabs growing coliforms. No other adverse events occurred.

Of the microbiology swabs taken from the 12 Control sinuses, 4 grew *P. aeruginosa*, 3 grew coliforms, and swabs from the remaining 5 Control sinuses

showed no growth on cultures. All 4 *P. aeruginosa* isolates were sensitive to all 4 phages comprising the CT-PA phage cocktail. All except one sinus inoculated with *P. aeruginosa* grew *P. aeruginosa* on swab cultures, of these sinuses, 4 concurrently grew coliforms. The remaining *P. aeruginosa*-inoculated sinus had no growth on swab culture.

Macroscopic examination of the frontal sinus mucosa revealed inflamed mucosa in all sinuses inoculated with *P. aeruginosa*, with frank purulent secretions observed in all sinuses inoculated with 2mL x $10^8$ CFU/mL, and in one sinus inoculated with 1mL x $10^8$ CFU/mL. Only 2 Control sinuses from one sheep displayed mucosal erythema, of which one sinus contained frank pus, which swabbed positive for coliforms. The remainder of Control sinuses had macroscopically normal mucosa.

BacLight staining of infected sinuses showed the presence of biofilm structures consisting of green-fluorescing rod-shaped organisms (Figure 1). FISH biofilm detection using the *E. coli*/*P. aeruginosa* probe confirmed the presence of biofilm structures comprised of red-fluorescing rods, consistent with *P. aeruginosa* biofilms (Figure 2). Quantification of the biomass of BacLight-stained live biofilm structures by COMSTAT analysis of z-stack images showed that sinuses inoculated with *P. aeruginosa* grew significantly more biofilm compared to Control sinuses (mixed model analysis, p = 0.001, 0.02, and 0.004 for 1 mL x $10^7$, 1 mL x $10^8$, and 2 mL x $10^8$ CFU/mL respectively). There were no significant differences in biomass between the different amounts of *P. aeruginosa* inoculated. However, the sinuses inoculated with 2mL x $10^8$ CFU/mL *P.*

*aeruginosa* had biofilms distributed more evenly within the pieces of mucosa examined. Figure 3 shows the biomass of BacLight-stained biofilms in the different groups.

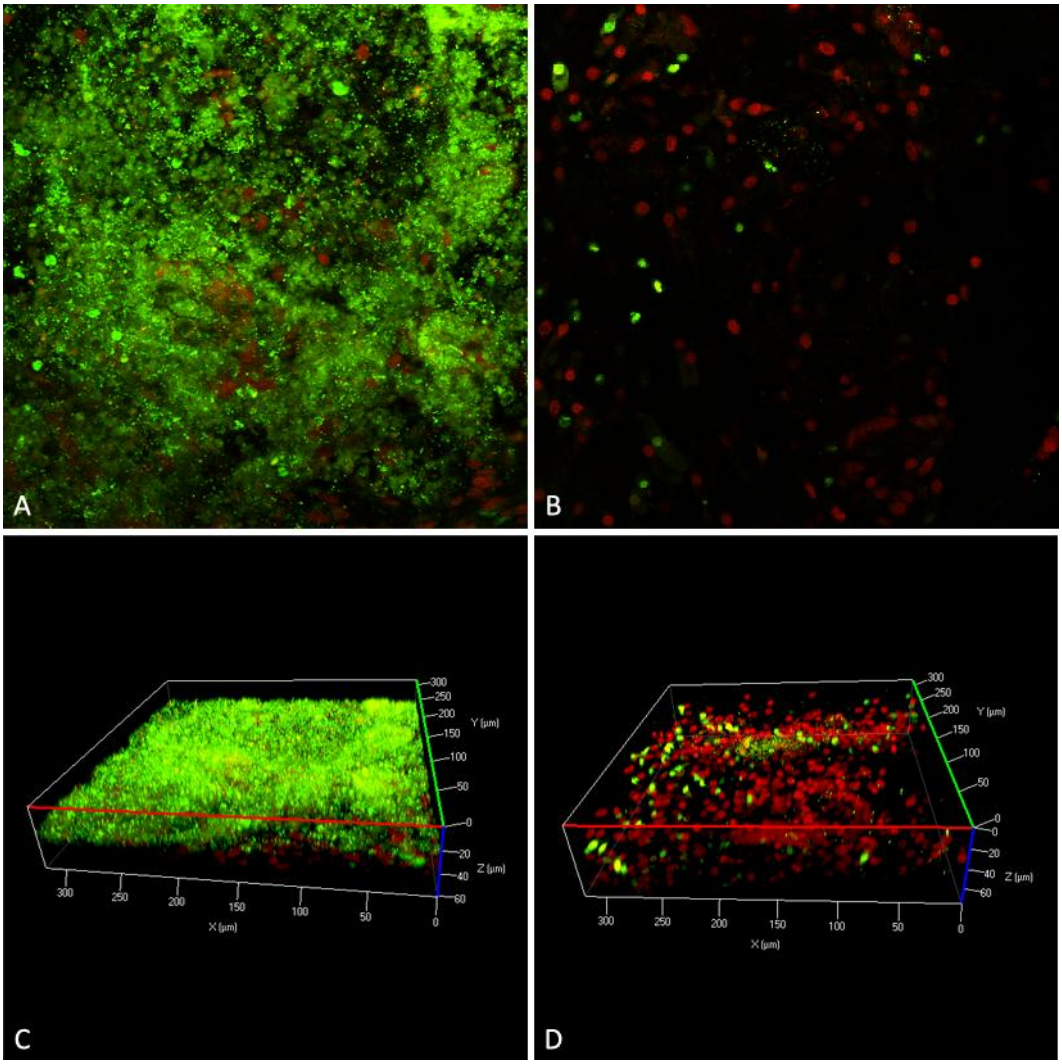


**Figure 1.** Optimisation arm: 2D cross-sectional images (A & B) and 3D reconstructions of z-stack images (C & D) (taken at 20x magnification) of BacLight-stained biofilm in a sinus inoculated with 2 mL x $10^8$ CFU/mL *P. aeruginosa* (A & C), and in a matched control sinus inoculated with 2 mL sterile 0.9% saline (B & D)

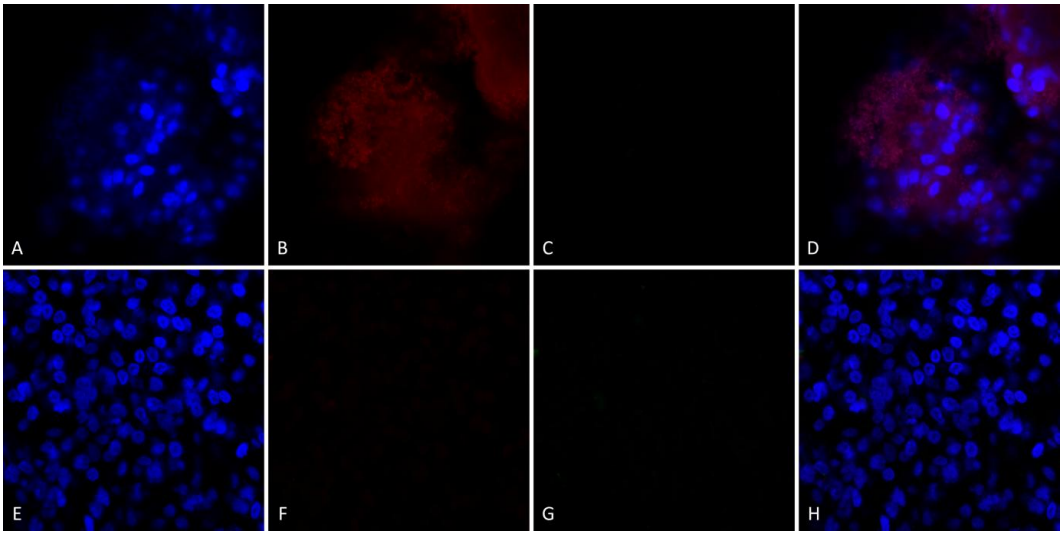


**Figure 2.** Optimisation arm: split channel images showing: epithelial cell nuclei counterstained blue with DAPI (A & E), Texas Red-tagged PNA FISH probe for *P. aeruginosa* (B & F), fluorescein-tagged PNA FISH probe for *E. coli* (C & G), and merged images (D & H) from a sinus inoculated with 2 mL x $10^8$ CFU/mL *P. aeruginosa* (A, B, C & D) and a matched control sinus inoculated with 2 mL sterile 0.9% saline (E, F, G & H).

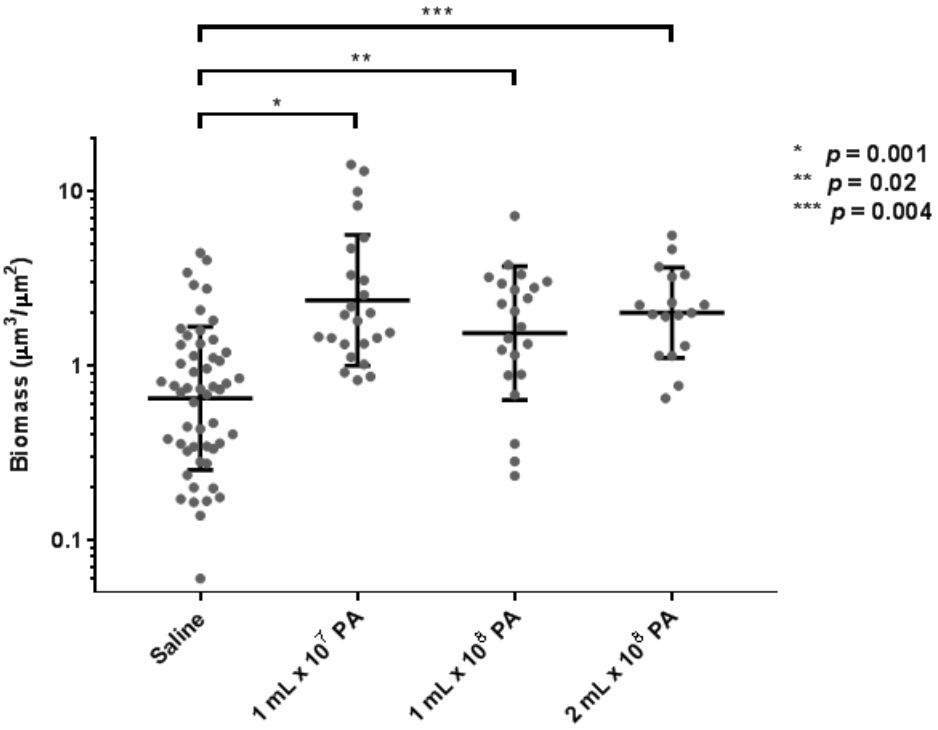


**Figure 3. Optimisation arm: biomass of sinus biofilms by dose of *P. aeruginosa* inoculated into frontal sinus.** The complete area of two randomly selected 1 $cm^2$ pieces of sinus mucosa from each frontal sinus was assessed for biofilms (n = 44 samples). Each data point represents the biomass of each of the three largest biofilms (as measured by Comstat quantification of biofilm biomass) from each piece of sinus mucosa. Log-transformed biofilm biomass data were analysed using a mixed effects model with piece nested in side and sheep. Error bars represent geometric mean ± geometric standard deviation. PA: *Pseudomonas aeruginosa*

**Efficacy arm**

*Pre-inoculation sinus microbiology*

The swab of pre-inoculation frontal sinus lavage was obtained bilaterally from all sheep in the $10^8$ and $10^{10}$ PFU/mL CT-PA and Saline treatment groups, and in 5 out of 6 sinuses in the $10^9$ PFU/mL CT-PA treatment group (23 sinus cavities in total). Overall, 15 of 23 sinuses had growth on the pre-inoculation swab; of these, 9 sinuses grew *P. aeruginosa*. Four swabs grew *Pseudomonas fluorescens*, 1 grew coliforms, and 1 grew skin flora. The outcome of pre-inoculation swabs is shown in Table 1.

**Table 1: Efficacy arm: Pre-inoculation and post-treatment sinus swab microbiology**

| Treatment group | Sheep # | Side | Pre-inoculation | Post-treatment* | Post-treatment isolate spot test assay CT-PA titre** (PFU/mL) |
|---|---|---|---|---|---|
| **Saline** | 17 | Right | No growth | No growth | n/a |
| | | Left | *P. aeruginosa* | *P. aeruginosa* | n/a |
| | 18 | Right | No growth | *P. aeruginosa* | n/a |
| | | Left | No growth | *P. aeruginosa* | n/a |
| | 19 | Right | *P. fluorescens* | *P. aeruginosa* | n/a |
| | | Left | *P. fluorescens* | *P. aeruginosa* | n/a |
| | | | | | |
| **$10^8$ CT-PA** | 21 | Right | *P. fluorescens* | *P. aeruginosa* | 1.0E+06 |
| | | Left | *P. fluorescens* | *P. aeruginosa* | 2.0E+06 |
| | 22 | Right | *P. aeruginosa* | Coliforms | n/a |
| | | Left | Skin flora | *P. aeruginosa* | Not tested |
| | 23 | Right | No growth | *P. aeruginosa* | 4.0E+06 |
| | | Left | No growth | Coliforms | n/a |
| | | | | | |
| **$10^9$ CT-PA** | 13 | Right | *P. aeruginosa* | Skin flora | n/a |
| | | Left | No growth | *P. aeruginosa* | 1.2E+07 |
| | 14 | Right | *P. aeruginosa* | *P. aeruginosa* | 1.0E+08 |
| | | Left | *P. aeruginosa* | *P. aeruginosa* | 1.0E+08 |
| | 15 | Right | No growth | *P. aeruginosa* | 1.0E+08 |
| | | Left | Not collected | *P. aeruginosa* | 1.0E+04 |
| | | | | | |
| **$10^{10}$ CT-PA** | 10 | Right | Coliforms | *P. aeruginosa* | 1.0E+08 |
| | | Left | *P. aeruginosa* | No growth | n/a |
| | 11 | Right | *P. aeruginosa* | Coliforms | n/a |
| | | Left | *P. aeruginosa* | *P. aeruginosa*, coliforms | 1.0E+08 |
| | 12 | Right | *P. aeruginosa* | Coliforms | n/a |
| | | Left | No growth | Coliforms | n/a |

n/a: not applicable

* Post-treatment swabs were taken following experimental inoculation with *P. aeruginosa* and the 7-day treatment period

** Tested against $10^8$ PFU/mL CT-PA

*Biofilm biomass*

The complete area of two randomly selected 1 $cm^2$ pieces of sinus mucosa from each frontal sinus was assessed for biofilms. The biomass of each of the three largest biofilms (as measured by Comstat quantification of biofilm biomass) from

each piece of sinus mucosa was analysed using a mixed effects model following log transformation of the data, with piece nested in side and sheep. Mean biomass was significantly lower in the three CT-PA treatment groups when compared to saline ($10^{10}$ PFU/mL: $p = 0.0007$; $10^{9}$ PFU/mL: $p = 0.049$; $10^{8}$ PFU/mL: $p = 0.003$). The three treatment groups did not differ significantly from each other. The biomass of frontal sinus biofilms following treatment for each group is displayed in Figure 4.

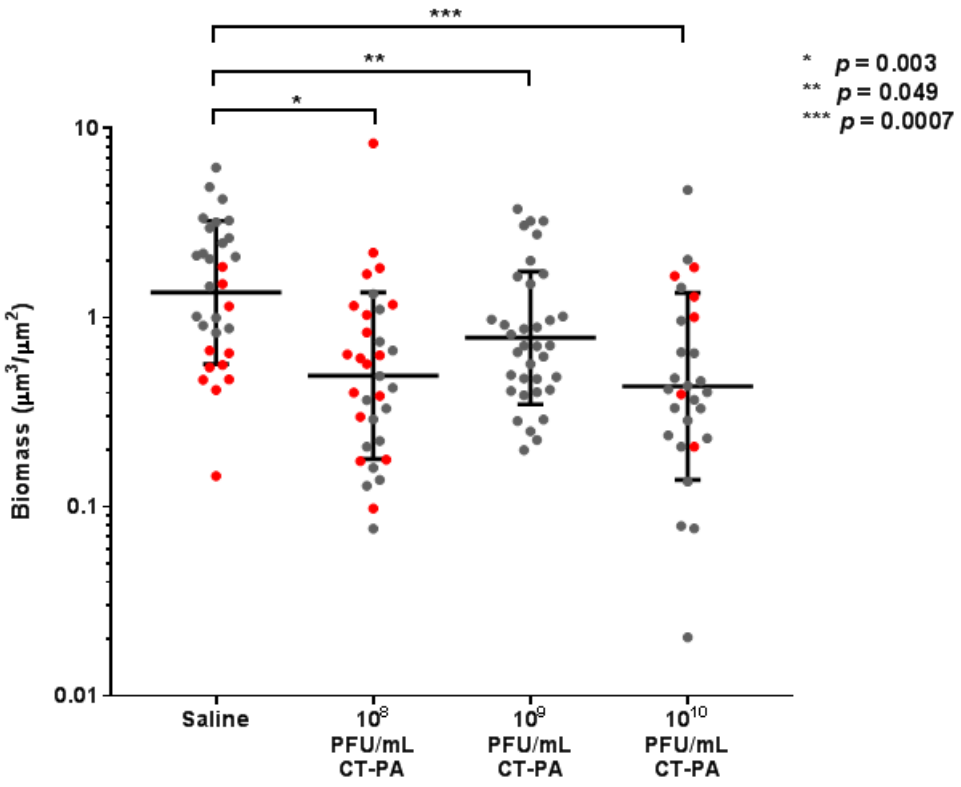


**Figure 4. Efficacy arm: biomass of frontal sinus biofilms following treatment.** The complete area of two randomly located 1 $cm^2$ pieces of sinus mucosa from each frontal sinus was assessed for bacterial biofilms using LIVE/DEAD BacLight staining (n = 48 samples). Each data point represents the biomass of each of the three largest biofilms (as measured by Comstat quantification of biofilm biomass) from each piece of sinus mucosa. Red data points represent data from sinuses that contained non-*Pseudomonas aeruginosa* bacterial species prior to inoculation with *P. aeruginosa*, whilst grey data points are from sinuses that lacked non-*P. aeruginosa* bacterial species prior to inoculation. Log-transformed biofilm biomass data from all sinuses were analysed using a linear effects model with piece nested in side and sheep. Error bars represent geometric mean ± geometric SD.

Regarding the effect of commensal sinus bacteria cultured from pre-inoculation lavage on post-treatment biofilm biomass, type III tests did not show a significant interaction between the presence of commensal *P. aeruginosa* prior to inoculation and treatment effect (p = 0.195). However, a significant interaction was present between the presence of non-*P. aeruginosa* bacteria prior to inoculation and treatment effect (p = 0.004). Post hoc comparisons demonstrate significant reductions in biofilm with all CT-PA treatments compared to saline when bacterial species other than *P. aeruginosa* were absent, but a lack of significant reduction when they were present (Table 2).

**Table 2: Efficacy arm: Post hoc comparisons between groups with and without bacterial species other than *P. aeruginosa* present prior to inoculation**

| **Other bacterial species present prior to inoculation** | **Comparison between groups** | | **Ratio of geometric means (95% CI)** | **p value** |
|---|---|---|---|---|
| No | $10^8$ CT-PA | Saline | 0.15 (0.07-0.35) | **<0.0001** |
| | $10^9$ CT-PA | Saline | 0.36 (0.18-0.73) | **0.006** |
| | $10^{10}$ CT-PA | Saline | 0.17 (0.08-0.35) | **<0.0001** |
| | $10^{10}$ CT-PA | $10^8$ CT-PA | 1.08 (0.49-2.38) | 0.846 |
| | $10^{10}$ CT-PA | $10^9$ CT-PA | 0.46 (0.24-0.89) | **0.022** |
| | $10^8$ CT-PA | $10^9$ CT-PA | 0.42 (0.20-0.90) | **0.027** |
| Yes* | $10^8$ CT-PA | Saline | 1.11 (0.45-2.75) | 0.813 |
| | $10^{10}$ CT-PA | Saline | 1.22 (0.36-4.06) | 0.744 |
| | $10^{10}$ CT-PA | $10^8$ CT-PA | 1.09 (0.36-3.36) | 0.873 |

* The $10^9$ CT-PA treatment group did not have any bacterial species other than *P. aeruginosa* grown on swabs of pre-inoculation lavage samples, thus no comparisons with this group could be performed for this outcome.

*Post-treatment sinus microbiology*

Post-treatment sinus swabs showed growth of *P. aeruginosa* in 4, 5, and 2 out of 6 sinuses in the $10^8$, $10^9$, and $10^{10}$ PFU/mL CT-PA treatment groups respectively, compared to 5 out of 6 sinuses treated with saline only. Of the CT-PA-treated sinuses where *P. aeruginosa* was not detected on the post-treatment swab, other bacteria (coliforms or skin flora) were isolated in all except one sinus. These bacterial species were also isolated on pre-inoculation swabs in both the Optimisation and Efficacy arms, although not necessarily in the same individual sheep. Of note, whilst *Pseudomonas fluorescens* was isolated from the pre-inoculation swabs of 4 sheep, it was not isolated from any of the post-treatment swabs. The outcome of post-treatment swabs is shown in Table 1.

All tested post-treatment *P. aeruginosa* isolates from CT-PA-treated sheep were sensitive to CT-PA on the spot test assays. One post-treatment *P. aeruginosa* isolate was unable to be tested as it was not retained by the clinical laboratory. In the $10^{10}$ PFU/mL treatment group, all isolates retained full sensitivity to CT-PA, whilst in the $10^9$ PFU/mL treatment group, one out of five isolates displayed a significantly decreased titre ($10^4$ PFU/mL), suggesting a decrease in sensitivity. Titres in the $10^8$ PFU/mL treatment group were slightly decreased, in the order of $10^6$ PFU/mL. Titres measured from the post-treatment spot test assays are shown in Table 1.

*Sinus histopathology*

There were no statistically significant differences in the degree of sinus mucosal inflammation, composition of sinus inflammatory cell population, epithelial hyperplasia, or fibrosis between groups. Degree of inflammation ranged from mild to severe in all groups. Figure 5 displays a breakdown of degree of sinus mucosal inflammation by treatment group. The inflammatory cell population was predominantly (≥ 95%) lymphoplasmacytic in 18 of 24 (75%) sinuses. Of the sinuses that displayed a neutrophilic infiltrate, 3 were treated with saline, 2 with $10^8$ PFU/mL CT-PA, and 1 with $10^{10}$ PFU/mL CT-PA. These results are summarised in Table 3, with representative sections displayed in Figure 6. None of the sinuses were found to have any detectable increase in fibrous tissue.

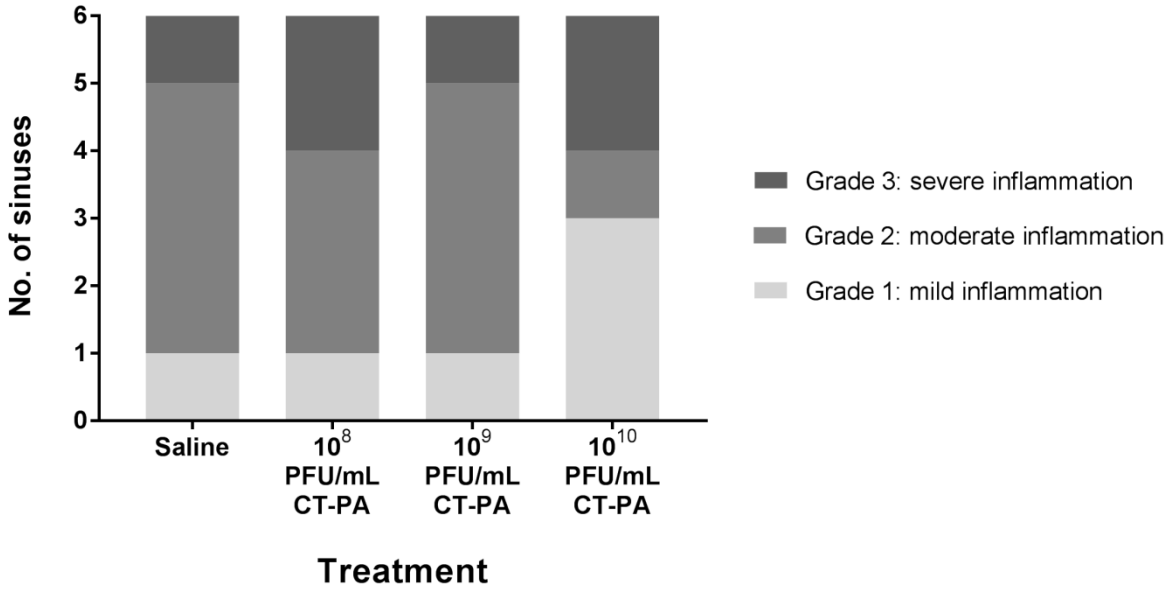


**Figure 5. Efficacy arm: Degree of histopathological sinus inflammation by treatment group**

Grade 1: <30% of superficial half of stroma occupied by inflammatory cells at a density of 50+ cells/high powered field

Grade 2: 30% - 60% of superficial half of stroma occupied by inflammatory cells at a density of 50+ cells/high powered field

Grade 3: >60% of superficial half of stroma occupied by inflammatory cells at a density of 50+ cells/high powered field

**Table 3: Efficacy arm: Composition of inflammatory cell population of frontal sinuses**

| Treatment | Sheep # | Frontal sinus side | Predominant type of inflammation | Other inflammatory cells detected |
|---|---|---|---|---|
| Saline | 17 | L | 95% LPC | 5% N |
| | | R | 90% LPC | 10% N |
| | 18 | L | 95% LPC | 5% N |
| | | R | 95% LPC | 5% N |
| | 19 | L | 60% N | 40% PC |
| | | R | 60% N | 40% PC |
| $10^8$ PFU/mL CT-PA | 21 | L | 95% LC | 5% N |
| | | R | 95% LC | 5% N |
| | 22 | L | 60% LC | 40% N |
| | | R | 60% LC | 40% N |
| | 23 | L | 95% LC | 5% N |
| | | R | 95% LC | 5% N |
| $10^9$ PFU/mL CT-PA | 13 | L | 95% LPC | 5% N |
| | | R | 95% LPC | 5% N |
| | 14 | L | 95% LPC | 5% N |
| | | R | 95% LPC | 5% N |
| | 15 | L | 95% LPC | 5% N |
| | | R | 95% LPC | 5% N |
| $10^{10}$ PFU/mL CT-PA | 10 | L | 95% LPC | 5% N |
| | | R | 95% LPC | 5% N |
| | 11 | L | 95% LPC | 5% N |
| | | R | 95% LPC | 5% N |
| | 12 | L | 50% LC; 50% N | n/a |
| | | R | 95% LPC | 5% N |

L: left side
R: right side
LPC: lymphoplasmacytic
N: neutrophilic
LC: lymphocytic
PC: plasmacytic
n/a: not applicable

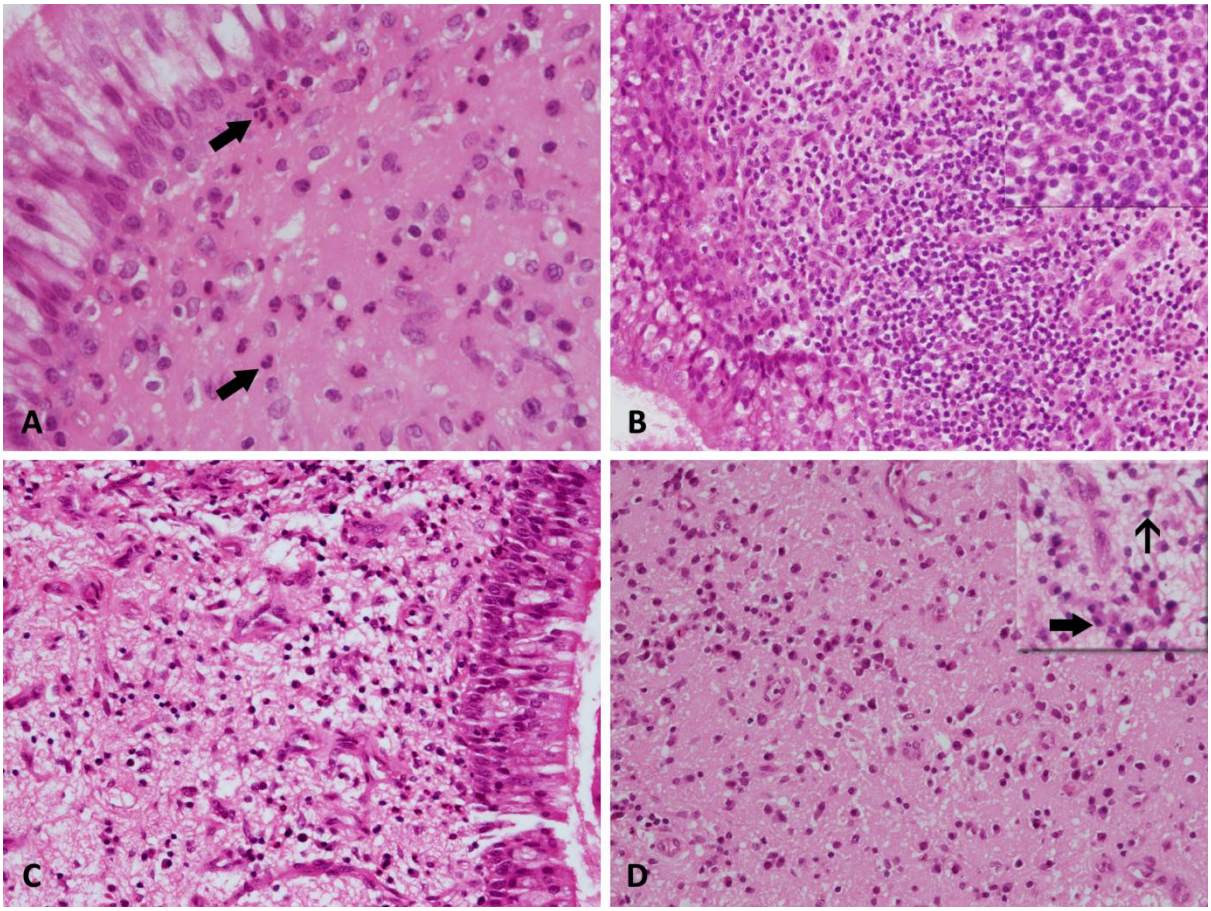


**Figure 6.** Representative H&E stained sections containing hyperplastic pseudostratified respiratory epithelium and underlying lamina propria, with the latter infiltrated by predominantly: (A) neutrophilic (neutrophils indicated by arrows), (B) lymphocytic (higher power view in inset), and (C) lymphoplasmacytic inflammatory cell populations.

(D) Lymphoplasmacytic inflammation of lamina propria, with higher power view in inset showing lymphocytes (arrow) and plasma cells (thick arrow).

*Local and systemic distribution of phage*

Sheep were humanely killed between 16 and 18 hours following administration of the last sinus flush. Measurable phage titres were detected in the sinus tissue of 13 out of 18 CT-PA-treated sinuses, with an additional 4 sinuses showing the presence of phage after enrichment. Post-treatment phage titres are shown in Table 4.

**Table 4: Efficacy arm: Sinus phage titres 16 to 18 hours following final flush**

| Treatment | Sheep # | Side | Sinus mucosa phage titre (PFU/cm$^2$) |
|---|---|---|---|
| $10^8$ PFU/mL CT-PA | 21 | R | 4.00E+03 |
| | | L | 1.95E+05 |
| | 22 | R | 1.34E+06 |
| | | L | * |
| | 23 | R | * |
| | | L | 4.00E+03 |
| $10^9$ PFU/mL CT-PA | 13 | R | 3.00E+03 |
| | | L | 1.85E+04 |
| | 14 | R | 6.00E+03 |
| | | L | 4.00E+03 |
| | 15 | R | 8.25E+04 |
| | | L | 5.85E+06 |
| $10^{10}$ PFU/mL CT-PA | 10 | R | * |
| | | L | - |
| | 11 | R | 7.63E+04 |
| | | L | 7.27E+06 |
| | 12 | R | * |
| | | L | 4.00E+03 |

R: right
L: left
- : phage not detected
*: phage detected after enrichment

Phage was detected in blood samples from Sheep #13 ($10^9$ PFU/mL CT-PA treatment group) on Day 7 of treatment, at all time points (titres of $8.8x10^3$,

$1.24x10^4$, $3.8x10^3$, and $3.0x10^3$ PFU/mL at 1, 2, 4, and 8 h post-dose respectively). Phage was detected after sample enrichment in blood samples from Sheep #10 and #11 ($10^{10}$ PFU/mL CT-PA treatment group) on Day 1 of treatment. No sheep had detectable phage in faecal samples taken prior to initiation of sinus flushes, and all CT-PA-treated sheep had phage detected in Day 7 faecal samples.

**Safety arm**

All Safety arm sheep completed 21 days of treatment. One sheep (# 30) was found deceased on the morning of Day 22; pathological findings were consistent with exacerbation of a background interstitial pneumonia by aspiration of the sinus flush. Two sheep experienced minor infection around the trephine sites, without associated loss of appetite, fever, or other signs of systemic illness. No other adverse events occurred.

*Haematology and biochemistry*

No clinically significant changes in complete blood count, white blood cell differential count, serum biochemistry, liver function tests, cholesterol, calcium, phosphate, albumin, or globulins were noted between blood samples taken prior to the first flush (Day 1) and following the completion of flushes (Day 22).

*Histopathology*

There was no statistically significant difference in degree of inflammation or composition of inflammatory cell population between frontal sinuses in the CT-

PA and vehicle control-treated groups. The degree of epithelial hyperplasia was significantly lower in the CT-PA group compared to the vehicle control group (p = 0.043). None of the sinuses were found to have any detectable increase in fibrous tissue. Histopathology results are summarised in Table 5.

**Table 5: Frontal sinus histopathology (Safety arm)**

| Treatment | Sheep # | Frontal sinus side | Degree of inflammation (grade*) | Predominant type of inflammation | Other inflammatory cells detected | Epithelial hyperplasia (grade*) |
|---|---|---|---|---|---|---|
| Vehicle control | 31 | L | 3 | 95% LPC | 5% N | 1 |
| | | R | 2 | 95% LPC | 5% N | 1 |
| | 32 | L | 1 | 60% PC | 40% N | 1 |
| | | R | 3 | 70% LPC | 30% N | 4 |
| | 33 | L | 2 | 95% LC | 5% PC | 1 |
| | | R | 3 | 100% LC | n/a | 1 |
| $10^{10}$ PFU/mL CT-PA | 28 | L | 1 | 80% PC | 20% N | 1 |
| | | R | 3 | 90% LC | 5% PC, 5% N | 1 |
| | 29 | L | 2 | 95% LPC | 5% N | 1 |
| | | R | 1 | 95% LPC | 5% N | 0 |
| | 30 | L | 2 | 95% LPC | 5% N | 0 |
| | | R | 1 | 80% N | 20% PC | 0 |

* Graded as described in Method
L: left side
R: right side
LPC: lymphoplasmacytic
N: neutrophilic
LC: lymphocytic
PC: plasmacytic
n/a: not applicable

No clinically significant histopathological findings were noted in any of the brain, heart, kidney, liver, or spleen samples. All lung samples from both CT-PA and vehicle control-treated sheep were noted to have changes consistent with mild

alveolar interstitial pneumonia, likely a residual lesion of lungworm infestation, which is common in Australian sheep.

*Sinus ciliary integrity*

One sinus treated with the phage cocktail (# 28 left) and one sinus treated with the vehicle control (# 33 left) showed some cells possessing microvilli, as well as cells with normal cilia. Another sinus treated with vehicle control (# 32 left) had an obscuring overlying mucus layer covering the majority of the sample, showing mostly cells with microvilli or short cilia in the areas not covered by the mucus layer. Cilia were preserved in all other sinuses. Representative images of each sinus are shown in Figure 7.

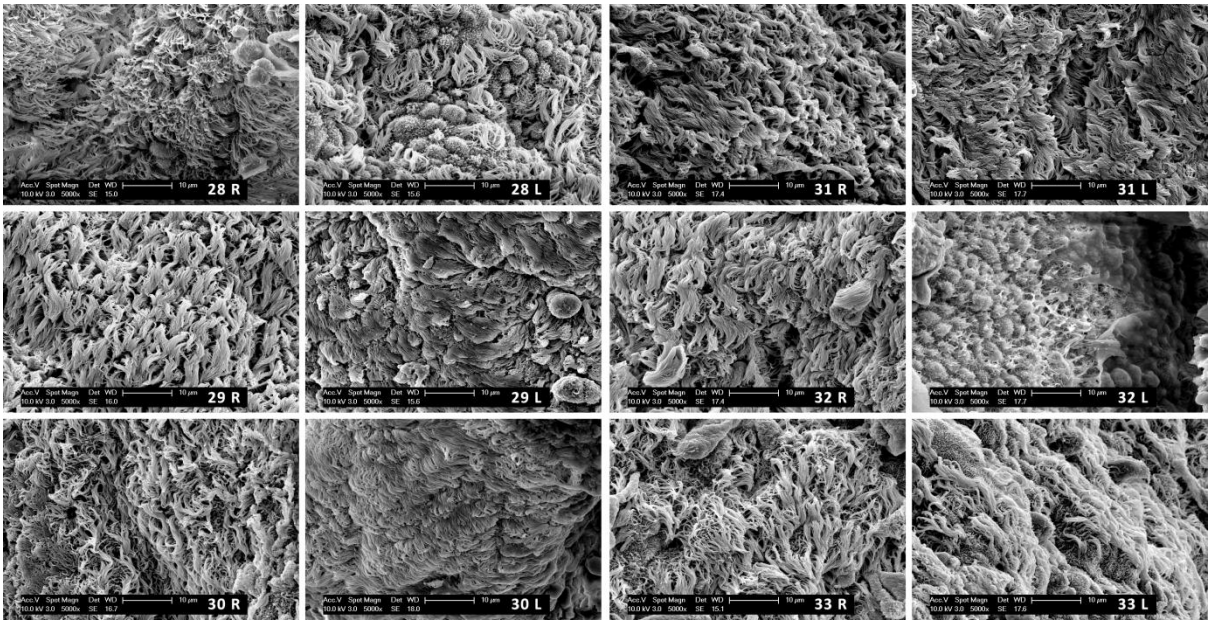


**Figure 7:** SEM images of frontal sinus mucosal samples from each sheep in the Safety arm (# 28, 29 & 30: CT-PA-treated sheep; # 31, 32 & 33: vehicle control-treated sheep; R: right frontal sinus; L: left frontal sinus). 5 of 6 sinuses in the CT-PA-treated group show intact cilia, with 1 sinus (# 28 left) showing a mix of ciliated cells and cells possessing microvilli. 4 of 6 sinuses in the vehicle control-treated group show intact cilia, with 1 sinus (# 33 left) showing a mix of ciliated cells and cells possessing microvilli, and another sinus (# 32 left) having an obscuring overlying mucus layer and cells possessing microvilli or short cilia.

*Local and systemic distribution of phage*

Phage was detected in the faeces of all CT-PA-treated sheep at all time points throughout treatment, with average titres of 6.87 x $10^3$, 5.64 x $10^4$, and 2.03 x $10^4$ PFU/g on Days 8, 15, and 22 respectively. Phage was not detected in any of the faecal samples from vehicle control-treated sheep.

Phage was only detected after sample enrichment in the blood of one CT-PA-treated sheep (# 30) on Day 22. Phage was not detected in blood samples from any of the other 5 sheep.

On phage titration of organ samples, phage was not detected in any samples from vehicle control-treated sheep. For CT-PA-treated sheep, phage was not detected in any brain samples. Measurable phage titres were found in only one lung sample (# 28) at a titre of $3.06 \times 10^4$ PFU/g. Phage was not detected in any other lung samples, even after sample enrichment. Phage was detected only after enrichment in one liver, one spleen, one kidney, and 2 heart samples (# 28 and 30).

Phage was detected after enrichment in 3 of 6 sinus samples from CT-PA-treated sheep, but not at detectable levels prior to enrichment.

## 3.5 Discussion

To our knowledge, this is the first study examining safety and efficacy of a novel treatment for biofilm-associated *P. aeruginosa* sinus infection in a large animal model. Whilst models of *P. aeruginosa* biofilm on airway epithelial cells *in vitro*, and in rabbit maxillary sinuses *in vivo*, have been developed, the sheep rhinosinusitis model enables simulation of treatment application via a sinus flush in frontal sinuses similar in size to the human paranasal sinuses[512, 513].

The ability of the phage cocktail to reduce the biomass of *P. aeruginosa* biofilms *in vivo* is in concordance with numerous studies demonstrating *P. aeruginosa* biofilm removal by bacteriophages *in vitro*[416, 418, 421, 514-519]. Phages have also

shown efficacy in treating *P. aeruginosa* biofilm-associated infections *in vivo*, such as canine chronic otitis and a murine model of keratitis[432, 433]. Of particular relevance to CF-associated CRS, phages have also demonstrated *in vivo* efficacy against *P. aeruginosa* CF isolates in murine models of lung infection[437, 439, 520].

Clinical trials of phage cocktails for treatment of *P. aeruginosa* infections in humans have had mixed results. Wright et al found a statistically significant improvement in symptom severity and clinician-assessed disease severity, as well as a drop in *P. aeruginosa* counts, in a double-blind randomised placebo-controlled trial in patients with antibiotic-resistant chronic *P. aeruginosa* otitis[443]. Conversely, a clinical trial of a phage cocktail designed to target both *P. aeruginosa* and *S. aureus*, applied to colonised burn wounds, did not find any advantage over standard of care[445]. It is worth noting that the authors describe several pitfalls of the study design, which may explain their results.

One aspect of phage therapy for CRS that we have attempted to address is whether targeting *P. aeruginosa* alone, in the context of co-infection with other bacterial species, is likely to have any beneficial effects. Our statistical analysis suggests that the presence of another bacterial species on pre-treatment sinus cultures predicts a poor outcome in terms of biofilm removal by CT-PA. The LIVE/DEAD BacLight stain does not differentiate between bacterial species with similar morphology (eg. *P. aeruginosa* and *E. coli*), so the presence of biofilms consisting partially or completely of non-*P. aeruginosa* species in these sinuses cannot be excluded. Given the species specificity of the phage cocktail, non-*P. aeruginosa* species are unlikely to be sensitive to the phage cocktail, which may

explain the lack of biofilm removal in these sinuses. *In vitro* studies of mixed species biofilms (*P. aeruginosa* with *Klebsiella pneumoniae* or *Escherichia coli*) have demonstrated a protective effect against phages targeted at a single species alone[521, 522]. The aforementioned studies suggest that simultaneous treatment with other anti-bacterial or anti-biofilm matrix agents such as xylitol or Tween 20 may overcome this effect[521, 522].

We were not able to detect any significant differences in histopathological severity of sinus inflammation between treatment groups in the Efficacy arm. Whilst correlations between tissue eosinophilia and radiological or endoscopic severity of CRS have been previously reported, the relationship between the severity of microscopic tissue inflammation, biofilms, and CRS treatment outcomes is unclear[523-525]. As such, the sheep *P. aeruginosa* rhinosinusitis model was developed primarily to assess efficacy of biofilm removal, rather than any effect on tissue inflammation. Potential confounding factors for analysis of tissue inflammation include the proximity of mucosal samples to the site of trephination, and the amount and species of commensal bacteria present in the sinuses. In addition, whilst the presence of *P. aeruginosa* is very uncommon in cultures from the healthy human sinonasal tract, we routinely found *P. aeruginosa* within the sinuses of sheep prior to experimental inoculation without clinical signs of infection [337-339]. *P. aeruginosa* may form part of the commensal flora of the paranasal sinuses in sheep and thus may not induce overt signs of infection and inflammation, even when artificially instilled.

Persisting high phage titres in the sinuses of Efficacy arm sheep, up to 18 hours following the final flush, implies phage amplification by successful phage replication (and therefore lysis of host bacteria). We did not assess whether regrowth of biofilms occurs upon cessation of phage flushes, however the continued presence of and bacterial sensitivity to the phage suggests that there is potential for an ongoing treatment effect. The finding of detectable phage in faeces during the treatment period was not unexpected, as many sheep were observed to swallow some of the sinus flushes. *P. aeruginosa* is found in the gastrointestinal microflora of healthy sheep, suggesting that phage amplification may also have occurred in the sheep gastrointestinal tract[526]. A human clinical trial of oral anti-*E. coli* phage T4 also detected faecal phage when subjects were administered $10^5$ PFU/mL phage in drinking water[463].

The sporadic detection of phage in sheep blood and organ samples, in most cases only after sample enrichment, suggests that phage may reach the systemic circulation at low levels after topical application into the paranasal sinuses. There are few other studies of the penetration of topically-administered phage into the systemic circulation. Bogovazova et al identified the presence of phage in the blood and internal organs of mice within 24 hours following the intranasal administration of *Klebsiella pneumoniae* phage[527]. Conversely, Drilling et al did not find measurable phage titres in the blood or internal organs of sheep administered *S. aureus* phage in the sheep rhinosinusitis model[461]. Studies of intravenous administration of phage suggest that neutralising antibody formation and the reticuloendothelial system may play a role in inactivation and

clearance of phage from the circulation, which could explain the variation in findings between studies[415, 528].

The pseudostratified respiratory epithelium lining the sinonasal mucosa is a complex epithelium containing ciliated, non-ciliated, mucous (goblet), and basal cells. The SEM appearance of some cells with microvilli or short cilia in 3 of 12 sinuses in the Safety arm is consistent with changes seen in regenerating human sinus mucosa, up to 6 months following sinus surgery[529]. Given the 3 week time frame between trephine insertion and the collection of samples for SEM, it is possible that the observed changes are due to healing of mucosal trauma incited during trephine insertion. Similar findings have been noted in other studies utilising the sheep rhinosinusitis model, where a small number of sinuses in both treatment and control arms demonstrated some areas of loss of cilia[461, 511].

In conclusion, the CT-PA phage cocktail has demonstrated efficacy in decreasing biofilm biomass in sinuses infected with *P. aeruginosa* biofilms, with no treatment-related adverse effects noted within the limitations of the sheep model. Given increasing concerns regarding antibiotic resistance and overuse, further investigation of the therapeutic potential of phage therapy in CRS would be valuable.

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# Chapter 4: Genomic variation and changes in antibiotic susceptibility in *Pseudomonas aeruginosa* clinical respiratory isolates with acquired resistance to a bacteriophage cocktail

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## Contextual statement

The prior work in this thesis has explored bacteriophage therapy as an alternative to conventional antibiotics for treatment of *Pseudomonas aeruginosa* infection in CRS and CF. Phage therapy has been suggested as a potential way to combat antibiotic-resistant bacteria, which have developed as a result of the widespread use of antibiotics. This raises the question of whether the use of phage as an alternative to conventional antibiotics would lead to phage-resistant bacteria. As such, the aims of this study were to elucidate any genetic changes that may result in phage resistance, and to determine if the development of a phage-resistant phenotype leads to changes in bacterial susceptibility to conventional antibiotics.

# Statement of authorship

| | |
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| Title of Paper | Genomic variation and changes in antibiotic susceptibility in *Pseudomonas aeruginosa* clinical respiratory isolates with acquired resistance to a bacteriophage cocktail |
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## Principal Author

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| Name of Principal Author (Candidate) | Dr. Stephanie Anne Fong | | |
| Contribution to the Paper | Study design, execution of experiments, data analysis, and writing of manuscript | | |
| Overall percentage (%) | 75% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
| Signature | | Date | 18/1/2020 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

the candidate's stated contribution to the publication is accurate (as detailed above);

permission is granted for the candidate in include the publication in the thesis; and

the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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| Signature | | Date | 9/1/2020 |

## 4.1 Abstract

*Pseudomonas aeruginosa* is an opportunistic pathogen that can cause sinus infections and serious lung infections in cystic fibrosis (CF) patients. It has a propensity for antibiotic resistance, and thus alternatives to conventional antibiotics for treating *P. aeruginosa* infections are under investigation. One such treatment is bacteriophage therapy. Whilst virulent (lytic) bacteriophages have shown promise in treating *P. aeruginosa* biofilm infections, the appearance of bacteriophage insensitive mutants (BIMs) following the exposure of bacteria to phages has been described.

The aim of this study was to examine the genetic changes associated with the bacteriophage insensitive phenotype, as well as to determine if there were any changes in antibiotic sensitivity in the BIMs.

Microtitre plate biofilms of 5 genetically distinct *P. aeruginosa* strains, including ATCC 15692 (PAO1) and 4 clinical respiratory isolates (2 CF and 2 non-CF) were grown for 7 days, and treated with either $4 \times 10^8$ PFU/mL of the phage cocktail (CT-PA) or a vehicle control for 7 consecutive days. BIMs were detected by streak assays and resistance to CT-PA was confirmed using spot test assays, followed by whole genome sequencing of the recovered BIMs and the vehicle control-treated phage sensitive isolates. Antibiotic sensitivity of planktonic BIMs and phage sensitive isolates was measured by minimum inhibitory concentration assays.

One BIM was isolated from vehicle control-treated biofilms in 1 strain, whilst BIMs were isolated from CT-PA-treated biofilms in all strains. Whole genome sequencing of the BIMs and phage-sensitive controls revealed a high number of mutations in CF strain BIMs, as well as variants in prophage Pf1 that differed in genotype between BIMs and phage-sensitive isolates. There were variable changes in conventional antibiotic sensitivities in the BIMs compared to phage-sensitive parent strains, particularly for beta-lactam antibiotics.

## 4.2 Introduction

*Pseudomonas aeruginosa* is an aerobic gram-negative bacterium, considered to be an opportunistic pathogen. It is ubiquitous in the environment, and capable of causing life-threatening sepsis, infective endocarditis, osteomyelitis, soft tissue infections, and respiratory infections[207]. *Pseudomonas aeruginosa* is of particular significance in cystic fibrosis-associated upper and lower respiratory tract infections. Chronic *P. aeruginosa* lung infection is associated with worsening lung function in cystic fibrosis (CF) patients. Phenotypic changes in the infecting strain over time can result in conversion to antibiotic-resistant mucoid phenotypes, which has been correlated with increased morbidity and mortality[530, 531]. Patients with CF are also frequently affected by chronic rhinosinusitis, in which colonisation of the paranasal sinuses with *P. aeruginosa* has been found to be a reservoir for recurrent lung infection[350, 353-355].

Treatment of *P. aeruginosa* infections is complicated by high rates of single-drug and multi-drug resistance[332, 333]. One potential treatment that is being investigated as an alternative to antibiotics is bacteriophage (phage) therapy. Virulent (lytic) bacteriophages are viruses that infect bacteria, replicate within the bacteria, and then cause bacterial lysis and death, releasing phage progeny in the process. Anti-*P. aeruginosa* bacteriophages have shown promise in several *in vitro* and *in vivo* pre-clinical studies[416-421, 532]. Some of the advantages of phage therapy over conventional antibiotics include activity against multi-drug resistant strains, the ability to target a specific bacterial species, and activity against biofilms[424, 425, 434, 503].

However, several studies have described the emergence of phage-resistant bacterial isolates, also known as bacteriophage insensitive mutants (BIMs), following treatment of *P. aeruginosa* biofilms with bacteriophages[516, 519]. Acquired resistance to bacteriophages may represent a potential barrier to the use of phage therapy in treating *P. aeruginosa* infections, but it may also be harnessed to decrease antibiotic resistance or as a target for the combination of phage with other anti-microbial therapies[533]. Chan et al demonstrated that

resistance to a phage that uses outer membrane porin M (OprM) of *P. aeruginosa* multi-drug efflux systems as a surface receptor produces an evolutionary trade-off in multi-drug resistant *P. aeruginosa*, resulting in increased sensitivity to conventional antibiotics[534].

In order to utilise anti-*P. aeruginosa* bacteriophage therapy in the most efficacious way, it is important that we understand the mechanisms underlying acquired phage resistance. The primary aim of this study was to examine genome variation in *P. aeruginosa* clinical isolates from the upper and lower respiratory tract that have acquired phage resistance following repeated exposure to a phage cocktail. A *in vitro* biofilm model was chosen as *P. aeruginosa* biofilms have been demonstrated in chronic respiratory infections, and are associated with recurrent and treatment-recalcitrant disease[158, 159, 486, 535]. Our secondary aim was to determine if the development of phage resistance resulted in associated changes in antibiotic susceptibility.

## 4.3 Methods

### Ethics

This study was approved by the Human Research Ethics Committee of The Queen Elizabeth Hospital, Adelaide, South Australia (approval number HREC/13/TQEHLMH/277).

### Bacterial strains

Four clinical *P. aeruginosa* strains (Aus 4, Aus 16, Ned 5, USA 2) were isolated from the sinuses of CRS patients, as previously described[503]. Two clinical isolates (Aus 16 and Ned 5) were from CF patients. *P. aeruginosa* laboratory reference strain ATCC 15692 (PAO1) was obtained from American Type Culture Collection (Manassas, VA, USA). All 5 strains exhibited a high degree of sensitivity to the bacteriophage cocktail (CT-PA) when tested using the spot test assay described by Mazzocco et al[475, 503]. All strains were stored in 25% glycerol in nutrient broth

at -80°C, and grown on 1.5% nutrient agar or in nutrient broth (Oxoid, Hants, UK) at 37°C. Cation-adjusted Mueller-Hinton broth (Oxoid, Hants, UK) was used for minimum inhibitory concentration assays.

**Bacteriophage cocktail**

Stocks of 4 anti-*P. aeruginosa* bacteriophages (Pa193, Pa204, Pa222, and Pa223) in phosphate-buffered saline with 0.01M magnesium sulphate (PBS+Mg) were supplied by AmpliPhi Biosciences (Brookvale, New South Wales, Australia). Pa193 and Pa204 are of the Myoviridae family, and Pa222 and Pa223 are of the Podoviridae family. All 4 phages have been characterised as strictly lytic by genome sequencing (unpublished data). Prior to each assay, the stock suspension of each bacteriophage was titrated against a selected *P. aeruginosa* bacterial strain using the soft agar overlay small drop assay, as described by Mazzocco et al[(475)]. Equal concentrations of each bacteriophage were combined to form the bacteriophage cocktail (CT-PA) at a concentration of $4 \times 10^8$ PFU/mL.

**Generation of bacteriophage insensitive mutants**

Biofilms of each *P. aeruginosa* strain were grown on separate clear polystyrene 96-well plates, as previously described[(503)]. A total of 48 wells, comprised of 2 blocks containing 24 wells each separated by PBS-filled wells, were inoculated in each plate. Plates were incubated on a gyratory mixer in a 37°C incubator for 7 days to encourage biofilm formation, with 50 µL/well nutrient broth replenished at 24 hour intervals. After the first 7 days, every 24 hours 50 µL of liquid media was aspirated from each well immediately substituted with 50 µL of either $4 \times 10^8$ PFU/mL CT-PA or PBS+Mg as a vehicle control. For each strain, one "block" of 24 wells was treated with CT-PA every 24 hours, whilst the other "block" was treated with PBS+Mg every 24 hours, for 7 consecutive days. Following 7 days of treatment, liquid contents of wells were gently aspirated and discarded. Biofilms were gently washed with PBS twice to remove any remaining planktonic cells. The method for isolating bacteriophage insensitive mutants (BIMs) was adapted from Buckling et al[(536)]. 200 µL of $10^{10}$ PFU/mL CT-PA was spread evenly onto 1.5% nutrient agar plates, and excess liquid allowed to evaporate. A sterile cotton

swab was used to streak bacteria from each row of wells in each CT-PA-treated "block" separately onto these agar plates. The same process was used to streak bacteria from the PBS+Mg-treated blocks onto 1.5% nutrient agar plates that had not been inoculated with phages. Agar plates were then incubated at 37°C overnight. Streaks that displayed bacterial growth without phage plaques were subcultured onto fresh 1.5% agar plates, and sensitivity or resistance to CT-PA was confirmed using the spot test assay.

**Minimum inhibitory concentration assays**

Minimum inhibitory concentration of ciprofloxacin, gentamicin, amikacin, piperacillin and ceftazidime (Sigma-Aldrich, Castle Hill, NSW, Australia) was tested for parent strains and BIMs using the broth microdilution method described by Wiegand et al[474].

**Bacterial DNA extraction and whole genome sequencing**

Bacterial genomic DNA extraction and purification was performed as previously described[503]. The quality of the purified DNA was assessed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham MA, USA) and gel electrophoresis. DNA samples were sent to an external laboratory (Australian Genome Research Facility, Melbourne, Australia) for TruSeq Nano (Illumina, San Diego, CA) library preparation and whole genome sequencing on the MiSeq platform (Illumina) with 250 bp paired-end reads. Read quality was assessed using FastQC (version 0.11.7)[537]. Adapter sequences were trimmed using cutadapt (version 1.16) in paired-end mode, with quality trimming with a threshold of 20[538]. Bwa (version 0.7.17-r1188) was used to align reads against the *P. aeruginosa* PAO1 reference genome (RefSeq accession number NC_002516.2)[539]. Duplicate reads were marked with Picard MarkDuplicates (version 2.8.1)[540]. Mapped read depth was calculated using bedtools genomecov (version 2.26.0)[541].

**Variant calling and annotation**

Freebayes (version 1.0.0) was used to call variants in the 10 samples with a minimum mapping quality of 20, minimum base quality of 30, and minimum alternate fraction of 0.5, with ploidy set to 1[542]. Variants were annotated using SnpEff (version 4.3t) based on the *Pseudomonas aeruginosa* PAO1 reference genome[543]. Genes containing variants that differed between the vehicle-treated sample and BIM for each strain were cross-referenced with the Antimicrobial Resistance (AMR) Gene Predictions from the Comprehensive Antibiotic Resistance Database, as listed on the Pseudomonas Genome Database (www.pseudomonas.com). Structural variant detection was performed using Delly2 (version 0.7.9) with default parameters[544]. The resulting variant call format (VCF) files were merged using Picard MergeVcfs (version 2.8.1). Biopet VcfFilter (version 0.1) was used to filter for variants that differed in genotype between phage sensitive vehicle-treated strains and BIMs[545].

**Genome-wide association analysis**

Consensus fasta genome sequences were generated from assemblies, and annotated with Prokka (version 1.13.3) using the *P. aeruginosa* PAO1 reference genome annotations (Refseq accession number NC_002516.2) as well as Prokka's inbuilt databases[546]. Roary (version 3.12.0) was then used to determine gene presence or absence for all samples[547]. Scoary (version 1.6.16) was then used to determine if there were any statistically significant associations between gene presence/absence or the variants detected by Freebayes, and resistance to the phage cocktail or antibiotic resistance[548]. Scoary analysis was performed using a *p* value cut-off of 0.05 with Benjamini-Hochberg correction.

## 4.4 Results

### Generation of BIMs

BIMs were isolated from all CT-PA-treated biofilm plates, and confirmed as resistant to CT-PA by the complete absence of spots and plaques on the spot test assay (Figure 1). One BIM was also isolated from vehicle-treated PAO1 biofilms. No BIMs were detected in any of the other vehicle-treated biofilms.

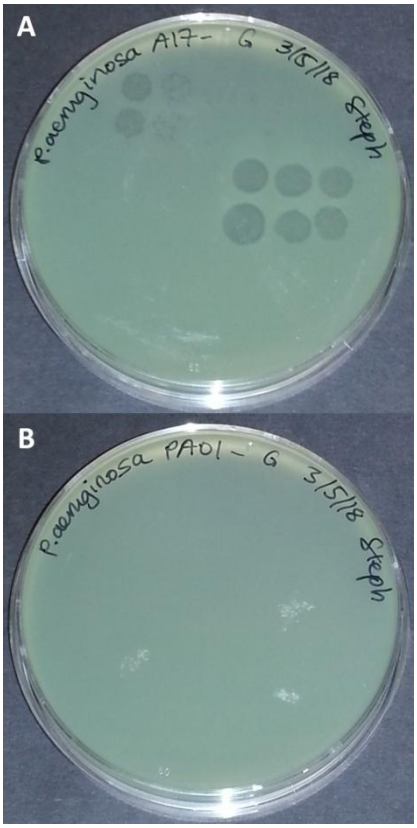


**Figure 1: Representative spot test assays showing (A) phage lysis spots on the bacterial lawn of a phage sensitive vehicle-treated isolate and (B) absence of phage lysis spots on the bacterial lawn of a bacteriophage insensitive mutant (BIM) generated following treatment with CT-PA for 7 days**

### Sensitivity of BIMs to conventional antibiotics

The MIC values of several conventional antibiotics for the isolated BIMs, compared to each BIM's respective parent strain, are shown in Table 1.

Sensitivity or resistance to the aminoglycosides tested (gentamicin and amikacin) remained largely stable between parent isolates and BIMs for all strains. In one isolate (USA 2), the BIM displayed intermediate sensitivity to ciprofloxacin, whilst its parent isolate was resistant. There were a variety of changes observed in MICs of piperacillin and ceftazidime between parent and BIM isolates. In one isolate (Aus 4), MICs remained stable for both antibiotics. In the two other non-CF isolates and PAO1 reference strain, MICs for both piperacillin and ceftazidime moved from being sensitive into the intermediate sensitivity range. In the two CF isolates, sensitivity or resistance to ceftazidime remained largely stable. One CF parent isolate (Aus 16) that was sensitive to piperacillin developed intermediate sensitivity to piperacillin in the BIM, and in the other CF parent isolate (Ned 5) that was resistant to piperacillin, sensitivity to piperacillin was observed in the BIM.

**Table 1: Minimum inhibitory concentration (mg/L) of various conventional antibiotics against BIMs, compared to parent strains**

| Antibiotic | Non-CF isolates | | | | | | CF isolates | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | PAO1 parent | PAO1 BIM | Aus 4 parent | Aus 4 BIM | USA 2 parent | USA 2 BIM | Ned 5 parent | Ned 5 BIM | Aus 16 parent | Aus 16 BIM |
| Ciprofloxacin | 0.25 | 1 | 0.25 | 1 | 64 | 1 | >128 | 32 | 2 | 2 |
| Amikacin | 8 | 16 | 16 | 16 | 4 | 16 | >128 | >128 | 128 | 64 |
| Gentamicin | 2 | 4 | 4 | 4 | 2 | 4 | >128 | 128 | 32 | 16 |
| Piperacillin | 8 | 32 | 16 | 16 | 1 | 32 | >128 | 4 | 8 | 32 |
| Ceftazidime | 8 | 16 | 16 | 16 | 1 | 16 | 64 | 32 | 8 | 8 |

Sensitive *
Intermediate sensitivity *
Resistant *

* according to Clinical and Laboratory Standards Institute breakpoints (M100, 29th edition)

## Whole genome sequencing

Mean read depth and genome coverage of the assembled genomes are displayed in Table 2. Mean read depth of >90 was achieved across all samples. Genome coverage was >99% for all samples except the CF strain BIMs (Ned 5 and Aus 16).

**Table 2: Mean read depth and genome coverage of assembled genomes**

| Isolate | Vehicle-treated | | BIM | |
|---|---|---|---|---|
| | Mean read depth | Genome coverage (%) | Mean read depth | Genome coverage (%) |
| PAO1 | 92 | 99.1 | 97 | 99.1 |
| Aus 4 | 98 | 99.1 | 99 | 99.1 |
| USA 2 | 115 | 99.1 | 95 | 99.1 |
| Ned 5 | 92 | 99.1 | 95 | 96.2 |
| Aus 16 | 97 | 99.1 | 90 | 95.1 |

**Variant calling and annotation**

Overall amongst all samples, 41603 variants and 98 structural variants were identified by Freebayes and Delly2 respectively as meeting the criteria described above. A large number of variants were identified in CF strain BIMs, with the Ned 5 BIM having 27076 variants and the Aus 16 BIM having 27987 variants, compared to 119 and 122 variants respectively for the vehicle-treated Ned 5 and Aus 16 samples (Figure 2A). In contrast, the number of variants identified in non-CF strain vehicle-treated samples and BIMs were similar and ranged from 124 to 157 (Figure 2A). Both CF strain BIMs had a higher proportion of SNPs, and a lower proportion of indels and mixed variants, compared to the other samples (Figure 2B). Inversions and duplications were only identified in the CF strain BIM samples.

**Figure 2A: Total variants per sample**

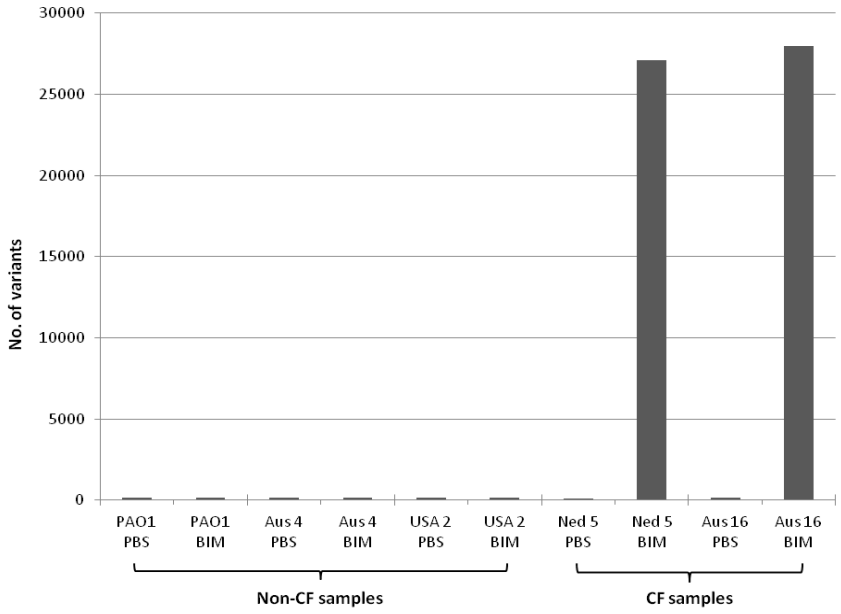


**Figure 2B: Breakdown of variant types by sample**

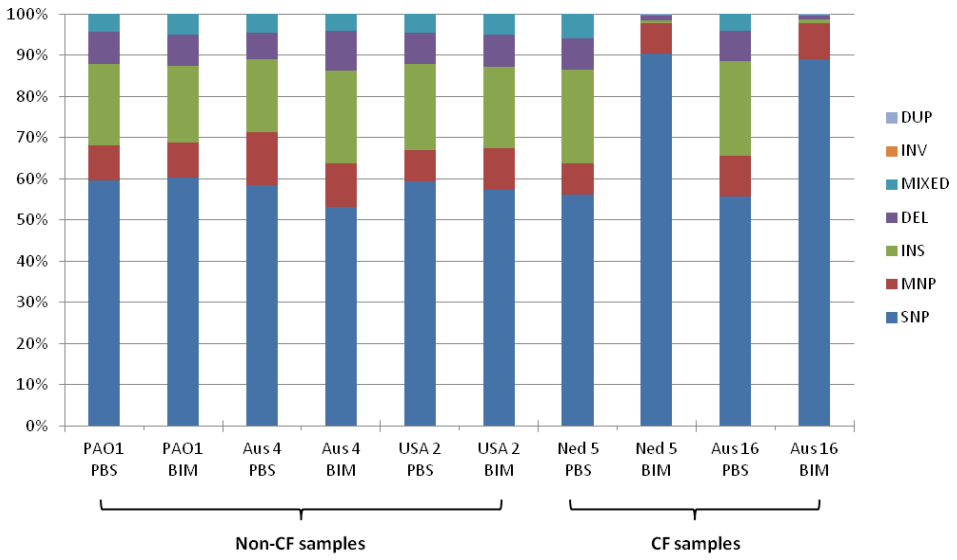


**Figure 2:** (A) Total number of variants and (B) breakdown of proportion of variant types per sample for cystic fibrosis (CF) and non-CF strains treated with vehicle control (labelled PBS) vs. CT-PA (labelled BIM) for 7 days

DUP: duplication; INV: inversion; MIXED: mixed variant; DEL: deletion; INS: insertion; MNP: multi-nucleotide polymorphism; SNP: single-nucleotide polymorphism; BIM: bacteriophage insensitive mutant

Nine sites of genome variation were identified as consistently having differing genotypes between phage-sensitive vehicle-treated isolates and BIMs across all strains. These sites were all clustered in the 789979 to 794064 bp region of the *P. aeruginosa* genome (Figure 3). Seven single-nucleotide polymorphisms and 3 multi-nucleotide polymorphisms were identified at these sites, including 7 synonymous variants and 2 missense variants. SnpEff predicted effects on 20 genes, tabulated in Table 3.

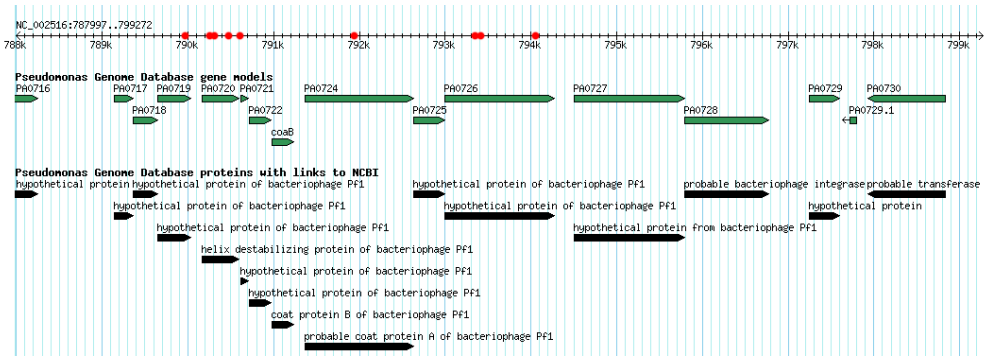


**Figure 3: 787997 to 799272 bp region of the *P. aeruginosa* PAO1 reference genome as displayed on Gbrowse, with sites of consistent genome variation between phage-sensitive vehicle-treated isolates and BIMs indicated by red markers.**

**Table 3: Predicted effects of variants where genotype differed between phage-sensitive isolates and BIMs across all strains**

| Gene Name | Gene Id | Description | Variant impact | Variant effect |
|---|---|---|---|---|
| PA0714 | PA0714 | protein_coding | MODIFIER | Downstream |
| PA0715 | PA0715 | protein_coding | MODIFIER | Downstream |
| PA0716 | PA0716 | protein_coding | MODIFIER | Downstream |
| PA0717 | PA0717 | protein_coding | MODIFIER | Downstream |
| PA0718 | PA0718 | protein_coding | MODIFIER | Downstream |
| PA0719 | PA0719 | protein_coding | LOW, MODERATE, MODIFIER | Downstream, missense, synonymous |
| PA0720 | PA0720 | protein_coding | LOW, MODERATE, MODIFIER | Downstream, missense, synonymous, upstream |
| PA0721 | PA0721 | protein_coding | MODIFIER | Downstream, upstream |
| PA0722 | PA0722 | protein_coding | MODIFIER | Downstream, upstream |
| PA0724 | PA0724 | protein_coding | LOW, MODIFIER | Downstream, synonymous, upstream |
| PA0725 | PA0725 | protein_coding | MODIFIER | Downstream, upstream |
| PA0726 | PA0726 | protein_coding | LOW, MODIFIER | Synonymous, upstream |
| PA0727 | PA0727 | protein_coding | MODIFIER | Upstream |
| PA0728 | PA0728 | protein_coding | MODIFIER | Upstream |
| PA0729 | PA0729 | protein_coding | MODIFIER | Upstream |
| PA0729.1 | PA0729.1 | protein_coding | MODIFIER | Downstream |
| PA0730 | PA0730 | protein_coding | MODIFIER | Downstream |
| coaB | PA0723 | protein_coding | MODIFIER | Downstream, upstream |
| phrD | PA0714.1 | lincRNA | MODIFIER | Downstream |
| tRNA | EBG00001435503 | protein_coding | MODIFIER | Upstream |

Cross-referencing of variant-containing genes in which the genotype differed between the vehicle-treated sample and the BIM for each strain against the Antimicrobial Resistance Gene Predictions revealed variants in several antimicrobial resistance genes, listed in Table 4.

**Table 4: Antimicrobial resistance gene variants in which genotypes differed between vehicle-treated isolate and BIM for each strain**

| Gene or Product Name | Locus Tag | PAO1 | Aus 4 | USA 2 | Ned 5 | Aus 16 |
|---|---|---|---|---|---|---|
| gyrB | PA0004 | | | | | |
| triA | PA0156 | | | | | |
| triB | PA0157 | | | | | |
| triC | PA0158 | | | | | |
| mexA | PA0425 | | | | | |
| mexB | PA0426 | | | | | |
| oprM | PA0427 | | | | | |
| cat | PA0706 | | | | | |
| alaS | PA0903 | | | | | |
| alanyl-phosphatidylglycerol synthase | PA0920 | | | | | |
| fosA | PA1129 | | | | | |
| farB | PA1236 | | | | | |
| lfrA | PA1282 | | | | | |
| lfrA | PA1316 | | | | | |
| mexM | PA1435 | | | | | |
| mexN | PA1436 | | | | | |
| cysB | PA1754 | | | | | |
| eptA | PA1972 | | | | | |
| mexY | PA2018 | | | | | |
| mexX | PA2019 | | | | | |
| pvdR | PA2389 | | | | | |
| pvdT | PA2390 | | | | | |
| opmQ | PA2391 | | | | | |
| mexS | PA2491 | | | | | |
| mexE | PA2493 | | | | | |
| mexF | PA2494 | | | | | |
| oprN | PA2495 | | | | | |
| opmB | PA2525 | | | | | |
| muxC | PA2526 | | | | | |
| muxB | PA2527 | | | | | |
| opmA | PA2837 | | | | | |
| mfd | PA3002 | | | | | |
| uup | PA3019 | | | | | |
| farB | PA3137 | | | | | |
| gyrA | PA3168 | | | | | |
| opmE | PA3521 | | | | | |
| mexQ | PA3522 | | | | | |
| mexP | PA3523 | | | | | |
| arnC | PA3553 | | | | | |
| arnA | PA3554 | | | | | |
| nalD | PA3574 | | | | | |
| mexK | PA3676 | | | | | |
| mexJ | PA3677 | | | | | |
| mexL | PA3678 | | | | | |
| opmI | PA3894 | | | | | |
| rocS1 | PA3946 | | | | | |
| ampC | PA4110 | | | | | |
| aph | PA4119 | | | | | |
| mexG | PA4205 | | | | | |
| mexH | PA4206 | | | | | |
| mexI | PA4207 | | | | | |
| opmD | PA4208 | | | | | |
| tufA | PA4265 | | | | | |
| tufB | PA4277 | | | | | |
| mexV | PA4374 | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| **mexW** | PA4375 | | | | |
| **ileS** | PA4560 | | | | |
| **yjjK** | PA4595 | | | | |
| **oprJ** | PA4597 | | | | |
| **mexD** | PA4598 | | | | |
| **mexC** | PA4599 | | | | |
| **nfxB** | PA4600 | | | | |
| **opmH** | PA4974 | | | | |
| **emrE** | PA4990 | | | | |
| **msbA** | PA4997 | | | | |
| **opmG** | PA5158 | | | | |
| **emrB** | PA5160 | | | | |
| **OXA-50** | PA5514 | | | | |
| **ybaL** | PA5518 | | | | |

Shaded cells indicate variant genotype differed between vehicle-treated isolate and BIM

**Genome-wide association analyses**

Prokka identified between 5668 and 5673 coding sequences per isolate consensus genome, and annotated 98% of coding sequences identified for all genomes. Roary pangenome analysis found 5667 core genes, 59 shell genes and 49 cloud genes across all samples.

Genome-wide association analyses using Scoary did not find any significant association between the presence or absence of any particular gene and resistance to the phage cocktail. Genome-wide analyses of variants identified a SNP at 794106 bp (in the PAO1 reference genome) (Figure 3) as potentially being associated with resistance to the phage cocktail (naïve p=0.03), however this was not significant once the Benjamini-Hochberg correction was applied (Benjamini-Hochberg-adjusted p=1.0).

No variants were significantly associated with isolate resistance to piperacillin, ceftazidime or ciprofloxacin. A set of 14169 genes was identified for potential association with resistance to both amikacin and gentamicin (naïve p=0.02; Benjamini-Hochberg-adjusted p=0.06), however this was likely confounded by the fact that only the CF strains displayed aminoglycoside resistance.

## 4.5 Discussion

In this study, BIMs were generated through repeated exposure of *P. aeruginosa* biofilms to a phage cocktail. We identified genome variants in all vehicle-treated and phage-treated samples of the 5 strains tested, but in particular, a large number of variants in the two CF strains treated with phages. The large number of variants in the phage-treated CF strains may reflect hypermutability, which is commonly found in CF clinical isolates[549]. Studies of *Salmonella* Typhimurium have demonstrated activation of an SOS response in association with phage resistance, which enables cell survival in the presence of DNA damage[550]. An SOS response could also explain the persistence of numerous genetic mutations in the CF strain BIMs.

BIMs were identified in *P. aeruginosa* 7-day biofilms exposed to phage for 7 days across all strains tested, whilst a BIM was identified in the vehicle-treated control biofilms from one strain only. All 5 strains were strongly sensitive to the phage cocktail when previously tested in planktonic form and in 48-hour biofilms ([503] and unpublished data). It is possible that within the initial bacterial population, BIMs were already present, and that the extended biofilm status and/or continuous exposure to phage selected for and enriched BIMs. Alternatively, we cannot exclude *de novo* development of phage resistance during the treatment phase. The appearance of BIMs in bacterial cultures exposed to infecting bacteriophages has been described in numerous *in vitro* studies[536, 551, 552]. Betts et al have provided evidence of the development of phage resistance in *P. aeruginosa* through coevolution[553]. Various mechanisms of bacterial resistance to phage have been described. Pal et al have shown that the rate of spontaneous mutations is increased in bacteria that have co-evolved with lytic phages[554]. These mutations may affect the expression of bacterial cell surface molecules that are used by phages as receptors, resulting in phage resistance[555]. Other bacterial defence mechanisms against phage include superinfection exclusion systems, which prevent injection or translocation of phage genetic material, and

restriction-modification and CRISPR-Cas systems that recognise and remove foreign genetic material[394-399].

The region of the *P. aeruginosa* genome where variant genotypes differed between vehicle-treated and phage-exposed BIMs was located in prophage Pf1 and adjacent regions of the genome. Lim et al similarly identified multiple SNPs in the prophage Pf1 region of small colony variants isolated from a PAO1 culture following infection by a lytic phage, PB1[556]. Prophages occur where a temperate bacteriophage integrates its genome into the host bacterial genome, entering a lysogenic cycle rather than causing lysis of the host[557]. *P. aeruginosa* prophages have been found to resist superinfection of their host by other phages, via mechanisms including phage repressor proteins and modification of cell-surface phage receptor molecules[558, 559]. Bacteriophage Pf1 is a filamentous *P. aeruginosa* phage[560]. It is not known to be associated with virulence or virulence factors, however infection of *P. aeruginosa* strain K by phage Pf1 has been found to increase sensitivity to antibiotics (carbenicillin and gentamicin)[561-563]. Prophage Pf1 genes are upregulated during anaerobic growth in the presence of $NO_2^-$, and in *P. aeruginosa* biofilms, suggesting that they may play a role in adaptation to growth conditions[564, 565].

We identified a SNP in gene PA0726, which encodes a hypothetical protein of bacteriophage Pf1, as being potentially associated with phage resistance, although this was not statistically significant. PA0726 shares homology with a zonula occludens toxin (Zot) encoded by a filamentous phage of *V. cholerae*[566, 567]. Aside from its toxin effect of increasing small intestine mucosal permeability, Zot also appears to play a role in phage morphogenesis[566, 568]. Zot homologues include a family of proteins encoded by plasmids and filamentous phages of *E. coli*, *Pseudomonas* and *Xanthomonas* species[569]. One such homologue is GpI, a transmembrane protein involved in phage assembly and exit from the host cell[570]. To our knowledge, this family of proteins has not been previously investigated in the context of host resistance to infection by lytic phages. Further studies would be required to determine the relationship between PA0726 mutations and the development of BIMs.

The clinical significance of the acquisition of resistance to phage by bacterial isolates or the selection of such variants during phage therapy is unclear, as the majority of clinical studies of anti-*P. aeruginosa* phage therapy published do not report the emergence of BIMs as an outcome measure. A decrease in the proportion of *E. coli* colonies susceptible to T4 phage was described by Bruttin et al after healthy adults ingested the phage in drinking water for two 2-day courses over 3 weeks[463]. Aslam et al describe the detection of an isolate with resistance to a *P. aeruginosa* phage cocktail following a successful 2-week course of intravenous and nebulised treatment for *P. aeruginosa* pneumonia in a bilateral lung transplant patient[451]. This patient subsequently received a successful treatment course of the same phage cocktail with the addition of 1 new phage for a further episode of pneumonia.

Chan et al's work suggests that the development of BIMs could be exploited for therapeutic intervention by the application of phages that use receptors related to antibiotic resistance, in order to exert negative selection pressure against antibiotic resistant traits[534]. Our antibiotic susceptibility testing results suggest that following exposure to a phage cocktail, individual bacterial strains respond differently. This is not unexpected, as the 5 strains used in this study have different levels of sensitivity to each phage in the phage cocktail[503]. Of note, USA 2 and Ned 5 strains were initially resistant to ciprofloxacin and piperacillin respectively, and both became sensitive to the aforementioned antibiotics following exposure to the phage cocktail. There were several variants in AMR genes, however we are unable to determine from this study if these variants are responsible for the changes in antibiotic sensitivity. We did not assess gene expression, which may also affect antibiotic susceptibility. This study only analysed a small number of strains, thus we are unable to draw broader conclusions regarding the effect of the phage cocktail on antibiotic resistance.

In conclusion, we have identified genomic changes that appear to be associated with the development of phage resistance in clinical *P. aeruginosa* isolates following repeated exposure to a phage cocktail. Whilst the functional significance of these variants is unclear, there also appear to be alterations in

susceptibility to conventional antibiotics following repeated exposure to the phage cocktail, including the development of sensitivity to selected antibiotics in previously resistant isolates. The therapeutic potential of these changes in antibiotic susceptibility in antibiotic-resistant isolates should be further investigated.

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# Chapter 5: Summary of thesis and future directions

The role of *Pseudomonas aeruginosa* as a pathogen in CRS and CF is well-established, with evidence of an association with increased severity and disease recalcitrance or progression in both disease processes. Antibiotics have traditionally been a mainstay of management in both chronic conditions. Biofilm formation, induction of a mucoid phenotype, and both innate and acquired resistance often mean that *P. aeruginosa* is unable to be completely eradicated, and repeated courses of antibiotics are required. Aside from limited efficacy, other concerns with the ongoing use of antibiotics include treatment side effects, the impact of antibiotics on the microbiome, and the development of antimicrobial resistance.

The potential of lytic bacteriophages as antimicrobial agents against *P. aeruginosa* has been examined by multiple investigators using *in vitro* and *in vivo* animal studies. Several clinical studies in humans have been performed, with a favourable safety profile and mixed efficacy results reported. In more recent years, several clinical case reports describing the successful use of anti-*P. aeruginosa* bacteriophages to treat multidrug-resistant *P. aeruginosa* infections have been published.

Despite these promising results, several hurdles need to be overcome prior to the adoption of anti-*P. aeruginosa* phage therapy for mainstream medical use. From a medical and pharmacological point of view, clear indications for treatment and effective dosing regimes need to be defined. From a biotechnology and regulatory point of view, standards for quality control and systems for screening and approval of phage preparations must be created.

This thesis provides an in-depth investigation of the suitability of an anti-*P. aeruginosa* lytic bacteriophage cocktail (CT-PA) for the treatment of *P. aeruginosa* sinus infections in patients with CRS and/or CF. 40 clinical isolates from CRS and CF patients across 3 continents were collected and strain typed,

which provided important insights into the diversity of *P. aeruginosa* strains found in CRS and CF patients. We also detected several multidrug-resistant isolates. CT-PA was able to lyse 89% of the clinical isolates *in vitro*, and importantly, all of the multidrug-resistant isolates. These results indicate that CT-PA has a broad host range amongst *P. aeruginosa* clinical isolates. CT-PA was also able to remove biofilms of the *P. aeruginosa* clinical isolates *in vitro*, resulting in significant reductions in biofilm biomass after 48 hours of exposure.

Given our findings *in vitro*, we then undertook safety and efficacy testing of CT-PA in an animal model of *P. aeruginosa* sinusitis. This included assays for toxicity to sinuses and other organs. Our results supported the favourable safety profile of phage therapy that has been demonstrated thus far in scientific literature. We were also able to gain important insights into the pharmacokinetics of CT-PA sinus flushes and factors affecting efficacy. High phage titres were detected on CT-PA-treated sinus mucosa harvested 16 to 18 hours following the final sinus flush. This indicates persistence of viable phage for a significant period of time following topical application to *P. aeruginosa* infected sinuses. This may help to guide dosing regimes, given that there is currently very sparse data on the pharmacokinetics of phage therapy. Whilst a significant reduction in biofilm biomass was found in all CT-PA-treated groups compared to the saline control in the Efficacy arm, we found a statistically significant interaction between the presence of non-*P. aeruginosa* bacterial species prior to inoculation and the efficacy of biofilm biomass reduction. Although this was not an unexpected finding given that bacteriophages are generally species specific, it does highlight a potential pitfall in treating CRS patients with phage therapy targeted at a single bacterial species. At the same time, there is evidence that infection with *P. aeruginosa* is independently associated with poorer disease-related quality of life and recalcitrant disease, and thus being able to target *P. aeruginosa* without eradicating potentially helpful bacteria may lead to improvement in these parameters. Given that this was an animal model, we were not able to assess quality of life or disease recalcitrance in a meaningful way.

Although significant reductions in biofilm biomass were achieved in the animal study, biofilms were not completely eradicated from the sheep's sinuses. There are several possible explanations for this finding, including insufficient dosing or duration of treatment, growth of non-*P. aeruginosa* bacterial biofilms, or the development of resistance to the phage cocktail. In all CT-PA-treated sinuses tested, sensitivity of *P. aeruginosa* isolated at the end of the treatment period to phage was maintained, however in two sinuses the titre was decreased, suggesting decreased sensitivity. Given that other studies have noted the emergence of bacteriophage insensitive mutants (BIMs), we decided to investigate mechanisms of acquired resistance to phage in *P. aeruginosa*.

Whilst we were only able to perform whole genome sequencing on a small number of strains, we detected a shared region of genetic variation across the clinical isolates. Similar findings have been previously reported by Lim et al, in a study of a phage-resistant small colony variants derived from *P. aeruginosa* PAO1. Whether these genetic variants are of any functional significance is unclear, and further studies of specific mutations would be required to determine this. Perhaps of greater clinical significance, our study showed that changes in antibiotic susceptibility in BIMs varied between strains. This has potential implications when considering the possible use of phage therapy to "re-sensitise" bacteria to conventional antibiotics.

Several avenues for further investigation have arisen from this work. Firstly, human clinical trials are required to confirm the safety of CT-PA and explore its efficacy. Whilst we were able to confirm the growth of *P. aeruginosa* biofilms within sheep sinuses in our sheep rhinosinusitis model, the ovine sinus microbiome and immune response to bacteria are likely to differ significantly from humans with CRS. Furthermore, we only tested a single *P. aeruginosa* strain in the animal model, compared to the diversity of strains revealed in our *in vitro* study of human CRS clinical isolates. Dosing regimes, alternative drug delivery vehicles, and duration of treatment could also be appropriately explored in a clinical trial setting.

Secondly, a method for rapid and detailed characterisation of both phages and the bacteria they target in terms of phage virulence, bacterial sensitivity to phage, and propensity for bacteria to develop resistance to phage would assist in real-world application of phage therapy by rapidly screening for likely efficacy and safety. If genetic or molecular markers can be identified, polymerase chain reaction, high-throughput genetic sequencing, transcriptome analysis, or molecular analysis could be used to determine which phage or phage cocktail should be prescribed to treat a patient.

Thirdly, given the rapidly expanding knowledge base around microbial dysbiosis in CRS, studies of species-specific bacteriophage therapy and its resultant effects on the sinus microbiome, as well as clinical outcomes, could help clarify the role of specific bacterial species and interactions between species in CRS.

Fourthly, the use of bacteriophages to address multidrug resistance in bacteria, and the use of phages in combination with conventional antibiotics both warrant further investigation. Numerous *in vitro* and *in vivo* animal studies have demonstrated synergy between phage and antibiotics, reductions in antibiotic minimum inhibitory concentration, or reductions in resistance, when both phage and antibiotics are administered in combination compared to antibiotic or phage monotherapy[571]. However, no clinical trials addressing the question of phage-antibiotic synergy have been performed.

In conclusion, bacteriophage therapy shows great potential as a novel treatment for *P. aeruginosa* infections in CRS. We have the demonstrated *in vitro* and *in vivo* efficacy and safety of an anti-*P. aeruginosa* phage cocktail, and attempted to gain a further understanding of the way *P. aeruginosa* clinical isolates respond when exposed to the phage cocktail. Further preclinical and clinical studies to expand our understanding of the science and pharmacology of bacteriophages will ensure that bacteriophage therapy can reach its full potential in clinical use.

# Bibliography


1. Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, et al. Rhinosinusitis: Establishing definitions for clinical research and patient care. Journal of Allergy and Clinical Immunology. 2004;114(6, Supplement):155-212.
2. Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, et al. Rhinosinusitis: Developing guidance for clinical trials. Journal of Allergy and Clinical Immunology. 2006;118(5, Supplement):S17-S61.
3. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. Rhinology. 2012;50(1):1-12.
4. Australian Institute of Health and Welfare. Chronic respiratory diseases in Australia: their prevalence, consequences and prevention. Canberra: AIHW; 2005.
5. Rudmik L, Smith TL. Quality of Life in Patients with Chronic Rhinosinusitis. Current Allergy and Asthma Reports. [journal article]. 2011;11(3):247-52.
6. Macdonald KI, McNally JD, Massoud E. Quality of life and impact of surgery on patients with chronic rhinosinusitis. J Otolaryngol Head Neck Surg. 2009;38(2):286-93.
7. Teul I, Zbislawski W, Baran S, Czerwinski F, Lorkowski J. Quality of life of patients with diseases of sinuses. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society. 2007;58 Suppl 5(Pt 2):691-7.
8. Fu Q-L, Ma J-X, Ou C-Q, Guo C, Shen S-Q, Xu G, et al. Influence of Self-Reported Chronic Rhinosinusitis on Health-Related Quality of Life: A Population-Based Survey. PLoS ONE. 2015;10(5):e0126881.
9. Lange B, Holst R, Thilsing T, Baelum J, Kjeldsen A. Quality of life and associated factors in persons with chronic rhinosinusitis in the general population: A prospective questionnaire and clinical cross-sectional study. Clinical Otolaryngology. 2013;38(6):474-80.
10. Kountakis SE, Arango P, Bradley D, Wade ZK, Borish L. Molecular and Cellular Staging for the Severity of Chronic Rhinosinusitis. The Laryngoscope. 2004;114(11):1895-905.
11. Bachert C, Gevaert P, Holtappels G, Cuvelier C, Paul van C. Nasal Polyposis: From Cytokines to Growth. Am J Rhinol. 2000;14(5):279-90.
12. Stoop AE, van der Heijden HAMD, Biewenga J, van der Baan S. Eosinophils in nasal polyps and nasal mucosa: An immunohistochemical study. Journal of Allergy and Clinical Immunology. 1993;91(2):616-22.
13. Van Bruaene N, Derycke L, Perez-Novo CA, Gevaert P, Holtappels G, De Ruyck N, et al. TGF-β signaling and collagen deposition in chronic rhinosinusitis. Journal of Allergy and Clinical Immunology. 2009;124(2):253-9.e2.
14. Van Bruaene N, C P-N, Van Crombruggen K, De Ruyck N, Holtappels G, Van Cauwenberge P, et al. Inflammation and remodelling patterns in early stage chronic rhinosinusitis. Clinical & Experimental Allergy. 2012;42(6):883-90.
15. Van Zele T, Claeys S, Gevaert P, Van Maele G, Holtappels G, Van Cauwenberge P, et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. Allergy. 2006;61(11):1280-9.
16. Van Bruaene N, Pérez-Novo CA, Basinski TM, Van Zele T, Holtappels G, De Ruyck N, et al. T-cell regulation in chronic paranasal sinus disease. Journal of Allergy and Clinical Immunology. 2008;121(6):1435-41.e3.

17. Van Zele T, Gevaert P, Holtappels G, Beule A, Wormald PJ, Mayr S, et al. Oral steroids and doxycycline: Two different approaches to treat nasal polyps. Journal of Allergy and Clinical Immunology. 2010;125(5):1069-76.e4.
18. Schalek P, Petráš P, Klement V, Hahn A. Short-term antibiotics treatment in patients with nasal polyps and enterotoxins producing Staphylococcus aureus strains. Eur Arch Otorhinolaryngol. [journal article]. 2009;266(12):1909.
19. Wallwork B, Coman W, Feron F, Mackay-Sim A, Cervin A. Clarithromycin and Prednisolone Inhibit Cytokine Production in Chronic Rhinosinusitis. The Laryngoscope. 2002;112(10):1827-30.
20. Inamura K, Ohta N, Fukase S, Kasajima N, Aoyagi M. The effects of erythromycin on human peripheral neutrophil apoptosis. Rhinology. 2000;38(3):124-9.
21. Wallwork B, Coman W, Mackay-Sim A, Greiff L, Cervin A. A Double-Blind, Randomized, Placebo-Controlled Trial of Macrolide in the Treatment of Chronic Rhinosinusitis. The Laryngoscope. 2006;116(2):189-93.
22. Videler WJ, Badia L, Harvey RJ, Gane S, Georgalas C, van der Meulen FW, et al. Lack of efficacy of long-term, low-dose azithromycin in chronic rhinosinusitis: a randomized controlled trial. Allergy. 2011;66(11):1457-68.
23. Marom Z, Shelhamer J, Alling D, Kaliner M. The effects of corticosteroids on mucous glycoprotein secretion from human airways in vitro. The American review of respiratory disease. 1984;129(1):62-5.
24. Shimura S, Sasaki T, Ikeda K, Yamauchi K, Sasaki H, Takishima T. Direct Inhibitory Action of Glucocorticoid on Glycoconjugate Secretion from Airway Submucosal Glands. American Review of Respiratory Disease. 1990;141(4_pt_1):1044-9.
25. Qvarnberg Y, Kantola O, Salo J, Toivanen M, Valtonen H, Vuori E. Influence of topical steroid treatment on maxillary sinusitis. Rhinology. 1992;30(2):103-12.
26. Anti-inflammatory steroid action: basic and clinical aspects. Schleimer RP, Claman HN, Oronsky A, editors. San Diego: Academic Press; 1989.
27. Ristimäki A, Narko K, Enholm B, Joukov V, Alitalo K. Proinflammatory Cytokines Regulate Expression of the Lymphatic Endothelial Mitogen Vascular Endothelial Growth Factor-C. Journal of Biological Chemistry. 1998;273(14):8413-8.
28. Ecevit MC, Erdag TK, Dogan E, Sutay S. Effect of steroids for nasal polyposis surgery: A placebo-controlled, randomized, double-blind study. Laryngoscope. 2015;125(9):2041-5.
29. Hissaria P, Smith W, Wormald PJ, Taylor J, Vadas M, Gillis D, et al. Short course of systemic corticosteroids in sinonasal polyposis: a double-blind, randomized, placebo-controlled trial with evaluation of outcome measures. J Allergy Clin Immunol. 2006;118(1):128-33.
30. Vaidyanathan S, Barnes M, Williamson P, Hopkinson P, Donnan PT, Lipworth B. Treatment of chronic rhinosinusitis with nasal polyposis with oral steroids followed by topical steroids: a randomized trial. Annals of internal medicine. 2011;154(5):293-302.
31. Kirtsreesakul V, Wongsritrang K, Ruttanaphol S. Does oral prednisolone increase the efficacy of subsequent nasal steroids in treating nasal polyposis? Am J Rhinol Allergy. 2012;26(6):455-62.
32. Möller W, Schuschnig U, Saba GK, Meyer G, Junge-Hülsing B, Keller M, et al. Pulsating aerosols for drug delivery to the sinuses in healthy volunteers. Otolaryngology–Head and Neck Surgery. 2010;142(3):382-8.
33. Rudman KL, O'Brien EK, Leopold DA. Radiographic distribution of drops and sprays within the sinonasal cavities. Am J Rhinol Allergy. 2011;25(2):94-7.
34. Leclerc L, Pourchez J, Aubert G, Leguellec S, Vecellio L, Cottier M, et al. Impact of Airborne Particle Size, Acoustic Airflow and Breathing Pattern on Delivery of

Nebulized Antibiotic into the Maxillary Sinuses Using a Realistic Human Nasal Replica. Pharmaceutical Research. 2014;31(9):2335-43.
35. Wormald P-J, Cain T, Oates L, Hawke L, Wong I. A Comparative Study of Three Methods of Nasal Irrigation. The Laryngoscope. 2004;114(12):2224-7.
36. Ural A, Oktemer TK, Kizil Y, Ileri F, Uslu S. Impact of isotonic and hypertonic saline solutions on mucociliary activity in various nasal pathologies: clinical study. The Journal of Laryngology & Otology. 2008;123(5):517-21.
37. Shoseyov D, Bibi H, Shai P, Shoseyov N, Shazberg G, Hurvitz H. Treatment with hypertonic saline versus normal saline nasal wash of pediatric chronic sinusitis. Journal of Allergy and Clinical Immunology. 1998;101(5):602-5.
38. Rabago D, Pasic T, Zgierska A, Mundt M, Barrett B, Maberry R. The Efficacy of Hypertonic Saline Nasal Irrigation for Chronic Sinonasal Symptoms. Otolaryngology - Head and Neck Surgery. 2005;133(1):3-8.
39. Culig J, Leppee M, Vceva A, Djanic D. Efficiency of hypertonic and isotonic seawater solutions in chronic rhinosinusitis. Medicinski glasnik : official publication of the Medical Association of Zenica-Doboj Canton, Bosnia and Herzegovina. 2010;7(2):116-23.
40. Liang K-L, Su M-C, Tseng H-C, Jiang R-S. Impact of pulsatile nasal irrigation on the prognosis of functional endoscopic sinus surgery. Journal Of Otolaryngology - Head & Neck Surgery = Le Journal D'oto-Rhino-Laryngologie Et De Chirurgie Cervico-Faciale. 2008;37(2):148-53.
41. Freeman SRM, Sivayoham ESG, Jepson K, De Carpentier J. A preliminary randomised controlled trial evaluating the efficacy of saline douching following endoscopic sinus surgery. Clinical Otolaryngology. 2008;33(5):462-5.
42. Chong LY, Head K, Hopkins C, Philpott C, Schilder AGM, Burton MJ. Intranasal steroids versus placebo or no intervention for chronic rhinosinusitis. Cochrane Database of Systematic Reviews. 2016(4).
43. Steinke JW, Payne SC, Tessier ME, Borish LO, Han JK, Borish LC. Pilot study of budesonide inhalant suspension irrigations for chronic eosinophilic sinusitis. Journal of Allergy and Clinical Immunology. 2009;124(6):1352-4.e7.
44. Sykes DA, Chan KL, Wilson R, Mackay IS, Cole PJ. Relative importance of antibiotic and improved clearance in topical treatment of chronic mucopurulent rhinosinusitis. The Lancet. 1986;328(8503):359-60.
45. Desrosiers MY, Salas-Prato M. Treatment of Chronic Rhinosinusitis Refractory to Other Treatments with Topical Antibiotic Therapy Delivered by Means of a Large-Particle Nebulizer: Results of a Controlled Trial. Otolaryngology–Head and Neck Surgery. 2001;125(3):265-9.
46. Videler WJ, van Drunen CM, Reitsma JB, Fokkens WJ. Nebulized bacitracin/colimycin: a treatment option in recalcitrant chronic rhinosinusitis with Staphylococcus aureus? A double-blind, randomized, placebo-controlled, cross-over pilot study. Rhinology. 2008;46(2):92-8.
47. Jervis-Bardy J, Boase S, Psaltis A, Foreman A, Wormald P-J. A randomized trial of mupirocin sinonasal rinses versus saline in surgically recalcitrant staphylococcal chronic rhinosinusitis. The Laryngoscope. 2012;122(10):2148-53.
48. Rosenfeld RM, Piccirillo JF, Chandrasekhar SS, Brook I, Ashok Kumar K, Kramper M, et al. Clinical Practice Guideline (Update): Adult Sinusitis. Otolaryngology–Head and Neck Surgery. 2015;152(2_suppl):S1-S39.
49. Simmen D, Jones NS. Manual of Endoscopic Sinus Surgery. New York, UNITED STATES: Thieme; 2011.
50. Lund VJ, MacKay IS. Outcome assessment of endoscopic sinus surgery. Journal of the Royal Society of Medicine. 1994;87(2):70-2.

51. Senior BA, Kennedy DW, Tanabodee J, Kroger H, Hassab M, Lanza D. Long-term Results of Functional Endoscopic Sinus Surgery. The Laryngoscope. 1998;108(2):151-7.
52. Damm M, Quante G, Jungehuelsing M, Stennert E. Impact of Functional Endoscopic Sinus Surgery on Symptoms and Quality of Life in Chronic Rhinosinusitis. The Laryngoscope. 2002;112(2):310-5.
53. Lund VJ, Scadding GK. Objective assessment of endoscopic sinus surgery in the management of chronic rhinosinusitis: an update. J Laryngol Otol. 1994;108(9):749-53.
54. Abdel-Hak B, Gunkel A, Kanonier G, Schrott-Fischer A, Ulmer H, Thumfart W. Ciliary beat frequency, olfaction and endoscopic sinus surgery. ORL J Otorhinolaryngol Relat Spec. 1998;60(4):202-5.
55. Hartog B, van Benthem PP, Prins LC, Hordijk GJ. Efficacy of sinus irrigation versus sinus irrigation followed by functional endoscopic sinus surgery. Ann Otol Rhinol Laryngol. 1997;106(9):759-66.
56. Ragab SM, Lund VJ, Scadding G. Evaluation of the Medical and Surgical Treatment Of Chronic Rhinosinusitis: A Prospective, Randomised, Controlled Trial. The Laryngoscope. 2004;114(5):923-30.
57. Benninger MS, Ferguson BJ, Hadley JA, Hamilos DL, Jacobs M, Kennedy DW, et al. Adult Chronic Rhinosinusitis: Definitions, Diagnosis, Epidemiology, and Pathophysiology. Otolaryngology -- Head and Neck Surgery. 2003;129(3 suppl):S1-S32.
58. Lehrer RI, Barton A, Daher KA, Harwig SS, Ganz T, Selsted ME. Interaction of human defensins with Escherichia coli. Mechanism of bactericidal activity. The Journal of clinical investigation. 1989;84(2):553-61.
59. Sørensen O, Arnljots K, Cowland JB, Bainton DF, Borregaard N. The Human Antibacterial Cathelicidin, hCAP-18, Is Synthesized in Myelocytes and Metamyelocytes and Localized to Specific Granules in Neutrophils. Blood. 1997;90(7):2796-803.
60. Bals R, Wang X, Zasloff M, Wilson JM. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proc Natl Acad Sci U S A. 1998;95(16):9541-6.
61. Kim ST, Cha HE, Kim DY, Han GC, Chung YS, Lee YJ, et al. Antimicrobial peptide LL-37 is upregulated in chronic nasal inflammatory disease. Acta Otolaryngol. 2003;123(1):81-5.
62. Oren Z, Shai Y. Mode of action of linear amphipathic alpha-helical antimicrobial peptides. Biopolymers. 1998;47(6):451-63.
63. De Y, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, et al. Ll-37, the Neutrophil Granule–And Epithelial Cell–Derived Cathelicidin, Utilizes Formyl Peptide Receptor–Like 1 (Fprl1) as a Receptor to Chemoattract Human Peripheral Blood Neutrophils, Monocytes, and T Cells. The Journal of Experimental Medicine. 2000;192(7):1069-74.
64. Niyonsaba F, Hirata M, Ogawa H, Nagaoka I. Epithelial cell-derived antibacterial peptides human beta-defensins and cathelicidin: multifunctional activities on mast cells. Current drug targets Inflammation and allergy. 2003;2(3):224-31.
65. Ellison RT, Giehl TJ. Killing of gram-negative bacteria by lactoferrin and lysozyme. Journal of Clinical Investigation. 1991;88(4):1080-91.
66. Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilm development. Nature. 2002;417(6888):552-5.
67. Hiemstra PS. Novel roles of protease inhibitors in infection and inflammation. Biochemical Society transactions. 2002;30(2):116-20.

68. Hiemstra PS, Maassen RJ, Stolk J, Heinzel-Wieland R, Steffens GJ, Dijkman JH. Antibacterial activity of antileukoprotease. Infect Immun. 1996;64(11):4520-4.
69. Fahey JV, Wira CR. Effect of menstrual status on antibacterial activity and secretory leukocyte protease inhibitor production by human uterine epithelial cells in culture. J Infect Dis. 2002;185(11):1606-13.
70. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. [10.1038/nri1391]. 2004;4(7):499-511.
71. Bingle CD, Craven CJ. PLUNC: A novel family of candidate host defence proteins expressed in the upper airways and nasopharynx. Human Molecular Genetics. 2002;11(8):937-43.
72. Stanley PJ, Wilson R, Greenstone MA, Mackay IS, Cole PJ. Abnormal nasal mucociliary clearance in patients with rhinitis and its relationship to concomitant chest disease. British journal of diseases of the chest. 1985;79(1):77-82.
73. Ohashi Y, Nakai Y. Functional and morphological pathology of chronic sinusitis mucous membrane. Acta oto-laryngologica Supplementum. 1983;397:11-48.
74. Ohashi Y, Nakai Y. Reduced Ciliary Action in Chronic Sinusitis. Acta Oto-Laryngologica. 1983;95(sup397):3-9.
75. Vamanshankar H, Kumar SC, Preetham AP, Feshan M. Mucociliary function: Its variations with disease. Clinical Rhinology. 2016;9(2):68-73.
76. Soyka MB, Wawrzyniak P, Eiwegger T, Holzmann D, Treis A, Wanke K, et al. Defective epithelial barrier in chronic rhinosinusitis: The regulation of tight junctions by IFN-γ and IL-4. Journal of Allergy and Clinical Immunology. 2012;130(5):1087-96.e10.
77. Lee SH, Kim JE, Lee HM, Lim HH, Choi JO. Antimicrobial Defensin Peptides of the Human Nasal Mucosa. Ann Otol Rhinol Laryngol. 2002;111(2):135-41.
78. Kim ST, Cha HE, Kim DY, Han GC, Chung Y-s, Young JL, et al. Antimicrobial Peptide LL-37 is Upregulated in Chronic Nasal Inflammatory Disease. Acta Oto-Laryngologica. 2003;123(1):81-5.
79. Ooi EH, Wormald P-J, Carney AS, James CL, Tan LW. Fungal allergens induce cathelicidin LL-37 expression in chronic rhinosinusitis patients in a nasal explant model. Am J Rhinol. 2007;21(3):367-72.
80. Fukami M, Stierna P, Veress B, Carlsöö B. Lysozyme and lactoferrin in human maxillary sinus mucosa during chronic sinusitis. Eur Arch Otorhinolaryngol. [journal article]. 1993;250(3):133-9.
81. Psaltis AJ, Bruhn MA, Ooi EH, Tan LW, Wormald P-J. Nasal Mucosa Expression of Lactoferrin in Patients With Chronic Rhinosinusitis. The Laryngoscope. 2007;117(11):2030-5.
82. Seshadri S, Lin DC, Rosati M, Carter RG, Norton JE, Suh L, et al. Reduced Expression of Antimicrobial PLUNC Proteins in Nasal Polyp Tissues of patients with Chronic Rhinosinusitis. Allergy. 2012;67(7):920-8.
83. Hirschberg A, Kiss M, Kadocsa E, Polyanka H, Szabo K, Razga Z, et al. Different activations of toll-like receptors and antimicrobial peptides in chronic rhinosinusitis with or without nasal polyposis. Eur Arch Otorhinolaryngol. [journal article]. 2016;273(7):1779-88.
84. Zhao CY, Wang X, Liu M, Jin DJ. Microarray Gene Analysis of Toll-Like Receptor Signaling Elements in Chronic Rhinosinusitis with Nasal Polyps. International archives of allergy and immunology. 2011;156(3):297-304.
85. Wang X, Zhang N, Bo M, Holtappels G, Zheng M, Lou H, et al. Diversity of TH cytokine profiles in patients with chronic rhinosinusitis: A multicenter study in

Europe, Asia, and Oceania. Journal of Allergy and Clinical Immunology. 2016;138(5):1344-53.
86. Derycke L, Eyerich S, Van Crombruggen K, Pérez-Novo C, Holtappels G, Deruyck N, et al. Mixed T Helper Cell Signatures In Chronic Rhinosinusitis with and without Polyps. PLoS ONE. 2014;9(6):e97581.
87. Shaw JL, Fakhri S, Citardi MJ, Porter PC, Corry DB, Kheradmand F, et al. IL-33–Responsive Innate Lymphoid Cells Are an Important Source of IL-13 in Chronic Rhinosinusitis with Nasal Polyps. American Journal of Respiratory and Critical Care Medicine. 2013;188(4):432-9.
88. Nagarkar DR, Poposki JA, Tan BK, Comeau MR, Peters AT, Hulse KE, et al. Thymic stromal lymphopoietin activity is increased in nasal polyps of patients with chronic rhinosinusitis. Journal of Allergy and Clinical Immunology. 2013;132(3):593-600.e12.
89. Kato A, Peters A, Suh L, Carter R, Harris KE, Chandra R, et al. Evidence of a role for B cell–activating factor of the TNF family in the pathogenesis of chronic rhinosinusitis with nasal polyps. Journal of Allergy and Clinical Immunology. 2008;121(6):1385-92.e2.
90. Tan BK, Li Q-Z, Suh L, Kato A, Conley DB, Chandra RK, et al. Evidence for intranasal antinuclear autoantibodies in patients with chronic rhinosinusitis with nasal polyps. Journal of Allergy and Clinical Immunology. 2011;128(6):1198-206.e1.
91. Feldman S, Kasjanski R, Poposki J, Hernandez D, Chen JN, Norton JE, et al. Chronic airway inflammation provides a unique environment for B cell activation and antibody production. Clinical & Experimental Allergy. 2017;47(4):457-66.
92. Lau A, Lester S, Moraitis S, Ou J, Psaltis AJ, McColl S, et al. Tertiary lymphoid organs in recalcitrant chronic rhinosinusitis. Journal of Allergy and Clinical Immunology. 2017;139(4):1371-3.e6.
93. Lam K, Schleimer R, Kern RC. The Etiology and Pathogenesis of Chronic Rhinosinusitis: a Review of Current Hypotheses. Current Allergy and Asthma Reports. [journal article]. 2015;15(7):41.
94. Seidman MD, Gurgel RK, Lin SY, Schwartz SR, Baroody FM, Bonner JR, et al. Clinical Practice Guideline: Allergic Rhinitis. Otolaryngology—Head and Neck Surgery. 2015;152(1_suppl):S1-S43.
95. Corey JP, Bumsted RM, Panje WR, Shaw GY, Conley D. Allergy and Fungal Screens in Chronic Sinusitis. Am J Rhinol. 1990;4(1):25-8.
96. Emanuel IA, Shah SB. Chronic rhinosinusitis: allergy and sinus computed tomography relationships. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery. 2000;123(6):687-91.
97. Gutman M, Torres A, Keen KJ, Houser SM. Prevalence of Allergy in Patients with Chronic Rhinosinusitis. Otolaryngology—Head and Neck Surgery. 2004;130(5):545-52.
98. Sedaghat AR, Phipatanakul W, Cunningham MJ. Characterization of aeroallergen sensitivities in children with allergic rhinitis and chronic rhinosinusitis. Allergy & Rhinology. 2014;5(3):e143-e5.
99. Batra PS, Tong L, Citardi MJ. Analysis of comorbidities and objective parameters in refractory chronic rhinosinusitis. The Laryngoscope. 2013;123:S1-S11.
100. Houser SM, Keen KJ. The Role of Allergy and Smoking in Chronic Rhinosinusitis and Polyposis. The Laryngoscope. 2008;118(9):1521-7.

101. Li QC, Cheng KJ, Wang F, Zhou SH. Role of atopy in chronic rhinosinusitis with nasal polyps: does an atopic condition affect the severity and recurrence of disease? The Journal of Laryngology & Otology. 2016;130(7):640-4.
102. Ferreira D, Kaushik S, Smith C, Benke G, Thompson B, Dharmage S, et al. Persistently high asthma and atopy prevalence among aging adults in Melbourne. Respirology. 2015;20:66.
103. Sadoway T, Nelson V, Pathmanapan S, Patel P, Salapatek AM, Couroux P. Prevalence of skin prick and IgE positive subjects in the Greater Toronto Area (GTA), Canada. Allergy: European Journal of Allergy and Clinical Immunology. 2015;70:565-6.
104. Feng M, Yang Z, Li J. Marked disparity in environment, infections, and asthma and atopy prevalence between rural and urban children in China. European Respiratory Journal. 2014;44.
105. Leo G, Piacentini E, Incorvaia C, Consonni D, Frati F. Chronic rhinosinusitis and allergy. Pediatric Allergy and Immunology. 2007;18:19-21.
106. Salo PM, Arbes SJ, Jaramillo R, Calatroni A, Weir CH, Sever ML, et al. Prevalence of allergic sensitization in the U.S.: Results from the National Health and Nutrition Examination Survey (NHANES) 2005–2006. The Journal of allergy and clinical immunology. 2014;134(2):350-9.
107. Corbo GM, Foresi A, Bonfitto P, Mugnano A, Agabiti N, Cole PJ. Measurement of nasal mucociliary clearance. Archives of disease in childhood. 1989;64(4):546-50.
108. Pelikan Z, Pelikan-Filipek M. Role of nasal allergy in chronic maxillary sinusitis--diagnostic value of nasal challenge with allergen. J Allergy Clin Immunol. 1990;86(4 Pt 1):484-91.
109. Gwaltney JM, Hendley JO, Phillips CD, Bass CR, Mygind N, Winther B. Nose Blowing Propels Nasal Fluid into the Paranasal Sinuses. Clinical Infectious Diseases. 2000;30(2):387-91.
110. Harlin SL, Ansel DG, Lane SR, Myers J, Kephart GM, Gleich GJ. A clinical and pathologic study of chronic sinusitis: The role of the eosinophil. Journal of Allergy and Clinical Immunology. 1988;81(5, Part 1):867-75.
111. Suzuki M, Watanabe T, Suko T, Mogi G. Comparison of sinusitis with and without allergic rhinitis: characteristics of paranasal sinus effusion and mucosa. Am J Otolaryngol. 1999;20(3):143-50.
112. Hamilos DL, Leung DYM, Wood R, Bean DK, et al. Eosinophil infiltration in nonallergic chronic hyperplastic sinusitis with nasal polyposis (CHS/NP) is associated with endothelial VCAM-1 upregulation and expression of TNF-alpha. American Journal of Respiratory Cell and Molecular Biology. 1996;15(4):443-50.
113. Ramadan HH, Fornelli R, Ortiz AO, Rodman S. Correlation of Allergy and Severity of Sinus Disease. Am J Rhinol. 1999;13(5):345-7.
114. Robinson S, Douglas R, Wormald PJ. The relationship between atopy and chronic rhinosinusitis. Am J Rhinol. 2006;20(6):625-8.
115. Pearlman AN, Chandra RK, Chang D, Conley DB, Tripathi-Peters A, Grammer LC, et al. Relationships between severity of chronic rhinosinusitis and nasal polyposis, asthma, and atopy. Am J Rhinol Allergy. 2009;23(2):145-8.
116. Kowalski ML, Asero R, Bavbek S, Blanca M, Blanca-Lopez N, Bochenek G, et al. Classification and practical approach to the diagnosis and management of hypersensitivity to nonsteroidal anti-inflammatory drugs. Allergy. 2013;68(10):1219-32.
117. Rajan JP, Wineinger NE, Stevenson DD, White AA. Prevalence of aspirin-exacerbated respiratory disease among asthmatic patients: A meta-analysis of

the literature. Journal of Allergy and Clinical Immunology. 2015;135(3):676-81.e1.
118. Jang DW, Comer BT, Lachanas VA, Kountakis SE. Aspirin sensitivity does not compromise quality-of-life outcomes in patients with samter's triad. The Laryngoscope. 2014;124(1):34-7.
119. Awad OG, Lee JH, Fasano MB, Graham SM. Sinonasal Outcomes After Endoscopic Sinus Surgery in Asthmatic Patients With Nasal Polyps: A Difference Between Aspirin-Tolerant and Aspirin-Induced Asthma? The Laryngoscope. 2008;118(7):1282-6.
120. Robinson JL, Griest S, James KE, Smith TL. Impact of Aspirin Intolerance on Outcomes of Sinus Surgery. The Laryngoscope. 2007;117(5):825-30.
121. Sestini P, Armetti L, Gambaro G, Pieroni MG, Refini RM, Sala A, et al. Inhaled PGE2 prevents aspirin-induced bronchoconstriction and urinary LTE4 excretion in aspirin-sensitive asthma. American Journal of Respiratory and Critical Care Medicine. 1996;153(2):572-5.
122. Adamjee J, Suh YJ, Park HS, Choi JH, Penrose JF, Lam BK, et al. Expression of 5-lipoxygenase and cyclooxygenase pathway enzymes in nasal polyps of patients with aspirin-intolerant asthma. J Pathol. 2006;209(3):392-9.
123. Kowalski ML, Pawliczak R, Wozniak J, Suida K, Poniatowska M, Iwaszkiewicz J, et al. Differential Metabolism of Arachidonic Acid in Nasal Polyp Epithelial Cells Cultured from Aspirin-sensitive and Aspirin-tolerant Patients. American Journal of Respiratory and Critical Care Medicine. 2000;161(2):391-8.
124. Makowska JS, Grzegorczyk J, Bienkiewicz B, Wozniak M, Kowalski ML. Systemic responses after bronchial aspirin challenge in sensitive patients with asthma. J Allergy Clin Immunol. 2008;121(2):348-54.
125. Laidlaw TM, Kidder MS, Bhattacharyya N, Xing W, Shen S, Milne GL, et al. Cysteinyl leukotriene overproduction in aspirin-exacerbated respiratory disease is driven by platelet-adherent leukocytes. Blood. 2012;119(16):3790-8.
126. Sousa AR, Lams BE, Pfister R, Christie PE, Schmitz M, Lee TH. Expression of interleukin-5 and granulocyte-macrophage colony-stimulating factor in aspirin-sensitive and non-aspirin-sensitive asthmatic airways. Am J Respir Crit Care Med. 1997;156(5):1384-9.
127. Sanak M, Pierzchalska M, Bazan-Socha S, Szczeklik A. Enhanced expression of the leukotriene C(4) synthase due to overactive transcription of an allelic variant associated with aspirin-intolerant asthma. Am J Respir Cell Mol Biol. 2000;23(3):290-6.
128. Zhang Y, Huang H, Huang J, Xiang Z, Yang M, Tian C, et al. The −444A/C Polymorphism in the LTC4S Gene and the Risk of Asthma: A Meta-analysis. Archives of Medical Research. 2012;43(6):444-50.
129. Stankovic KM, Goldsztein H, Reh DD, Platt MP, Metson R. Gene Expression Profiling of Nasal Polyps Associated With Chronic Sinusitis and Aspirin-Sensitive Asthma. The Laryngoscope. 2008;118(5):881-9.
130. Su W-Y, Liu C, Hung S-Y, Tsai W-F. Bacteriological study in chronic maxillary sinusitis. The Laryngoscope. 1983;93(7):931-4.
131. Klossek J-M, Dubreuil L, Richet H, Richet B, Beutter P. Bacteriology of chronic purulent secretions in chronic rhinosinusitis. The Journal of Laryngology & Otology. 1998;112(12):1162-6.
132. Feazel LM, Robertson CE, Ramakrishnan VR, Frank DN. Microbiome complexity and Staphylococcus aureus in chronic rhinosinusitis. The Laryngoscope. 2012;122(2):467-72.

133. Cleland EJ, Bassiouni A, Vreugde S, Wormald PJ. The bacterial microbiome in chronic rhinosinusitis: Richness, diversity, postoperative changes, and patient outcomes. Am J Rhinol Allergy. 2016;30(1):37-43.
134. Ramakrishnan VR, Feazel LM, Gitomer SA, Ir D, Robertson CE, Frank DN. The Microbiome of the Middle Meatus in Healthy Adults. PLoS ONE. 2013;8(12):e85507.
135. Stephenson M-F, Mfuna L, Dowd SE, Wolcott RD, Barbeau J, Poisson M, et al. Molecular characterization of the polymicrobial flora in chronic rhinosinusitis. Journal Of Otolaryngology - Head & Neck Surgery = Le Journal D'oto-Rhino-Laryngologie Et De Chirurgie Cervico-Faciale. 2010;39(2):182-7.
136. Almadori G, Bastianini L, Bistoni F, Maurizi M, Ottaviani F, Paludetti G, et al. Microbial flora of nose and paranasal sinuses in chronic maxillary sinusitis. Rhinology. 1986;24(4):257-64.
137. Brook I, Thompson DH, Frazier EH. Microbiology and management of chronic maxillary sinusitis. Archives of Otolaryngology–Head & Neck Surgery. 1994;120(12):1317-20.
138. Doyle PW, Woodham JD. Evaluation of the microbiology of chronic ethmoid sinusitis. J Clin Microbiol. 1991;29(11):2396-400.
139. Hartog B, Degener JE, van Benthem PPG, Hordijk GJ. Microbiology of Chronic Maxillary Sinusitis in Adults: Isolated Aerobic and Anaerobic Bacteria and their Susceptibility to Twenty Antibiotics. Acta Oto-Laryngologica. 1995;115(5):672-7.
140. Cleland EJ, Bassiouni A, Wormald PJ. The bacteriology of chronic rhinosinusitis and the pre-eminence of Staphylococcus aureus in revision patients. Int Forum Allergy Rhinol. 2013;3(8):642-6.
141. Hsu J, Lanza DC, Kennedy DW. Antimicrobial resistance in bacterial chronic sinusitis. Am J Rhinol. [Comparative Study]. 1998;12(4):243-8.
142. Nadel DM, Lanza DC, Kennedy DW. Endoscopically guided cultures in chronic sinusitis. Am J Rhinol. 1998;12(4):233-41.
143. Brook I, Frazier EH. Correlation between Microbiology and Previous Sinus Surgery in Patients with Chronic Maxillary Sinusitis. Ann Otol Rhinol Laryngol. 2001;110(2):148-51.
144. Al-Shemari H, Abou-Hamad W, Libman M, Desrosiers M. Bacteriology of the sinus cavities of asymptomatic individuals after endoscopic sinus surgery. The Journal Of Otolaryngology. 2007;36(1):43-8.
145. Hamilos DL, Leung DYM, Wood R, Cunningham L, Bean DK, Yasruel Z, et al. Evidence for distinct cytokine expression in allergic versus nonallergic chronic sinusitis. Journal of Allergy and Clinical Immunology. 1995;96(4):537-44.
146. Chalermwatanachai T, Zhang N, Holtappels G, Bachert C, De Melo CB. Association of mucosal organisms with patterns of inflammation in chronic rhinosinusitis. PLoS ONE. 2015;10(8).
147. Bachert C, Gevaert P, Holtappels G, Johansson SGO, van Cauwenberge P. Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. Journal of Allergy and Clinical Immunology. 2001;107(4):607-14.
148. Tripathi A, Kern R, Conley DB, Seiberling K, Klemens JC, Harris KE, et al. Staphylococcal exotoxins and nasal polyposis: analysis of systemic and local responses. Am J Rhinol. 2005;19(4):327-33.
149. Bernstein JM, Ballow M, Schlievert PM, Rich G, Allen C, Dryja D. A superantigen hypothesis for the pathogenesis of chronic hyperplastic sinusitis with massive nasal polyposis. Am J Rhinol. 2003;17(6):321-6.
150. Patou J, Gevaert P, Van Zele T, Holtappels G, van Cauwenberge P, Bachert C. Staphylococcus aureus enterotoxin B, protein A, and lipoteichoic acid

stimulations in nasal polyps. Journal of Allergy and Clinical Immunology. 2008;121(1):110-5.
151. Xu G, Xia JH, Zhou H, Yu CZ, Zhang Y, Zuo KJ, et al. Interleukin-6 is essential for Staphylococcal exotoxin B-induced T regulatory cell insufficiency in nasal polyps. Clinical & Experimental Allergy. 2009;39(6):829-37.
152. Van Zele T, Holtappels G, Gevaert P, Bachert C. Differences in initial immunoprofiles between recurrent and nonrecurrent chronic rhinosinusitis with nasal polyps. Am J Rhinol Allergy. 2014;28(3):192-8.
153. Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as Complex Differentiated Communities. Annual Review of Microbiology. 2002;56(1):187-209.
154. Walker M, Singh A, Nazarali A, Gibson TWG, Rousseau J, Weese JS. Evaluation of the Impact of Methicillin-Resistant Staphylococcus pseudintermedius Biofilm Formation on Antimicrobial Susceptibility. Veterinary Surgery. 2016;45(7):968-71.
155. Chen H-H, Liu X, Ni C, Lu Y-P, Xiong G-Y, Lu Y-Y, et al. Bacterial biofilms in chronic rhinosinusitis and their relationship with inflammation severity. Auris Nasus Larynx. 2012;39(2):169-74.
156. Psaltis AJ, Ha KR, Beule AG, Tan LW, Wormald PJ. Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis. Laryngoscope. 2007;117(7):1302-6.
157. Pinto Bezerra TF, de Melo Pádua FG, Ogawa AI, Santiago Gebrim EMM, Nascimento Saldiva PH, Voegels RL. Biofilm in Chronic Sinusitis with Nasal Polyps: Pilot study. Brazilian Journal of Otorhinolaryngology (English Edition). [10.1016/S1808-8694(15)30538-3]. 2009(75):788-93.
158. Psaltis AJ, Weitzel EK, Ha KR, Wormald P-J. The effect of bacterial biofilms on post-sinus surgical outcomes. Am J Rhinol. 2008;22(1):1-6.
159. Singhal D, Psaltis AJ, Foreman A, Peter-John W. The impact of biofilms on outcomes after endoscopic sinus surgery. Am J Rhinol Allergy. 2010;24(3):169-74.
160. Prince AA, Steiger JD, Khalid AN, Dogrhamji L, Reger C, Eau Claire S, et al. Prevalence of biofilm-forming bacteria in chronic rhinosinusitis. Am J Rhinol. 2008;22(3):239-45.
161. Foreman A, Wormald PJ. Different biofilms, different disease? A clinical outcomes study. Laryngoscope. 2010;120(8):1701-6.
162. Bendouah Z, Barbeau J, Hamad WA, Desrosiers M. Biofilm formation by Staphylococcus aureus and Pseudomonas aeruginosa is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery. 2006;134(6):991-6.
163. Clement S, Vaudaux P, Francois P, Schrenzel J, Huggler E, Kampf S, et al. Evidence of an Intracellular Reservoir in the Nasal Mucosa of Patients with Recurrent Staphylococcus aureus Rhinosinusitis. The Journal of Infectious Diseases. 2005;192(6):1023-8.
164. Tan NCW, Foreman A, Jardeleza C, Douglas R, Tran H, Wormald PJ. The multiplicity of Staphylococcus aureus in chronic rhinosinusitis: Correlating surface biofilm and intracellular residence. The Laryngoscope. 2012;122(8):1655-60.
165. Hayes SM, Howlin R, Johnston DA, Webb JS, Clarke SC, Stoodley P, et al. Intracellular residency of Staphylococcus aureus within mast cells in nasal polyps:

A novel observation. Journal of Allergy and Clinical Immunology. 2015;135(6):1648-51.e5.
166. Tan NCW, Foreman A, Jardeleza C, Douglas R, Vreugde S, Wormald P-J. Intracellular Staphylococcus aureus: the Trojan horse of recalcitrant chronic rhinosinusitis? Int Forum Allergy Rhinol. 2013;3(4):261-6.
167. Ou J, Drilling A, Singhal D, Tan NCW, Wallis-Hill D, Vreugde S, et al. Association of intracellular Staphylococcus aureus with prognosis in chronic rhinosinusitis. Int Forum Allergy Rhinol. 2016;6(8):792-9.
168. Sachse F, Becker K, Von Eiff C, Metze D, Rudack C. Staphylococcus aureus invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells in vitro. Allergy. 2010;65(11):1430-7.
169. Perez AC, Pang B, King LB, Tan L, Murrah KA, Reimche JL, et al. Residence of Streptococcus pneumoniae and Moraxella catarrhalis within polymicrobial biofilm promotes antibiotic resistance and bacterial persistence in vivo. Pathogens and Disease. 2014;70(3):280-8.
170. Castro J, Machado D, Cerca N. Escherichia coli and Enterococcus faecalis are able to incorporate and enhance a pre-formed Gardnerella vaginalis biofilm. Pathogens and Disease. 2016;74(3):ftw007-ftw.
171. Abreu NA, Nagalingam NA, Song Y, Roediger FC, Pletcher SD, Goldberg AN, et al. Sinus Microbiome Diversity Depletion and Corynebacterium tuberculostearicum Enrichment Mediates Rhinosinusitis. Science Translational Medicine. 2012;4(151):151ra24-ra24.
172. Yan M, Pamp Sünje J, Fukuyama J, Hwang Peter H, Cho D-Y, Holmes S, et al. Nasal Microenvironments and Interspecific Interactions Influence Nasal Microbiota Complexity and S. aureus Carriage. Cell Host & Microbe. 2013;14(6):631-40.
173. Iwase T, Uehara Y, Shinji H, Tajima A, Seo H, Takada K, et al. Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. Nature. [10.1038/nature09074]. 2010;465(7296):346-9.
174. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol. [Review]. 2010;10(3):159-69.
175. Round JL, Mazmanian SK. Inducible Foxp3(+) regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(27):12204-9.
176. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446-50.
177. Cope EK, Goldberg AN, Pletcher SD, Lynch SV. A chronic rhinosinusitis–derived isolate of Pseudomonas aeruginosa induces acute and pervasive effects on the murine upper airway microbiome and host immune response. Int Forum Allergy Rhinol. 2016;6(12):1229-37.
178. Katzenstein AL, Sale SR, Greenberger PA. Allergic Aspergillus sinusitis: a newly recognized form of sinusitis. J Allergy Clin Immunol. 1983;72(1):89-93.
179. Bent JP, 3rd, Kuhn FA. Diagnosis of allergic fungal sinusitis. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery. 1994;111(5):580-8.
180. Schubert MS, Goetz DW. Evaluation and treatment of allergic fungal sinusitis. II. Treatment and follow-up. Journal of Allergy and Clinical Immunology. 1998;102(3):395-402.
181. Correll DP, Luzi SA, Nelson BL. Allergic Fungal Sinusitis. Head and Neck Pathology. [journal article]. 2015;9(4):488-91.

182. Shin S-H, Lee Y-H, Jeon C-H. Protease-dependent activation of nasal polyp epithelial cells by airborne fungi leads to migration of eosinophils and neutrophils. Acta Oto-Laryngologica. 2006;126(12):1286-94.
183. Ebert CS, McKinney KA, Urrutia G, Wu M, Rose AS, Fleischman GM, et al. Expression of protease-activated receptors in allergic fungal rhinosinusitis. Int Forum Allergy Rhinol. 2014;4(4):266-71.
184. Ferguson BJ, Seethala R, Wood WA. Eosinophilic bacterial chronic rhinosinusitis. Laryngoscope. 2007;117(11):2036-40.
185. Dutre T, Al Dousary S, Zhang N, Bachert C. Allergic fungal rhinosinusitis—more than a fungal disease? Journal of Allergy and Clinical Immunology. 2013;132(2):487-9.e1.
186. Boase S, Valentine R, Singhal D, Tan LW, Wormald P-J. A sheep model to investigate the role of fungal biofilms in sinusitis: fungal and bacterial synergy. Int Forum Allergy Rhinol. 2011;1(5):340-7.
187. Boase S, Jervis-Bardy J, Cleland E, Pant H, Tan L, Wormald PJ. Bacterial-induced epithelial damage promotes fungal biofilm formation in a sheep model of sinusitis. Int Forum Allergy Rhinol. 2013;3(5):341-8.
188. Gosepath J, Brieger J, Vlachtsis K, Mann WJ. Fungal DNA is present in tissue specimens of patients with chronic rhinosinusitis. Am J Rhinol. 2004;18(1):9-13.
189. Weschta M, Rimek D, Formanek M, Polzehl D, Podbielski A, Riechelmann H. Topical antifungal treatment of chronic rhinosinusitis with nasal polyps: A randomized, double-blind clinical trial. Journal of Allergy and Clinical Immunology. 2004;113(6):1122-8.
190. Shin S-H, Ponikau JU, Sherris DA, Congdon D, Frigas E, Homburger HA, et al. Chronic rhinosinusitis: An enhanced immune response to ubiquitous airborne fungi. Journal of Allergy and Clinical Immunology. 2004;114(6):1369-75.
191. Ebbens FA, Scadding GK, Badia L, Hellings PW, Jorissen M, Mullol J, et al. Amphotericin B nasal lavages: Not a solution for patients with chronic rhinosinusitis. Journal of Allergy and Clinical Immunology. 2006;118(5):1149-56.
192. Gerlinger I, Fittler A, Fonai F, Patzko A, Mayer A, Botz L. Postoperative application of amphotericin B nasal spray in chronic rhinosinusitis with nasal polyposis, with a review of the antifungal therapy. Eur Arch Otorhinolaryngol. 2009;266(6):847-55.
193. Kennedy DW, Kuhn FA, Hamilos DL, Zinreich SJ, Butler D, Warsi G, et al. Treatment of chronic rhinosinusitis with high-dose oral terbinafine: a double blind, placebo-controlled study. Laryngoscope. 2005;115(10):1793-9.
194. Liang KL, Su MC, Shiao JY, Tseng HC, Hsin CH, Lin JF, et al. Amphotericin B irrigation for the treatment of chronic rhinosinusitis without nasal polyps: a randomized, placebo-controlled, double-blind study. Am J Rhinol. 2008;22(1):52-8.
195. Ponikau JU, Sherris DA, Weaver A, Kita H. Treatment of chronic rhinosinusitis with intranasal amphotericin B: a randomized, placebo-controlled, double-blind pilot trial. J Allergy Clin Immunol. 2005;115(1):125-31.
196. Liao B, Hu C-Y, Liu T, Liu Z. Respiratory viral infection in the chronic persistent phase of chronic rhinosinusitis. The Laryngoscope. 2014;124(4):832-7.
197. Cho GS, Moon B-J, Lee B-J, Gong C-H, Kim NH, Kim Y-S, et al. High Rates of Detection of Respiratory Viruses in the Nasal Washes and Mucosae of Patients with Chronic Rhinosinusitis. Journal of Clinical Microbiology. 2013;51(3):979-84.
198. Rowan NR, Lee S, Sahu N, Kanaan A, Cox S, Phillips CD, et al. The role of viruses in the clinical presentation of chronic rhinosinusitis. Am J Rhinol Allergy. 2015;29(6):e197-e200.

199. Tacon CE, Wiehler S, Holden NS, Newton R, Proud D, Leigh R. Human Rhinovirus Infection Up-Regulates MMP-9 Production in Airway Epithelial Cells via NF-[kappa]B. American Journal of Respiratory Cell and Molecular Biology. 2010;43(2):201-9.
200. Wang JH, Kwon HJ, Jang YJ. Rhinovirus upregulates matrix metalloproteinase-2, matrix metalloproteinase-9, and vascular endothelial growth factor expression in nasal polyp fibroblasts. The Laryngoscope. 2009;119(9):1834-8.
201. Detwiller KY, Smith TL, Mace JC, Trune DR, Sautter NB. Steroid-independent upregulation of matrix metalloproteinase 9 in chronic rhinosinusitis patients with radiographic evidence of osteitis. Int Forum Allergy Rhinol. 2013;3(5):364-8.
202. Watelet J-B, Claeys C, Cauwenberge PV, Bachert C. Predictive and monitoring value of matrix metalloproteinase-9 for healing quality after sinus surgery. Wound Repair and Regeneration. 2004;12(4):412-8.
203. Wang JH, Kwon HJ, Jang YJ. Rhinovirus enhances various bacterial adhesions to nasal epithelial cells simultaneously. The Laryngoscope. 2009;119(7):1406-11.
204. Yeo N-K, Jang YJ. Rhinovirus infection-induced alteration of tight junction and adherens junction components in human nasal epithelial cells. The Laryngoscope. 2010;120(2):346-52.
205. Golebski K, van Tongeren J, van Egmond D, de Groot EJ, Fokkens WJ, van Drunen CM. Specific Induction of TSLP by the Viral RNA Analogue Poly(I:C) in Primary Epithelial Cells Derived from Nasal Polyps. PLOS ONE. 2016;11(4):e0152808.
206. Segata N, Haake SK, Mannon P, Lemon KP, Waldron L, Gevers D, et al. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. Genome Biology. 2012;13(6):R42-R.
207. D'Agata E. Pseudomonas aeruginosa and Other Pseudomonas species. In: Bennett JE, Dolin R, Blaser MJ, editors. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 8th ed. New York: Saunders; 2015.
208. Wahba AH, Darrell JH. The Identification of Atypical Strains of Pseudomonas aeruginosa. Microbiology. 1965;38(3):329-42.
209. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, et al. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Micro. [10.1038/nrmicro1384]. 2006;4(4):295-305.
210. Pritt B, O'Brien L, Winn W. Mucoid Pseudomonas in Cystic Fibrosis. American Journal of Clinical Pathology. 2007;128(1):32-4.
211. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Molecular Microbiology. 1998;30(2):295-304.
212. Hazlett LD, Moon MM, Singh A, Berk RS, Rudner XL. Analysis of adhesion, piliation, protease production and ocular infectivity of several P. aeruginosa strains. Current Eye Research. 1991;10(4):351-62.
213. Ramphal R, Sadoff JC, Pyle M, Silipigni JD. Role of pili in the adherence of Pseudomonas aeruginosa to injured tracheal epithelium. Infection and Immunity. 1984;44(1):38-40.
214. Sastry PA, Finlay BB, Pasloske BL, Paranchych W, Pearlstone JR, Smillie LB. Comparative studies of the amino acid and nucleotide sequences of pilin derived from Pseudomonas aeruginosa PAK and PAO. Journal of Bacteriology. 1985;164(2):571-7.
215. Kus JV, Tullis E, Cvitkovitch DG, Burrows LL. Significant differences in type IV pilin allele distribution among Pseudomonas aeruginosa isolates from cystic fibrosis (CF) versus non-CF patients. Microbiology. 2004;150(5):1315-26.

216. Comer JE, Marshall MA, Blanch VJ, Deal CD, Castric P. Identification of the Pseudomonas aeruginosa 1244 Pilin Glycosylation Site. Infection and Immunity. 2002;70(6):2837-45.
217. Mahenthiralingam E, Campbell ME, Speert DP. Nonmotility and phagocytic resistance of Pseudomonas aeruginosa isolates from chronically colonized patients with cystic fibrosis. Infection and Immunity. 1994;62(2):596-605.
218. Guzzo J, Pages JM, Duong F, Lazdunski A, Murgier M. Pseudomonas aeruginosa alkaline protease: evidence for secretion genes and study of secretion mechanism. Journal of Bacteriology. 1991;173(17):5290-7.
219. Duong F, Bonnet E, Géli V, Lazdunski A, Murgier M, Filloux A. The AprX protein of Pseudomonas aeruginosa: a new substrate for the Apr type I secretion system. Gene. 2001;262(1–2):147-53.
220. Létoffé S, Redeker V, Wandersman C. Isolation and characterization of an extracellular haem-binding protein from Pseudomonas aeruginosa that shares function and sequence similarities with the Serratia marcescens HasA haemophore. Molecular Microbiology. 1998;28(6):1223-34.
221. Bleves S, Viarre V, Salacha R, Michel GPF, Filloux A, Voulhoux R. Protein secretion systems in Pseudomonas aeruginosa: A wealth of pathogenic weapons. International Journal of Medical Microbiology. 2010;300(8):534-43.
222. Dacheux D, Goure J, Chabert J, Usson Y, Attree I. Pore-forming activity of type III system-secreted proteins leads to oncosis of Pseudomonas aeruginosa-infected macrophages. Molecular Microbiology. 2001;40(1):76-85.
223. Vance RE, Rietsch A, Mekalanos JJ. Role of the Type III Secreted Exoenzymes S, T, and Y in Systemic Spread of Pseudomonas aeruginosa PAO1 In Vivo. Infection and Immunity. 2005;73(3):1706-13.
224. Lee VT, Smith RS, Tümmler B, Lory S. Activities of Pseudomonas aeruginosa Effectors Secreted by the Type III Secretion System In Vitro and during Infection. Infection and Immunity. 2005;73(3):1695-705.
225. Shafikhani SH, Morales C, Engel J. The Pseudomonas aeruginosa type III secreted toxin ExoT is necessary and sufficient to induce apoptosis in epithelial cells. Cellular Microbiology. 2008;10(4):994-1007.
226. Barbieri JT. Pseudomonas aeruginosa exoenzyme S, a bifunctional type-III secreted cytotoxin. Int J Med Microbiol. 2000;290(4-5):381-7.
227. Garrity-Ryan L, Kazmierczak B, Kowal R, Comolli J, Hauser A, Engel JN. The Arginine Finger Domain of ExoT Contributes to Actin Cytoskeleton Disruption and Inhibition of Internalization of Pseudomonas aeruginosa by Epithelial Cells and Macrophages. Infection and Immunity. 2000;68(12):7100-13.
228. Goehring UM, Schmidt G, Pederson KJ, Aktories K, Barbieri JT. The N-terminal domain of Pseudomonas aeruginosa exoenzyme S is a GTPase-activating protein for Rho GTPases. J Biol Chem. 1999;274(51):36369-72.
229. Krall R, Schmidt G, Aktories K, Barbieri JT. Pseudomonas aeruginosa ExoT Is a Rho GTPase-Activating Protein. Infection and Immunity. 2000;68(10):6066-8.
230. Pederson KJ, Vallis AJ, Aktories K, Frank DW, Barbieri JT. The amino-terminal domain of Pseudomonas aeruginosa ExoS disrupts actin filaments via small-molecular-weight GTP-binding proteins. Mol Microbiol. 1999;32(2):393-401.
231. Rocha CL, Coburn J, Rucks EA, Olson JC. Characterization of Pseudomonas aeruginosa Exoenzyme S as a Bifunctional Enzyme in J774A.1 Macrophages. Infection and Immunity. 2003;71(9):5296-305.
232. Fraylick JE, La Rocque JR, Vincent TS, Olson JC. Independent and Coordinate Effects of ADP-Ribosyltransferase and GTPase-Activating Activities of Exoenzyme S on HT-29 Epithelial Cell Function. Infection and Immunity. 2001;69(9):5318-28.

233. Hauser AR. The Type III Secretion System of Pseudomonas aeruginosa: Infection by Injection. Nature reviews Microbiology. 2009;7(9):654-65.
234. Cowell BA, Chen DY, Frank DW, Vallis AJ, Fleiszig SMJ. ExoT of Cytotoxic Pseudomonas aeruginosa Prevents Uptake by Corneal Epithelial Cells. Infection and Immunity. 2000;68(1):403-6.
235. Kazmierczak BI, Engel JN. Pseudomonas aeruginosa ExoT Acts In Vivo as a GTPase-Activating Protein for RhoA, Rac1, and Cdc42. Infection and Immunity. 2002;70(4):2198-205.
236. Sato H, Frank DW, Hillard CJ, Feix JB, Pankhaniya RR, Moriyama K, et al. The mechanism of action of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU. The EMBO Journal. 2003;22(12):2959-69.
237. Yahr TL, Vallis AJ, Hancock MK, Barbieri JT, Frank DW. ExoY, an adenylate cyclase secreted by the Pseudomonas aeruginosa type III system. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(23):13899-904.
238. Hauser AR, Kang PJ, Engel JN. PepA, a secreted protein of Pseudomonas aeruginosa, is necessary for cytotoxicity and virulence. Molecular Microbiology. 1998;27(4):807-18.
239. Sawa T, Ohara M, Kurahashi K, Twining SS, Frank DW, Doroques DB, et al. In Vitro Cellular Toxicity Predicts Pseudomonas aeruginosa Virulence in Lung Infections. Infection and Immunity. 1998;66(7):3242-9.
240. Lee EJ, Evans DJ, Fleiszig SM. Role of Pseudomonas aeruginosa ExsA in penetration through corneal epithelium in a novel in vivo model. Invest Ophthalmol Vis Sci. 2003;44(12):5220-7.
241. Holder IA, Neely AN, Frank DW. Type III secretion/intoxication system important in virulence of Pseudomonas aeruginosa infections in burns. Burns. 2001;27(2):129-30.
242. Balachandran P, Dragone L, Garrity-Ryan L, Lemus A, Weiss A, Engel J. The ubiquitin ligase Cbl-b limits Pseudomonas aeruginosa exotoxin T–mediated virulence. Journal of Clinical Investigation. 2007;117(2):419-27.
243. Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, et al. Type III Protein Secretion Is Associated with Death in Lower Respiratory and Systemic Pseudomonas aeruginosa Infections. The Journal of Infectious Diseases. 2001;183(12):1767-74.
244. Hauser AR, Cobb E, Bodi M, Mariscal D, Valles J, Engel JN, et al. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by Pseudomonas aeruginosa. Critical care medicine. 2002;30(3):521-8.
245. El Solh AA, Akinnusi ME, Wiener-Kronish JP, Lynch SV, Pineda LA, Szarpa K. Persistent Infection with Pseudomonas aeruginosa in Ventilator-associated Pneumonia. American Journal of Respiratory and Critical Care Medicine. 2008;178(5):513-9.
246. Wilhelm S, Gdynia A, Tielen P, Rosenau F, Jaeger K-E. The Autotransporter Esterase EstA of Pseudomonas aeruginosa Is Required for Rhamnolipid Production, Cell Motility, and Biofilm Formation. Journal of Bacteriology. 2007;189(18):6695-703.
247. Kida Y, Higashimoto Y, Inoue H, Shimizu T, Kuwano K. A novel secreted protease from Pseudomonas aeruginosa activates NF-κB through protease-activated receptors. Cellular Microbiology. 2008;10(7):1491-504.

248. Ruer S, Ball G, Filloux A, de Bentzmann S. The 'P-usher', a novel protein transporter involved in fimbrial assembly and TpsA secretion. The EMBO Journal. 2008;27(20):2669-80.
249. Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, Gifford CA, et al. A Virulence Locus of Pseudomonas aeruginosa Encodes a Protein Secretion Apparatus. Science. 2006;312(5779):1526-30.
250. Sana TG, Berni B, Bleves S. The T6SSs of Pseudomonas aeruginosa Strain PAO1 and Their Effectors: Beyond Bacterial-Cell Targeting. Frontiers in Cellular and Infection Microbiology. [Mini Review]. 2016;6(61).
251. Pearson JP, Feldman M, Iglewski BH, Prince A. Pseudomonas aeruginosa Cell-to-Cell Signaling Is Required for Virulence in a Model of Acute Pulmonary Infection. Infection and Immunity. 2000;68(7):4331-4.
252. Wu H, Song Z, Givskov M, Doring G, Worlitzsch D, Mathee K, et al. Pseudomonas aeruginosa mutations in lasI and rhlI quorum sensing systems result in milder chronic lung infection. Microbiology. 2001;147(5):1105-13.
253. Rumbaugh KP, Griswold JA, Iglewski BH, Hamood AN. Contribution of Quorum Sensing to the Virulence of Pseudomonas aeruginosa in Burn Wound Infections. Infection and Immunity. 1999;67(11):5854-62.
254. Pesci EC, Milbank JBJ, Pearson JP, McKnight S, Kende AS, Greenberg EP, et al. Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(20):11229-34.
255. Das T, Manefield M. Pyocyanin Promotes Extracellular DNA Release in Pseudomonas aeruginosa. PLOS ONE. 2012;7(10):e46718.
256. Hassan HM, Fridovich I. Mechanism of the antibiotic action pyocyanine. Journal of Bacteriology. 1980;141(1):156-63.
257. Usher LR, Lawson RA, Geary I, Taylor CJ, Bingle CD, Taylor GW, et al. Induction of Neutrophil Apoptosis by the Pseudomonas aeruginosa Exotoxin Pyocyanin: A Potential Mechanism of Persistent Infection. The Journal of Immunology. 2002;168(4):1861-8.
258. Allen L, Dockrell DH, Pattery T, Lee DG, Cornelis P, Hellewell PG, et al. Pyocyanin Production by Pseudomonas aeruginosa Induces Neutrophil Apoptosis and Impairs Neutrophil-Mediated Host Defenses In Vivo. The Journal of Immunology. 2005;174(6):3643-9.
259. Bianchi SM, Prince LR, McPhillips K, Allen L, Marriott HM, Taylor GW, et al. Impairment of Apoptotic Cell Engulfment by Pyocyanin, a Toxic Metabolite of Pseudomonas aeruginosa. American Journal of Respiratory and Critical Care Medicine. 2008;177(1):35-43.
260. Shak S, Capon DJ, Hellmiss R, Marsters SA, Baker CL. Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. Proceedings of the National Academy of Sciences. 1990;87(23):9188-92.
261. Nutman J, Berger M, Chase PA, Dearborn DG, Miller KM, Waller RL, et al. Studies on the mechanism of T cell inhibition by the Pseudomonas aeruginosa phenazine pigment pyocyanine. The Journal of Immunology. 1987;138(10):3481-7.
262. Ulmer AJ, Pryjma J, Tarnok Z, Ernst M, Flad HD. Inhibitory and stimulatory effects of Pseudomonas aeruginosa pyocyanine on human T and B lymphocytes and human monocytes. Infection and Immunity. 1990;58(3):808-15.
263. Schalk IJ, Kyslik P, Prome D, van Dorsselaer A, Poole K, Abdallah MA, et al. Copurification of the FpvA ferric pyoverdin receptor of Pseudomonas aeruginosa

with its iron-free ligand: implications for siderophore-mediated iron transport. Biochemistry. 1999;38(29):9357-65.
264. Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML. Siderophore-mediated signaling regulates virulence factor production in Pseudomonas aeruginosa. Proceedings of the National Academy of Sciences. 2002;99(10):7072-7.
265. Fegan M, Francis P, Hayward AC, Davis GH, Fuerst JA. Phenotypic conversion of Pseudomonas aeruginosa in cystic fibrosis. Journal of Clinical Microbiology. 1990;28(6):1143-6.
266. Govan JR, Harris GS. Pseudomonas aeruginosa and cystic fibrosis: unusual bacterial adaptation and pathogenesis. Microbiological sciences. 1986;3(10):302-8.
267. Baltimore RS, Mitchell M. Immunologic Investigations of Mucoid Strains of Pseudomonas aeruginosa: Comparison of Susceptibility to Opsonic Antibody in Mucoid and Nonmucoid Strains. The Journal of Infectious Diseases. 1980;141(2):238-47.
268. Baltimore RS, Shedd DG. The Role of Complement in the Opsonization of Mucoid and Non-Mucoid Strains of Pseudomonas Aeruginosa. Pediatr Res. 1983;17(12):952-8.
269. Cabral DA, Loh BA, Speert DP. Mucoid Pseudomonas aeruginosa Resists Nonopsonic Phagocytosis by Human Neutrophils and Macrophages. Pediatr Res. 1987;22(4):429-31.
270. Krieg DP, Helmke RJ, German VF, Mangos JA. Resistance of mucoid Pseudomonas aeruginosa to nonopsonic phagocytosis by alveolar macrophages in vitro. Infection and Immunity. 1988;56(12):3173-9.
271. Meshulam T, Obedeanu N, Merzbach D, Sobel JD. Phagocytosis of mucoid and nonmucoid strains of Pseudomonas aeruginosa. Clinical Immunology and Immunopathology. 1984;32(2):151-65.
272. Oliver AM, Weir DM. Inhibition of bacterial binding to mouse macrophages by Pseudomonas alginate. Journal of clinical & laboratory immunology. 1983;10(4):221-4.
273. Schwarzmann S, Boring JR. Antiphagocytic Effect of Slime from a Mucoid Strain of Pseudomonas aeruginosa. Infection and Immunity. 1971;3(6):762-7.
274. Mai GT, Seow WK, Pier GB, McCormack JG, Thong YH. Suppression of lymphocyte and neutrophil functions by Pseudomonas aeruginosa mucoid exopolysaccharide (alginate): reversal by physicochemical, alginase, and specific monoclonal antibody treatments. Infection and Immunity. 1993;61(2):559-64.
275. Doig P, Smith NR, Todd T, Irvin RT. Characterization of the binding of Pseudomonas aeruginosa alginate to human epithelial cells. Infection and Immunity. 1987;55(6):1517-22.
276. Nicas TI, Hancock RE. Pseudomonas aeruginosa outer membrane permeability: isolation of a porin protein F-deficient mutant. Journal of Bacteriology. 1983;153(1):281-5.
277. Trias J, Dufresne J, Levesque RC, Nikaido H. Decreased outer membrane permeability in imipenem-resistant mutants of Pseudomonas aeruginosa. Antimicrobial agents and chemotherapy. 1989;33(8):1202-6.
278. Li X-Z, Zhang L, Poole K. Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of Pseudomonas aeruginosa. J Antimicrob Chemother. 2000;45(4):433-6.

279. Li XZ, Nikaido H, Poole K. Role of mexA-mexB-oprM in antibiotic efflux in Pseudomonas aeruginosa. Antimicrobial agents and chemotherapy. 1995;39(9):1948-53.
280. Mine T, Morita Y, Kataoka A, Mizushima T, Tsuchiya T. Expression in Escherichia coli of a New Multidrug Efflux Pump, MexXY, from Pseudomonas aeruginosa. Antimicrobial agents and chemotherapy. 1999;43(2):415-7.
281. Aires JR, Köhler T, Nikaido H, Plésiat P. Involvement of an Active Efflux System in the Natural Resistance of Pseudomonas aeruginosa to Aminoglycosides. Antimicrobial agents and chemotherapy. 1999;43(11):2624-8.
282. Srikumar R, Li XZ, Poole K. Inner membrane efflux components are responsible for beta-lactam specificity of multidrug efflux pumps in Pseudomonas aeruginosa. Journal of Bacteriology. 1997;179(24):7875-81.
283. Livermore DM, Yang Y-J. Beta-Lactamase Lability and Inducer Power of Newer Beta-Lactam Antibiotics in Relation to Their Activity against Beta-Lactamase-Inducibility Mutants of Pseudomonas aeruginosa. The Journal of Infectious Diseases. 1987;155(4):775-82.
284. Ramphal R, Lhermitte M, Filliat M, Roussel P. The binding of anti-pseudomonal antibiotics to macromolecules from cystic fibrosis sputum. J Antimicrob Chemother. 1988;22(4):483-90.
285. Weiner DJ, Bucki R, Janmey PA. The Antimicrobial Activity of the Cathelicidin LL37 Is Inhibited by F-actin Bundles and Restored by Gelsolin. American Journal of Respiratory Cell and Molecular Biology. 2003;28(6):738-45.
286. Potter JL, Matthews LW, Spector S, Lemm J. Complex formation between basic antibiotics and deoxyribonucleic acid in human pulmonary secretions. Pediatrics. 1965;36(5):714-20.
287. Chiang W-C, Nilsson M, Jensen PØ, Høiby N, Nielsen TE, Givskov M, et al. Extracellular DNA Shields against Aminoglycosides in Pseudomonas aeruginosa Biofilms. Antimicrobial agents and chemotherapy. 2013;57(5):2352-61.
288. Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA Chelates Cations and Induces Antibiotic Resistance in Pseudomonas aeruginosa Biofilms. PLoS pathogens. 2008;4(11):e1000213.
289. Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, et al. Alginate Overproduction Affects Pseudomonas aeruginosa Biofilm Structure and Function. Journal of Bacteriology. 2001;183(18):5395-401.
290. Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. Contributions of Antibiotic Penetration, Oxygen Limitation, and Low Metabolic Activity to Tolerance of Pseudomonas aeruginosa Biofilms to Ciprofloxacin and Tobramycin. Antimicrobial agents and chemotherapy. 2003;47(1):317-23.
291. Xu KD, McFeters GA, Stewart PS. Biofilm resistance to antimicrobial agents. Microbiology. 2000;146(3):547-9.
292. Xu KD, Stewart PS, Xia F, Huang C-T, McFeters GA. Spatial Physiological Heterogeneity in Pseudomonas aeruginosa Biofilm Is Determined by Oxygen Availability. Applied and environmental microbiology. 1998;64(10):4035-9.
293. Huang CT, Yu FP, McFeters GA, Stewart PS. Nonuniform spatial patterns of respiratory activity within biofilms during disinfection. Applied and environmental microbiology. 1995;61(6):2252-6.
294. Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T. Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. Molecular Microbiology. 2008;68(1):223-40.

295. Brooun A, Liu S, Lewis K. A Dose-Response Study of Antibiotic Resistance in Pseudomonas aeruginosa Biofilms. Antimicrobial agents and chemotherapy. 2000;44(3):640-6.
296. Haagensen JAJ, Klausen M, Ernst RK, Miller SI, Folkesson A, Tolker-Nielsen T, et al. Differentiation and Distribution of Colistin- and Sodium Dodecyl Sulfate-Tolerant Cells in Pseudomonas aeruginosa Biofilms. Journal of Bacteriology. 2007;189(1):28-37.
297. Spoering AL, Lewis K. Biofilms and Planktonic Cells of Pseudomonas aeruginosa Have Similar Resistance to Killing by Antimicrobials. Journal of Bacteriology. 2001;183(23):6746-51.
298. Harrison JJ, Turner RJ, Ceri H. Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic Pseudomonas aeruginosa. Environmental Microbiology. 2005;7(7):981-94.
299. De Groote VN, Verstraeten N, Fauvart M, Kint CI, Verbeeck AM, Beullens S, et al. Novel persistence genes in Pseudomonas aeruginosa identified by high-throughput screening. FEMS Microbiology Letters. 2009;297(1):73-9.
300. Viducic D, Ono T, Murakami K, Susilowati H, Kayama S, Hirota K, et al. Functional Analysis of spoT, relA and dksA Genes on Quinolone Tolerance in Pseudomonas aeruginosa under Nongrowing Condition. Microbiology and Immunology. 2006;50(4):349-57.
301. Murakami K, Ono T, Viducic D, Kayama S, Mori M, Hirota K, et al. Role for rpoS gene of Pseudomonas aeruginosa in antibiotic tolerance. FEMS Microbiology Letters. 2005;242(1):161-7.
302. Chiang W-C, Pamp SJ, Nilsson M, Givskov M, Tolker-Nielsen T. The metabolically active subpopulation in Pseudomonas aeruginosa biofilms survives exposure to membrane-targeting antimicrobials via distinct molecular mechanisms. FEMS Immunology & Medical Microbiology. 2012;65(2):245-56.
303. Gillis RJ, White KG, Choi K-H, Wagner VE, Schweizer HP, Iglewski BH. Molecular Basis of Azithromycin-Resistant Pseudomonas aeruginosa Biofilms. Antimicrobial agents and chemotherapy. 2005;49(9):3858-67.
304. Liao J, Schurr MJ, Sauer K. The MerR-Like Regulator BrlR Confers Biofilm Tolerance by Activating Multidrug Efflux Pumps in Pseudomonas aeruginosa Biofilms. Journal of Bacteriology. 2013;195(15):3352-63.
305. Mah T-F, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA. A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance. Nature. [10.1038/nature02122]. 2003;426(6964):306-10.
306. Livermore DM, Jones CS. Characterization of NPS-1, a novel plasmid-mediated beta-lactamase, from two Pseudomonas aeruginosa isolates. Antimicrobial agents and chemotherapy. 1986;29(1):99-103.
307. Simpson IN, Plested SJ, Budin-Jones MJ, Lees J, Hedges RW, Jacoby GA. Characterisation of a novel plasmid-mediated β-lactamase and its contribution to β-lactam resistance in Pseudomonas aeruginosa. FEMS Microbiology Letters. 1983;19(1):23-7.
308. Nordmann P, Guibert M. Extended-spectrum beta-lactamases in Pseudomonas aeruginosa. J Antimicrob Chemother. 1998;42(2):128-31.
309. Aubert D, Poirel L, Chevalier J, Leotard S, Pages J-M, Nordmann P. Oxacillinase-Mediated Resistance to Cefepime and Susceptibility to Ceftazidime in Pseudomonas aeruginosa. Antimicrobial agents and chemotherapy. 2001;45(6):1615-20.

310. Danel F, Hall LM, Gur D, Livermore DM. OXA-15, an extended-spectrum variant of OXA-2 beta-lactamase, isolated from a Pseudomonas aeruginosa strain. Antimicrobial agents and chemotherapy. 1997;41(4):785-90.
311. Kilic A, Aktas Z, Bedir O, Gumral R, Bulut Y, Stratton C, et al. Identification and characterization of OXA-48 producing, carbapenem-resistant Enterobacteriaceae isolates in Turkey. Annals of clinical and laboratory science. 2011;41(2):161-6.
312. Borah V, Saikia K, Hazarika N. First report on the detection of OXA-48 beta-lactamase gene in Escherichia coli and Pseudomonas aeruginosa co-infection isolated from a patient in a Tertiary Care Hospital in Assam. Indian Journal of Medical Microbiology. [Correspondence]. 2016;34(2):252-3.
313. Neyestanaki DK, Mirsalehian A, Rezagholizadeh F, Jabalameli F, Taherikalani M, Emaneini M. Determination of extended spectrum beta-lactamases, metallo-beta-lactamases and AmpC-beta-lactamases among carbapenem resistant Pseudomonas aeruginosa isolated from burn patients. Burns. 2014;40(8):1556-61.
314. Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, et al. Cloning and Characterization of bla VIM, a New Integron-Borne Metallo-β-Lactamase Gene from a Pseudomonas aeruginosa Clinical Isolate. Antimicrobial agents and chemotherapy. 1999;43(7):1584-90.
315. Giske CG, Libisch B, Colinon C, Scoulica E, Pagani L, Füzi M, et al. Establishing Clonal Relationships between VIM-1-Like Metallo-β-Lactamase-Producing Pseudomonas aeruginosa Strains from Four European Countries by Multilocus Sequence Typing. Journal of Clinical Microbiology. 2006;44(12):4309-15.
316. Jácome PRLdA, Alves LR, Cabral AB, Lopes ACS, Maciel MAV. First Report of KPC-Producing Pseudomonas aeruginosa in Brazil. Antimicrobial agents and chemotherapy. 2012;56(9):4990.
317. Livermore DM. Current Epidemiology and Growing Resistance of Gram-Negative Pathogens. Korean J Intern Med. 2012;27(2):128-42.
318. Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiology Reviews. 2011;35(5):736-55.
319. Miller GH, Sabatelli FJ, Hare RS, Glupczynski Y, Mackey P, Shlaes D, et al. The Most Frequent Aminoglycoside Resistance Mechanisms—Changes with Time and Geographic Area: A Reflection of Aminoglycoside Usage Patterns? Clinical Infectious Diseases. 1997;24(Supplement_1):S46-S62.
320. Yokoyama K, Doi Y, Yamane K, Kurokawa H, Shibata N, Shibayama K, et al. Acquisition of 16S rRNA methylase gene in Pseudomonas aeruginosa. The Lancet. 2003;362(9399):1888-93.
321. Doi Y, de Oliveira Garcia D, Adams J, Paterson DL. Coproduction of Novel 16S rRNA Methylase RmtD and Metallo-β-Lactamase SPM-1 in a Panresistant Pseudomonas aeruginosa Isolate from Brazil. Antimicrobial agents and chemotherapy. 2007;51(3):852-6.
322. Poole K, Gotoh N, Tsujimoto H, Zhao Q, Wada A, Yamasaki T, et al. Overexpression of the mexC–mexD–oprJ efflux operon in nfxB-type multidrug-resistant strains of Pseudomonas aeruginosa. Molecular Microbiology. 1996;21(4):713-25.
323. Ko¨hler T, Michéa-Hamzehpour M, Henze U, Gotoh N, Kocjancic Curty L, Pechère J-C. Characterization of MexE–MexF–OprN, a positively regulated multidrug efflux system of Pseudomonas aeruginosa. Molecular Microbiology. 1997;23(2):345-54.

324. Willmott CJ, Maxwell A. A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. Antimicrobial agents and chemotherapy. 1993;37(1):126-7.
325. Kureishi A, Diver JM, Beckthold B, Schollaardt T, Bryan LE. Cloning and nucleotide sequence of Pseudomonas aeruginosa DNA gyrase gyrA gene from strain PAO1 and quinolone-resistant clinical isolates. Antimicrobial agents and chemotherapy. 1994;38(9):1944-52.
326. Akasaka T, Tanaka M, Yamaguchi A, Sato K. Type II Topoisomerase Mutations in Fluoroquinolone-Resistant Clinical Strains of Pseudomonas aeruginosa Isolated in 1998 and 1999: Role of Target Enzyme in Mechanism of Fluoroquinolone Resistance. Antimicrobial agents and chemotherapy. 2001;45(8):2263-8.
327. Sader HS, Farrell DJ, Flamm RK, Jones RN. Antimicrobial susceptibility of Gram-negative organisms isolated from patients hospitalised with pneumonia in US and European hospitals: Results from the SENTRY Antimicrobial Surveillance Program, 2009–2012. International Journal of Antimicrobial Agents. 2014;43(4):328-34.
328. Gales AC, Castanheira M, Jones RN, Sader HS. Antimicrobial resistance among Gram-negative bacilli isolated from Latin America: results from SENTRY Antimicrobial Surveillance Program (Latin America, 2008–2010). Diagnostic Microbiology and Infectious Disease. 2012;73(4):354-60.
329. AURA 2016: first Australian report on antimicrobial use and resistance in human health. Sydney: Australian Commission on Safety and Quality in Health Care (ACSQHC); 2016.
330. ResistanceMap - Antibiotic Resistance. Center for Disease Dynamics, Economics & Policy; 2017 [cited 2017 26 June].
331. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiology and Infection. 2012;18(3):268-81.
332. Sader HS, Huband MD, Castanheira M, Flamm RK. Pseudomonas aeruginosa Antimicrobial Susceptibility Results from Four Years (2012 to 2015) of the International Network for Optimal Resistance Monitoring Program in the United States. Antimicrobial agents and chemotherapy. 2017;61(3).
333. Antibiotic Resistance Threats in the United States, 2013. Centers for Disease Control and Prevention; 2013.
334. Kingdom TT, Swain Jr RE. The microbiology and antimicrobial resistance patterns in chronic rhinosinusitis. Am J Otolaryngol. 2004;25(5):323-8.
335. Guss J, Abuzeid WM, Doghramji L, Edelstein PH, Chiu AG. Fluoroquinolone-resistant Pseudomonas aeruginosa in chronic rhinosinusitis. ORL : Journal for Oto - Rhino - Laryngology and Its Related Specialties. [Research Support, Non-U.S. Gov't]. 2009;71(5):263-7.
336. Genoway KA, Philpott CM, Javer AR. Pathogen yield and antimicrobial resistance patterns of chronic rhinosinusitis patients presenting to a tertiary rhinology centre. J Otolaryngol Head Neck Surg. 2011;40(3):232-7.
337. Morrison AJ, Jr., Wenzel RP. Epidemiology of infections due to Pseudomonas aeruginosa. Rev Infect Dis. 1984;6 Suppl 3:S627-42.
338. Köck R, Werner P, Friedrich AW, Fegeler C, Becker K, Bindewald O, et al. Persistence of nasal colonization with human pathogenic bacteria and associated antimicrobial resistance in the German general population. New Microbes and New Infections. 2016;9:24-34.

339. Kwashie ANA, Muibat AF, Modupe AI, Adejumoke BJM, Kehinde AO. A survey of bacterial isolates cultured from apparently healthy individuals in South-Western Nigeria. International Journal of Tropical Medicine. 2012;7(4):130-7.
340. Glück U, Gebbers J-O. Ingested probiotics reduce nasal colonization with pathogenic bacteria (Staphylococcus aureus, Streptococcus pneumoniae, and β-hemolytic streptococci). The American Journal of Clinical Nutrition. 2003;77(2):517-20.
341. Bhattacharyya N, Kepnes LJ. The microbiology of recurrent rhinosinusitis after endoscopic sinus surgery. Arch Otolaryngol Head Neck Surg. 1999;125(10):1117-20.
342. Boase S, Foreman A, Cleland E, Tan L, Melton-Kreft R, Pant H, et al. The microbiome of chronic rhinosinusitis: culture, molecular diagnostics and biofilm detection. BMC infectious diseases. 2013;13:210.
343. Chan J, Hadley J. The microbiology of chronic rhinosinusitis: results of a community surveillance study. Ear Nose Throat J. 2001;80(3):143-5.
344. Rickert SM, Rachakonda T, Hiltzik DH, Kacker A. Microbiology and antibiotic resistance of chronic rhinosinusitis in patients undergoing primary vs. revision endoscopic sinus surgery. The Laryngoscope. 2010;120(S4):S243-S.
345. Krzeski A, Kapiszewska-Dzedzej D, Górski NP, Jakubczyk I. Cystic Fibrosis in Rhinologic Practice. Am J Rhinol. 2002;16(3):155-60.
346. Khalid AN, Mace J, Smith TL. Outcomes of Sinus Surgery in Adults with Cystic Fibrosis. Otolaryngology -- Head and Neck Surgery. 2009;141(3):358-63.
347. Digoy GP, Dunn JD, Stoner JA, Christie A, Jones DT. Bacteriology of the paranasal sinuses in pediatric cystic fibrosis patients. Int J Pediatr Otorhinolaryngol. 2012;76(7):934-8.
348. Muhlebach MS, Miller MB, Moore C, Wedd JP, Drake AF, Leigh MW. Are lower airway or throat cultures predictive of sinus bacteriology in cystic fibrosis? Pediatric Pulmonology. 2006;41(5):445-51.
349. Rasmussen J, Aanaes K, Norling R, Nielsen KG, Johansen HK, von Buchwald C. CT of the paranasal sinuses is not a valid indicator for sinus surgery in CF patients. J Cyst Fibros. 2012;11(2):93-9.
350. Roby BB, McNamara J, Finkelstein M, Sidman J. Sinus surgery in Cystic Fibrosis patients: Comparison of sinus and lower airway cultures. Int J Pediatr Otorhinolaryngol. 2008;72(9):1365-9.
351. Shapiro ED, Milmoe GJ, Wald ER, Rodnan JB, Bowen AD. Bacteriology of the Maxillary Sinuses in Patients with Cystic Fibrosis. The Journal of Infectious Diseases. 1982;146(5):589-93.
352. Wise SK, Kingdom TT, McKean L, DelGaudio JM, Venkatraman G. Presence of fungus in sinus cultures of cystic fibrosis patients. Am J Rhinol. 2005;19(1):47-51.
353. Aanaes K. Bacterial sinusitis can be a focus for initial lung colonisation and chronic lung infection in patients with cystic fibrosis. J Cyst Fibros. 2013;12 Suppl 2:S1-20.
354. Aanaes K, von Buchwald C, Hjuler T, Skov M, Alanin M, Johansen HK. The effect of sinus surgery with intensive follow-up on pathogenic sinus bacteria in patients with cystic fibrosis. Am J Rhinol Allergy. 2013;27(1):e1-4.
355. Johansen HK, Aanaes K, Pressler T, Nielsen KG, Fisker J, Skov M, et al. Colonisation and infection of the paranasal sinuses in cystic fibrosis patients is accompanied by a reduced PMN response. J Cyst Fibros. 2012;11(6):525-31.
356. Jelsbak L, Johansen HK, Frost A-L, Thøgersen R, Thomsen LE, Ciofu O, et al. Molecular Epidemiology and Dynamics of Pseudomonas aeruginosa Populations in Lungs of Cystic Fibrosis Patients. Infection and Immunity. 2007;75(5):2214-24.

357. Munck A, Bonacorsi S, Mariani-Kurkdjian P, Lebourgeois M, Gérardin M, Brahimi N, et al. Genotypic characterization of Pseudomonas aeruginosa strains recovered from patients with cystic fibrosis after initial and subsequent colonization. Pediatric Pulmonology. 2001;32(4):288-92.
358. Taccetti G, Campana S, Festini F, Mascherini M, Döring G. Early eradication therapy against Pseudomonas aeruginosa in cystic fibrosis patients. European Respiratory Journal. 2005;26(3):458-61.
359. Mastella G, Hodson ME, Koch C, Navarro J, Rainisio M, Harms HK, et al. Factors affecting velocity of lung function deterioration in ERCF cystic fibrosis patients over 2–4 years follow-up. The Netherlands Journal of Medicine. 1999;54:S22.
360. Lee B, Haagensen JAJ, Ciofu O, Andersen JB, Høiby N, Molin S. Heterogeneity of Biofilms Formed by Nonmucoid Pseudomonas aeruginosa Isolates from Patients with Cystic Fibrosis. Journal of Clinical Microbiology. 2005;43(10):5247-55.
361. Li Z, Kosorok MR, Farrell PM, Laxova A, West SEH, Green CG, et al. Longitudinal development of mucoid Pseudomonas aeruginosa infection and lung disease progression in children with cystic fibrosis. JAMA. 2005;293(5):581-8.
362. Hansen SK, Rau MH, Johansen HK, Ciofu O, Jelsbak L, Yang L, et al. Evolution and diversification of Pseudomonas aeruginosa in the paranasal sinuses of cystic fibrosis children have implications for chronic lung infection. The ISME Journal. 2012;6(1):31-45.
363. Twort FW. An investigation on the nature of ultra-microscopic viruses. The Lancet. 1915;186(4814):1241-3.
364. d'Herelle F. [On an invisible microbe antagonistic to dysentery bacilli.]. Comptes Rendus Acad Sci. 1917(165):373-5.
365. Ackermann H-W. Phage Classification and Characterization. In: Clokie MRJ, Kropinski AM, editors. Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions. Totowa, NJ: Humana Press; 2009. p. 127-40.
366. Ackermann H. Bacteriophage taxonomy. Microbiology Australia. 2011(2):90-4.
367. Ackermann H-W. 5500 Phages examined in the electron microscope. Archives of Virology. [journal article]. 2007;152(2):227-43.
368. Edgar RS, Lielausis I. Some steps in the assembly of bacteriophage T4. Journal of molecular biology. 1968;32(2):263-76.
369. Kellenberger E. Control mechanisms in the morphogeneses of bacteriophage heads. Bio Systems. 1980;12(3-4):201-23.
370. De Rosier DJ, Klug A. Reconstruction of three dimensional structures from electron micrographs. Nature. 1968;217(5124):130-4.
371. Guttman B, Raya R, Kutter E. Basic Phage Biology. In: Kutter E, Sulakvelidze A, editors. Bacteriophages: Biology and Applications. Boca Raton: CRC Press; 2004.
372. Ackermann H-W. Tailed Bacteriophages: The Order Caudovirales. Advances in Virus Research. 1998;51:135-201.
373. Weinbauer MG. Ecology of prokaryotic viruses. FEMS Microbiology Reviews. 2004;28(2):127-81.
374. Paul JH, Jiang SC. Lysogeny and transduction. Methods in Microbiology. 2001;30:105-25.
375. Hagens S, Bläsi U. Genetically modified filamentous phage as bactericidal agents: a pilot study. Letters in Applied Microbiology. 2003;37(4):318-23.
376. Hagens S, Habel A, von Ahsen U, von Gabain A, Bläsi U. Therapy of Experimental Pseudomonas Infections with a Nonreplicating Genetically Modified Phage. Antimicrobial agents and chemotherapy. 2004;48(10):3817-22.

377. Moradpour Z, Sepehrizadeh Z, Rahbarizadeh F, Ghasemian A, Yazdi MT, Shahverdi AR. Genetically engineered phage harbouring the lethal catabolite gene activator protein gene with an inducer-independent promoter for biocontrol of Escherichia coli. FEMS Microbiology Letters. 2009;296(1):67-71.
378. Westwater C, Kasman LM, Schofield DA, Werner PA, Dolan JW, Schmidt MG, et al. Use of Genetically Engineered Phage To Deliver Antimicrobial Agents to Bacteria: an Alternative Therapy for Treatment of Bacterial Infections. Antimicrobial agents and chemotherapy. 2003;47(4):1301-7.
379. Lu TK, Collins JJ. Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(12):4629-34.
380. Yu P, Mathieu J, Li M, Dai Z, Alvarez PJJ. Isolation of Polyvalent Bacteriophages by Sequential Multiple-Host Approaches. Applied and environmental microbiology. 2016;82(3):808-15.
381. Souza KA, Ginoza HS, Haight RD. Isolation of a Polyvalent Bacteriophage for Escherichia coli, Klebsiella pneumoniae, and Aerobacter aerogenes. Journal of virology. 1972;9(5):851-6.
382. Ly-Chatain MH. The factors affecting effectiveness of treatment in phages therapy. Frontiers in Microbiology. 2014;5(FEB).
383. Bratbak G, Heldal M, Norland S, Thingstad TF. Viruses as Partners in Spring Bloom Microbial Trophodynamics. Applied and environmental microbiology. 1990;56(5):1400-5.
384. Heldal M, Bratbak G. Production and decay of viruses in aquatic environments. Marine Ecology Progress Series. 1991(72):205-12.
385. Steward GF, Wikner J, Smith DC, Cochlan WP, Azam F. Estimation of Virus Production in the Sea: I. Method Development. Marine Microbial Food Webs. 1992;6(2):57-78.
386. Santos SB, Carvalho C, Azeredo J, Ferreira EC. Population Dynamics of a Salmonella Lytic Phage and Its Host: Implications of the Host Bacterial Growth Rate in Modelling. PLOS ONE. 2014;9(7):e102507.
387. Abedon ST. Phage therapy dosing: The problem(s) with multiplicity of infection (MOI). Bacteriophage. 2016;6(3):e1220348.
388. Abedon S. Chapter 1 - Phage Therapy Pharmacology: Calculating Phage Dosing. In: Allen I. Laskin SS, Geoffrey MG, editors. Advances in Applied Microbiology: Academic Press; 2011. p. 1-40.
389. Krysiak-Baltyn K, Martin GJO, Stickland AD, Scales PJ, Gras SL. Computational models of populations of bacteria and lytic phage. Critical Reviews in Microbiology. 2016;42(6):942-68.
390. Suttle C. Cyanophages and their role in the ecology of cyanobacteria. In: Whitton B, Potts M, editors. The Ecology of Cyanobacteria. The Netherlands: Kluwer Academic Publishers; 2000. p. 563-89.
391. Uetake H, Luria SE, Burrous JW. Conversion of somatic antigens in Salmonella by phage infection leading to lysis or lysogeny. Virology. 1958;5(1):68-91.
392. Riede I, Eschbach M-L. Evidence that TraT interacts with OmpA of Escherichia coli. FEBS Letters. 1986;205(2):241-5.
393. Destoumieux-Garzón D, Duquesne S, Peduzzi J, Goulard C, Desmadril M, Letellier L, et al. The iron–siderophore transporter FhuA is the receptor for the antimicrobial peptide microcin J25: role of the microcin Val(11)–Pro(16) β-hairpin region in the recognition mechanism. Biochemical Journal. 2005;389(Pt 3):869-76.

394. Cornett JB. Spackle and Immunity Functions of Bacteriophage T4. Journal of virology. 1974;13(2):312-21.
395. Lu MJ, Henning U. The immunity (imm) gene of Escherichia coli bacteriophage T4. Journal of virology. 1989;63(8):3472-8.
396. Mahony J, McGrath S, Fitzgerald GF, van Sinderen D. Identification and Characterization of Lactococcal-Prophage-Carried Superinfection Exclusion Genes. Applied and environmental microbiology. 2008;74(20):6206-15.
397. Meselson M, Yuan R. DNA Restriction Enzyme from E. coli. Nature. 1968;217(5134):1110-4.
398. Linn S, Arber W. Host specificity of DNA produced by Escherichia coli, X. In vitro restriction of phage fd replicative form. Proceedings of the National Academy of Sciences of the United States of America. 1968;59(4):1300-6.
399. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, et al. CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. Science. 2007;315(5819):1709-12.
400. Dy RL, Richter C, Salmond GPC, Fineran PC. Remarkable Mechanisms in Microbes to Resist Phage Infections. Annual Review of Virology. 2014;1(1):307-31.
401. Parma DH, Snyder M, Sobolevski S, Nawroz M, Brody E, Gold L. The Rex system of bacteriophage lambda: tolerance and altruistic cell death. Genes & development. 1992;6(3):497-510.
402. Schmitt CK, Kemp P, Molineux IJ. Genes 1.2 and 10 of bacteriophages T3 and T7 determine the permeability lesions observed in infected cells of Escherichia coli expressing the F plasmid gene pifA. Journal of Bacteriology. 1991;173(20):6507-14.
403. Yu YT, Snyder L. Translation elongation factor Tu cleaved by a phage-exclusion system. Proc Natl Acad Sci U S A. 1994;91(2):802-6.
404. Levitz R, Chapman D, Amitsur M, Green R, Snyder L, Kaufmann G. The optional E. coli prr locus encodes a latent form of phage T4-induced anticodon nuclease. EMBO J. 1990;9(5):1383-9.
405. Bordet J, Ciuca M. Determinisme de l'autolyse microbienne transmissible. Compt Rend Soc Biol. 1921;84:276.
406. Kölsch E, Diller E, Weber G, Davies AJS. Genetics of the immune response. I. The immune response to the phage fd in high and low responding inbred strains of mice. European Journal of Immunology. 1971;1(3):201-10.
407. Fodor AR, Adams MH. Genetic Control of Serological Specificity in Bacteriophage. The Journal of Immunology. 1955;74(3):228-35.
408. Andrewes CH, Elford WJ. Observations on Anti-Phage Sera. I: "The Percentage Law". British journal of experimental pathology. 1933;14(6):367-76.
409. Burnet FM, Keogh EV, Lush D. Immunological reactions of filterable viruses. Aust J Exp Biol Med. 1937;15(3):227-368.
410. Lanni F, Lanni YT. Antigenic structure of bacteriophage. Cold Spring Harbor Symposia on Quantitative Biology. 1953;18:159-68.
411. Franklin NC. Serological study of tail structure and function in coliphages T2 and T4. Virology. 1961;14(4):417-29.
412. De Mars RI, Luria SE, Fisher H, Levinthal C. The production of incomplete bacteriophage particles by the action of proflavine and the properties of the incomplete particles. Ann Inst Pasteur. 1953;84:113.
413. Rountree PM. Serological studies of the multiplication of a staphylococcal bacteriophage. Aust J Exp Biol Med. 1952;30(6):567-86.

414. Stashak PW, Baker PJ, Roberson BS. The serum antibody response to bacteriophage ΦX174 in germ-free and conventionally reared mice: II. Kinetics of the serum antibody response following primary immunization. Immunology. 1970;18(2):307-17.
415. Łusiak-Szelachowska M, Zaczek M, Weber-Dabrowska B, Międzybrodzki R, Kłak M, Fortuna W, et al. Phage neutralization by sera of patients receiving phage therapy. Viral Immunology. 2014;27(6):295-304.
416. Holguin AV, Rangel G, Clavijo V, Prada C, Mantilla M, Gomez MC, et al. Phage PhiPan70, a Putative Temperate Phage, Controls Pseudomonas aeruginosa in Planktonic, Biofilm and Burn Mouse Model Assays. Viruses. 2015;7(8):4602-23.
417. Kwiatek M, Mizak L, Parasion S, Gryko R, Olender A, Niemcewicz M. Characterization of five newly isolated bacteriophages active against Pseudomonas aeruginosa clinical strains. Folia microbiologica. 2015;60(1):7-14.
418. Ahiwale S, Tamboli N, Thorat K, Kulkarni R, Ackermann H, Kapadnis B. In vitro management of hospital Pseudomonas aeruginosa biofilm using indigenous T7-like lytic phage. Current Microbiology. 2011;62(2):335-40.
419. Olszak T, Zarnowiec P, Kaca W, Danis-Wlodarczyk K, Augustyniak D, Drevinek P, et al. In vitro and in vivo antibacterial activity of environmental bacteriophages against Pseudomonas aeruginosa strains from cystic fibrosis patients. Applied microbiology and biotechnology. 2015;99(14):6021-33.
420. Saussereau E, Vachier I, Chiron R, Godbert B, Sermet I, Dufour N, et al. Effectiveness of bacteriophages in the sputum of cystic fibrosis patients. Clinical Microbiology and Infection. 2014;20(12):O983-O90.
421. Morales S, Mearns G, Cole R, Smithyman A. Bacteriophage treatment inhibits and reduces biofilm formation by Pseudomonas aeruginosa strains from cystic fibrosis patients. Clinical Microbiology and Infection. 2012;18:387-8.
422. Fu W, Forster T, Mayer O, Curtin JJ, Lehman SM, Donlan RM. Bacteriophage cocktail for the prevention of biofilm formation by Pseudomonas aeruginosa on catheters in an in vitro model system. Antimicrobial agents and chemotherapy. 2010;54(1):397-404.
423. Barr JJ, Auro R, Sam-Soon N, Kassegne S, Peters G, Bonilla N, et al. Subdiffusive motion of bacteriophage in mucosal surfaces increases the frequency of bacterial encounters. Proc Natl Acad Sci U S A. 2015;112(44):13675-80.
424. Hanlon GW, Denyer SP, Olliff CJ, Ibrahim LJ. Reduction in exopolysaccharide viscosity as an aid to bacteriophage penetration through Pseudomonas aeruginosa biofilms. Applied and environmental microbiology. 2001;67(6):2746-53.
425. Vilas Boas D, Almeida C, Sillankorva S, Nicolau A, Azeredo J, Azevedo NF. Discrimination of bacteriophage infected cells using locked nucleic acid fluorescent in situ hybridization (LNA-FISH). Biofouling. 2016;32(2):179-90.
426. Knezevic P, Obreht D, Curcin S, Petrusic M, Aleksic V, Kostanjsek R, et al. Phages of Pseudomonas aeruginosa: Response to environmental factors and in vitro ability to inhibit bacterial growth and biofilm formation. Journal of Applied Microbiology. 2011;111(1):245-54.
427. Krylov V, Shaburova O, Pleteneva E, Krylov S, Kaplan A, Burkaltseva M, et al. Selection of phages and conditions for the safe phage therapy against Pseudomonas aeruginosa infections. Virologica Sinica. 2015;30(1):33-44.
428. Pleteneva EA, Shaburova OV, Sykilinda NN, Miroshnikov KA, Krylov SV, Mesianzhinov VV, et al. [Study of the diversity in a group of phages of Pseudomonas aeruginosa species PB1 (Myoviridae) and their behavior in adsorbtion-resistant bacterial mutants]. Genetika. 2008;44(2):185-94.

429. Ceyssens P-J, Miroshnikov K, Mattheus W, Krylov V, Robben J, Noben J-P, et al. Comparative analysis of the widespread and conserved PB1-like viruses infecting Pseudomonas aeruginosa. Environmental Microbiology. 2009;11(11):2874-83.
430. Ceyssens PJ, Glonti T, Kropinski NM, Lavigne R, Chanishvili N, Kulakov L, et al. Phenotypic and genotypic variations within a single bacteriophage species. Virology journal. 2011;8:134.
431. Tan Y, Zhang K, Rao X, Jin X, Huang J, Zhu J, et al. Whole genome sequencing of a novel temperate bacteriophage of P. aeruginosa: evidence of tRNA gene mediating integration of the phage genome into the host bacterial chromosome. Cell Microbiol. 2007;9(2):479-91.
432. Fukuda K, Ishida W, Uchiyama J, Rashel M, Kato S, Morita T, et al. Pseudomonas aeruginosa keratitis in mice: effects of topical bacteriophage KPP12 administration. PLoS One. 2012;7(10):e47742.
433. Hawkins C, Harper D, Burch D, Anggard E, Soothill J. Topical treatment of Pseudomonas aeruginosa otitis of dogs with a bacteriophage mixture: a before/after clinical trial. Veterinary microbiology. 2010;146(3-4):309-13.
434. Khairnar K, Raut MP, Chandekar RH, Sanmukh SG, Paunikar WN. Novel bacteriophage therapy for controlling metallo-beta-lactamase producing Pseudomonas aeruginosa infection in catfish. BMC veterinary research. 2013;9:264.
435. Marza JA, Soothill JS, Boydell P, Collyns TA. Multiplication of therapeutically administered bacteriophages in Pseudomonas aeruginosa infected patients. Burns. 2006;32(5):644-6.
436. Watanabe R, Matsumoto T, Sano G, Ishii Y, Tateda K, Sumiyama Y, et al. Efficacy of bacteriophage therapy against gut-derived sepsis caused by Pseudomonas aeruginosa in mice. Antimicrobial agents and chemotherapy. 2007;51(2):446-52.
437. Alemayehu D, Casey PG, McAuliffe O, Guinane CM, Martin JG, Shanahan F, et al. Bacteriophages phiMR299-2 and phiNH-4 can eliminate Pseudomonas aeruginosa in the murine lung and on cystic fibrosis lung airway cells. MBio. 2012;3(2):e00029-12.
438. Debarbieux L, Leduc D, Maura D, Morello E, Criscuolo A, Grossi O, et al. Bacteriophages can treat and prevent Pseudomonas aeruginosa lung infections. J Infect Dis. 2010;201(7):1096-104.
439. Morello E, Saussereau E, Maura D, Huerre M, Touqui L, Debarbieux L. Pulmonary bacteriophage therapy on Pseudomonas aeruginosa cystic fibrosis strains: first steps towards treatment and prevention. PLoS One. 2011;6(2):e16963.
440. Bertoye A, Courtieu AL. [Treatment of infections caused by pyocyanic bacilli with bacteriophages adapted by selection]. Journal de medecine de Lyon. 1960;41:739-51.
441. Bertoye A, Gaillard L, Courtieu AL. [Adapted bacteriophages in the treatment of infections caused by antibiotic-resistant microorganisms]. Journal de medecine de Lyon. 1959;40(945):465-71.
442. Soothill J. Use of bacteriophages in the treatment of Pseudomonas aeruginosa infections. Expert Review of Anti-infective Therapy. 2013;11(9):909-15.
443. Wright A, Hawkins CH, Anggard EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. Clinical otolaryngology : official journal of ENT-UK ; official journal of Netherlands Society for Oto-Rhino-Laryngology & Cervico-Facial Surgery. 2009;34(4):349-57.
444. Ujmajuridze A, Jvania G, Chanishvili N, Goderdzishvili M, Sybesma W, Managadze L, et al. Phage therapy for the treatment for urinary tract infection:

Results of in-vitro screenings and in-vivo application using commercially available bacteriophage cocktails. European Urology, Supplements. 2016;15(3):e265.
445. Rose T, Verbeken G, de Vos D, Merabishvili M, Vaneechoutte M, Lavigne R, et al. Experimental phage therapy of burn wound infection: Difficult first steps. International Journal of Burns and Trauma. 2014;4(2):66-73.
446. MT DDR, MD RDW, PhD MAK, Wolcott BM, PhD LSW, PhD AS. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. Journal of Wound Care. 2009;18(6):237-43.
447. Kutateladze M. Experience of the Eliava Institute in bacteriophage therapy. Virologica Sinica. [journal article]. 2015;30(1):80-1.
448. Sulakvelidze A, Alavidze Z, Morris JG. Bacteriophage Therapy. Antimicrobial agents and chemotherapy. 2001;45(3):649-59.
449. Anpilov L, Prokudin A. Preventive effectiveness of dried polyvalent Shigella bacteriophage in organized collective farms. Voenno-meditsinskii zhurnal. 1984(5):39-40.
450. Babalova EG, Katsitadze KT, Sakvarelidze LA, Imnaishvili N, Sharashidze TG, Badashvili VA, et al. [Preventive value of dried dysentery bacteriophage]. Zhurnal mikrobiologii, epidemiologii, i immunobiologii. 1968;45(2):143-5.
451. Aslam S, Courtwright AM, Koval C, Lehman SM, Morales S, Furr CL, et al. Early clinical experience of bacteriophage therapy in 3 lung transplant recipients. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2019;19(9):2631-9.
452. Duplessis C, Biswas B, Hanisch B, Perkins M, Henry M, Quinones J, et al. Refractory Pseudomonas Bacteremia in a 2-Year-Old Sterilized by Bacteriophage Therapy. Journal of the Pediatric Infectious Diseases Society. 2017;7(3):253-6.
453. Ferry T, Boucher F, Fevre C, Perpoint T, Chateau J, Petitjean C, et al. Innovations for the treatment of a complex bone and joint infection due to XDR Pseudomonas aeruginosa including local application of a selected cocktail of bacteriophages. J Antimicrob Chemother. 2018;73(10):2901-3.
454. Jennes S, Merabishvili M, Soentjens P, Pang KW, Rose T, Keersebilck E, et al. Use of bacteriophages in the treatment of colistin-only-sensitive Pseudomonas aeruginosa septicaemia in a patient with acute kidney injury—a case report. Critical Care. 2017;21(1):129.
455. Law N, Logan C, Yung G, Furr CL, Lehman SM, Morales S, et al. Successful adjunctive use of bacteriophage therapy for treatment of multidrug-resistant Pseudomonas aeruginosa infection in a cystic fibrosis patient. Infection. 2019;47(4):665-8.
456. Chan BK, Turner PE, Kim S, Mojibian HR, Elefteriades JA, Narayan D. Phage treatment of an aortic graft infected with Pseudomonas aeruginosa. Evolution, Medicine, and Public Health. 2018;2018(1):60-6.
457. Shivshetty N, Hosamani R, Ahmed L, Oli AK, Sannauallah S, Sharanbassappa S, et al. Experimental protection of diabetic mice against lethal P. aeruginosa infection by bacteriophage. BioMed Research International. 2014;2014.
458. Pabary R, Singh C, Morales S, Bush A, Alshafi K, Bilton D, et al. Antipseudomonal Bacteriophage Reduces Infective Burden and Inflammatory Response in Murine Lung. Antimicrobial agents and chemotherapy. 2016;60(2):744-51.
459. Drilling AJ, Ooi ML, Miljkovic D, James C, Speck P, Vreugde S, et al. Long-Term Safety of Topical Bacteriophage Application to the Frontal Sinus Region. Frontiers in Cellular and Infection Microbiology. 2017;7:49.

460. Park K, Cha KE, Myung H. Observation of inflammatory responses in mice orally fed with bacteriophage T7. Journal of Applied Microbiology. 2014;117(3):627-33.
461. Drilling A, Morales S, Boase S, Jervis-Bardy J, James C, Jardeleza C, et al. Safety and efficacy of topical bacteriophage and ethylenediaminetetraacetic acid treatment of Staphylococcus aureus infection in a sheep model of sinusitis. Int Forum Allergy Rhinol. 2014;4(3):176-86.
462. McCallin S, Alam Sarker S, Barretto C, Sultana S, Berger B, Huq S, et al. Safety analysis of a Russian phage cocktail: From MetaGenomic analysis to oral application in healthy human subjects. Virology. 2013;443(2):187-96.
463. Bruttin A, Brüssow H. Human volunteers receiving Escherichia coli phage T4 orally: A safety test of phage therapy. Antimicrobial agents and chemotherapy. 2005;49(7):2874-8.
464. Międzybrodzki R, Borysowski J, Weber-Dąbrowska B, Fortuna W, Letkiewicz S, Szufnarowski K, et al. Chapter 3 - Clinical Aspects of Phage Therapy. In: Małgorzata Ł, Wacław S, editors. Advances in Virus Research: Academic Press; 2012. p. 73-121.
465. Ooi ML, Drilling AJ, Morales S, Fong S, Moraitis S, Macias-Valle L, et al. Safety and Tolerability of Bacteriophage Therapy for Chronic Rhinosinusitis Due to Staphylococcus aureus. JAMA Otolaryngology–Head & Neck Surgery. 2019;145(8):723-9.
466. de la Fuente-Núñez C, Reffuveille F, Fernández L, Hancock REW. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. Current Opinion in Microbiology. 2013;16(5):580-9.
467. Hanlon GW. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. International Journal of Antimicrobial Agents. 2007;30(2):118-28.
468. Curran B, Jonas D, Crundmann H, Pitt T, Dowson C. Development of a multilocus sequence typing scheme for the opportunistic pathogen Pseudomonas aeruginosa. J Clin Microbiol. 2004;42.
469. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular biology and evolution. 2013;30(12):2725-9.
470. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research. 2004;32(5):1792-7.
471. Felsenstein J. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. Evolution. 1985;39(4):783-91.
472. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(30):11030-5.
473. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data. Journal of Bacteriology. 2004;186(5):1518-30.
474. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protocols. 2008;3(2):163-75.
475. Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Enumeration of Bacteriophages Using the Small Drop Plaque Assay System. In: Clokie MRJ, Kropinski AM, editors. Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions. Totowa, NJ: Humana Press; 2009. p. 81-5.
476. O'Toole GA. Microtiter dish biofilm formation assay. J Visual Exp. 2011;47.

477. Drilling A, Morales S, Jardeleza C, Vreugde S, Speck P, Wormald P-J. Bacteriophage reduces biofilm of Staphylococcus aureus ex vivo isolates from chronic rhinosinusitis patients. Am J Rhinol Allergy. 2014;28(1):3-11.
478. Chan BK, Abedon ST. Chapter 1 - Phage Therapy Pharmacology: Phage Cocktails. In: Allen I. Laskin SS, Geoffrey MG, editors. Advances in Applied Microbiology: Academic Press; 2012. p. 1-23.
479. Hall AR, Vos D, Friman VP, Pirnay JP, Buckling A. Effects of sequential and simultaneous application of bacteriophages on populations of Pseudomonas aeruginosa in vitro and in waxmoth larvae. Appl Environ Microbiol. 2012;78(null):5646.
480. Worley-Morse TO, Zhang L, Gunsch CK. The long-term effects of phage concentration on the inhibition of planktonic bacterial cultures. Environmental Sciences: Processes and Impacts. 2014;16(1):81-7.
481. Kiewitz C, Tümmler B. Sequence Diversity of Pseudomonas aeruginosa: Impact on Population Structure and Genome Evolution. Journal of Bacteriology. 2000;182(11):3125-35.
482. Cramer N, Wiehlmann L, Ciofu O, Tamm S, Høiby N, Tümmler B. Molecular Epidemiology of Chronic Pseudomonas aeruginosa Airway Infections in Cystic Fibrosis. PLOS ONE. 2012;7(11):e50731.
483. Sasikala S, Sundararaj T. Characterization of Pseudomonas strains from sinusitis patients. BMC infectious diseases. [journal article]. 2012;12(1):P64.
484. Henwood CJ, Livermore DM, James D, Warner M, Pseudomonas Study Group t. Antimicrobial susceptibility of Pseudomonas aeruginosa: results of a UK survey and evaluation of the British Society for Antimicrobial Chemotherapy disc susceptibility test. J Antimicrob Chemother. 2001;47(6):789-99.
485. Doring G, Conway SP, Heijerman HG, Hodson ME, Hoiby N, Smyth A, et al. Antibiotic therapy against Pseudomonas aeruginosa in cystic fibrosis: a European consensus. European Respiratory Journal. 2000;16(4):749-67.
486. Ciofu O, Johansen HK, Aanaes K, Wassermann T, Alhede M, von Buchwald C, et al. P. aeruginosa in the paranasal sinuses and transplanted lungs have similar adaptive mutations as isolates from chronically infected CF lungs. J Cyst Fibros. 2013;12(6):729-36.
487. Nichols WW, Dorrington SM, Slack MP, Walmsley HL. Inhibition of tobramycin diffusion by binding to alginate. Antimicrobial agents and chemotherapy. 1988;32(4):518-23.
488. Govan JR, Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. Microbiological Reviews. 1996;60(3):539-74.
489. Haussler S, Tummler B, Weissbrodt H, Rohde M, Steinmetz I. Small-colony variants of Pseudomonas aeruginosa in cystic fibrosis. Clin Infect Dis. 1999;29(3):621-5.
490. Grobler A, Weitzel EK, Buele A, Jardeleza C, Cheong YC, Field J, et al. Pre- and Postoperative Sinus Penetration of Nasal Irrigation. The Laryngoscope. 2008;118(11):2078-81.
491. Beeton ML, Alves DR, Enright MC, Jenkins ATA. Assessing phage therapy against Pseudomonas aeruginosa using a Galleria mellonella infection model. International Journal of Antimicrobial Agents. 2015;46(2):196-200.
492. Heo YJ, Lee YR, Jung HH, Lee J, Ko G, Cho YH. Antibacterial efficacy of phages against Pseudomonas aeruginosa infections in mice and Drosophila melanogaster. Antimicrob Agents Chemother. 2009;53(6):2469.

493. Bhattacharyya N. The economic burden and symptom manifestations of chronic rhinosinusitis. Am J Rhinol. 2003;17(1):27-32.
494. Gliklich RE, Metson R. Economic implications of chronic sinusitis. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery. 1998;118(3 Pt 1):344-9.
495. Hastan D, Fokkens WJ, Bachert C, Newson RB, Bislimovska J, Bockelbrink A, et al. Chronic rhinosinusitis in Europe--an underestimated disease. A GA(2)LEN study. Allergy. 2011;66(9):1216-23.
496. Hirsch AG, Stewart WF, Sundaresan AS, Young AJ, Kennedy TL, Scott Greene J, et al. Nasal and sinus symptoms and chronic rhinosinusitis in a population-based sample. Allergy. 2017;72(2):274-81.
497. Shi JB, Fu QL, Zhang H, Cheng L, Wang YJ, Zhu DD, et al. Epidemiology of chronic rhinosinusitis: results from a cross-sectional survey in seven Chinese cities. Allergy. 2015;70(5):533-9.
498. Smith SS, Evans CT, Tan BK, Chandra RK, Smith SB, Kern RC. National burden of antibiotic use for adult rhinosinusitis. Journal of Allergy and Clinical Immunology. 2013;132(5):1230-2.
499. Sengupta S, Chattopadhyay MK, Grossart H-P. The multifaceted roles of antibiotics and antibiotic resistance in nature. Frontiers in Microbiology. 2013;4:47.
500. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010;8.
501. Prince AA, Steiger JD, Khalid AN, Dogrhamji L, Reger C, Steven Eau C, et al. Prevalence of biofilm-forming bacteria in chronic rhinosinusitis. Am J Rhinol. 2008;22(3):239-45.
502. Pires DP, Vilas Boas D, Sillankorva S, Azeredo J. Phage Therapy: a Step Forward in the Treatment of Pseudomonas aeruginosa Infections. Journal of virology. 2015;89(15):7449-56.
503. Fong SA, Drilling A, Morales S, Cornet ME, Woodworth BA, Fokkens WJ, et al. Activity of Bacteriophages in Removing Biofilms of Pseudomonas aeruginosa Isolates from Chronic Rhinosinusitis Patients. Frontiers in Cellular and Infection Microbiology. 2017;7(418).
504. Ha KR, Psaltis AJ, Tan L, Wormald P-J. A sheep model for the study of biofilms in rhinosinusitis. Am J Rhinol. 2007;21(3):339-45.
505. Singhal D, Jekle A, Debabov D, Wang L, Khosrovi B, Anderson M, et al. Efficacy of NVC-422 against Staphylococcus aureus biofilms in a sheep biofilm model of sinusitis. Int Forum Allergy Rhinol. 2012;2(4):309-15.
506. Singhal D, Boase S, Field J, Jardeleza C, Foreman A, Wormald P-J. Quantitative analysis of in vivo mucosal bacterial biofilms. Int Forum Allergy Rhinol. 2012;2(1):57-62.
507. Comstat 2. [cited 2016 25th August]. Available from: www.comstat.dk
508. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersbøll BK, et al. Quantification of biofilm structures by the novel computer program comstat. Microbiology. 2000;146(10):2395-407.
509. Vorregaard M. Comstat2 - a modern 3D image analysis environment for biofilms. Informatics and Mathematical Modelling. Kongens Lyngby, Denmark: Technical University of Denmark; 2008.
510. Foreman A, Psaltis AJ, Tan LW, Wormald PJ. Characterization of bacterial and fungal biofilms in chronic rhinosinusitis. Am J Rhinol Allergy. 2009;23(6):556-61.
511. Jardeleza C, Thierry B, Rao S, Rajiv S, Drilling A, Miljkovic D, et al. An in vivo safety and efficacy demonstration of a topical liposomal nitric oxide donor

treatment for Staphylococcus aureus biofilm–associated rhinosinusitis. Translational Research. 2015;166(6):683-92.
512. Woodworth BA, Tamashiro E, Bhargave G, Cohen NA, Palmer JN. An in vitro model of Pseudomonas aeruginosa biofilms on viable airway epithelial cell monolayers. Am J Rhinol. 2008;22(3):235-8.
513. Perloff JR, Palmer JN. Evidence of bacterial biofilms in a rabbit model of sinusitis. Am J Rhinol. 2005;19(1):1-6.
514. Alves DR, Perez-Esteban P, Kot W, Bean JE, Arnot T, Hansen LH, et al. A novel bacteriophage cocktail reduces and disperses Pseudomonas aeruginosa biofilms under static and flow conditions. Microbial biotechnology. 2015.
515. Danis-Wlodarczyk K, Olszak T, Arabski M, Wasik S, Majkowska-Skrobek G, Augustyniak D, et al. Characterization of the newly isolated lytic bacteriophages KTN6 and KT28 and their efficacy against pseudomonas aeruginosa biofilm. PLoS ONE. 2015;10(5).
516. Fu W, Forster T, Mayer O, Curtin JJ, Lehman SM, Donlan RM. Bacteriophage cocktail for the prevention of biofilm formation by Pseudomonas aeruginosa on catheters in an in vitro model system. Antimicrobial agents and chemotherapy. 2010;54(1):397-404.
517. Kim S, Rahman M, Seol SY, Yoon SS, Kim J. Pseudomonas aeruginosa bacteriophage PA1Ø requires type IV pili for infection and shows broad bactericidal and biofilm removal activities. Applied and environmental microbiology. 2012;78(17):6380-5.
518. Li LY, Yang HJ, Yue H. Isolation and classification of the bacteriophages of Pseudomonas aeruginosa and their application on biofilm control. Chinese Journal of Microbiology and Immunology (China). 2011;31(4):330-4.
519. Pires D, Sillankorva S, Faustino A, Azeredo J. Use of newly isolated phages for control of Pseudomonas aeruginosa PAO1 and ATCC 10145 biofilms. Research in Microbiology. 2011;162(8):798-806.
520. Pabary R, Singh C, Morales S, Bush A, Alshafi K, Bilton D, et al. S103 Anti-Pseudomonal Bacteriophage Cocktail Reduces Inflammatory Responses in the Murine Lung. Thorax. 2012;67(Suppl 2):A50-A1.
521. Chhibber S, Bansal S, Kaur S. Disrupting the mixed-species biofilm of klebsiella pneumoniae B5055 and pseudomonas aeruginosa PAO using bacteriophages alone or in combination with xylitol. Microbiology (United Kingdom). 2015;161(7):1369-77.
522. Kay MK, Erwin TC, McLean RJC, Aron GM. Bacteriophage ecology in Escherichia coli and pseudomonas aeruginosa mixed-biofilm communities. Applied and environmental microbiology. 2011;77(3):821-9.
523. Bhattacharyya N, Vyas DK, Fechner FP, Gliklich RE, Metson R. Tissue eosinophilia in chronic sinusitis: quantification techniques. Arch Otolaryngol Head Neck Surg. 2001;127(9):1102-5.
524. Soler ZM, Sauer DA, Mace J, Smith TL. Relationship Between Clinical Measures and Histopathologic Findings in Chronic Rhinosinusitis. Otolaryngology-Head and Neck Surgery. 2009;141(4):454-61.
525. Harvey RJ, Lund VJ. Biofilms and chronic rhinosinusitis: systematic review of evidence, current concepts and directions for research. Rhinology. [Review]. 2007;45(1):3-13.
526. Songer JG, Post KW. Veterinary Microbiology: Bacterial and Fungal Aspects of Animal Disease. St Louis: Saunders Elsevier; 2005.

527. Bogovazova GG, Voroshilova NN, Bondarenko VM. [The efficacy of Klebsiella pneumoniae bacteriophage in the therapy of experimental Klebsiella infection]. Zhurnal mikrobiologii, epidemiologii, i immunobiologii. 1991(4):5-8.
528. Nungester WJ, Watrous RM. Accumulation of Bacteriophage in Spleen and Liver Following Its Intravenous Inoculation. Proceedings of the Society for Experimental Biology and Medicine. 1934;31(8):901-5.
529. Toskala E, Rautiainen M. Electron Microscopy Assessment of the Recovery of Sinus Mucosa after Sinus Surgery. Acta Oto-Laryngologica. 2003;123(8):954-9.
530. Høiby N. Prospects for the Prevention and Control of Pseudomonal Infection in Children with Cystic Fibrosis. Paediatric Drugs. 2000;2(6):451-63.
531. Henry RL, Mellis CM, Petrovic L. Mucoid Pseudomonas aeruginosa is a marker of poor survival in cystic fibrosis. Pediatric Pulmonology. 1992;12(3):158-61.
532. Fong SA, Drilling AJ, Ooi ML, Paramasivan S, Finnie JW, Morales S, et al. Safety and efficacy of a bacteriophage cocktail in an in vivo model of Pseudomonas aeruginosa sinusitis. Translational research : the journal of laboratory and clinical medicine. 2019;206:41-56.
533. Gurney J, Kaltz O, Hochberg M, Brown S. Efficacy and evolutionary robustness of a phage-antibiotic combination therapy. Pediatric Pulmonology. 2019;54:S307.
534. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. Scientific Reports. [Article]. 2016;6:26717.
535. Cryer J, Schipor I, Perloff JR, Palmer JN. Evidence of bacterial biofilms in human chronic sinusitis. ORL J Otorhinolaryngol Relat Spec. 2004;66(3):155-8.
536. Buckling A, Rainey PB. Antagonistic coevolution between a bacterium and a bacteriophage. Proceedings of the Royal Society of London Series B: Biological Sciences. 2002;269(1494):931-6.
537. FastQC. Babraham Bioinformatics; 2010. Available from: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
538. Martin M. Cutadapt. 2018. Available from: https://cutadapt.readthedocs.io/en/stable/
539. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics. 2009;25(14):1754-60.
540. Picard Tools. Broad Institute; 2016. Available from: http://broadinstitute.github.io/picard/
541. Quinlan AR, Kindlon N. Bedtools. 2016. Available from: https://bedtools.readthedocs.io/en/latest/index.html
542. Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. 2012; arXiv: 1207.3907v2. Available from: http://arxiv.org/abs/1207.3907
543. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w(1118); iso-2; iso-3. Fly. 2012;6(2):80-92.
544. Rausch T, Zichner T, Schlattl A, Stütz AM, Benes V, Korbel JO. DELLY: structural variant discovery by integrated paired-end and split-read analysis. Bioinformatics (Oxford, England). 2012;28(18):i333-i9.
545. VcfFilter. Leids Universitair Medisch Centrum; 2017. Available from: https://github.com/biopet/biopet
546. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014;30(14):2068-9.

547. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, et al. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics. 2015;31(22):3691-3.
548. Brynildsrud O, Bohlin J, Scheffer L, Eldholm V. Rapid scoring of genes in microbial pan-genome-wide association studies with Scoary. Genome Biology. 2016;17(1):238.
549. Lutz L, Leão RS, Ferreira AG, Pereira DC, Raupp C, Pitt T, et al. Hypermutable Pseudomonas aeruginosa in Cystic fibrosis patients from two Brazilian cities. Journal of Clinical Microbiology. 2013;51(3):927-30.
550. Wang C, Nie T, Lin F, Connerton IF, Lu Z, Zhou S, et al. Resistance mechanisms adopted by a Salmonella Typhimurium mutant against bacteriophage. Virus research. 2019;273:197759.
551. Brockhurst MA, Morgan AD, Rainey PB, Buckling A. Population mixing accelerates coevolution. Ecology Letters. 2003;6(11):975-9.
552. Harrison E, Wood AJ, Dytham C, Pitchford JW, Truman J, Spiers A, et al. Bacteriophages Limit the Existence Conditions for Conjugative Plasmids. mBio. 2015;6(3):e00586-15.
553. Betts A, Kaltz O, Hochberg ME. Contrasted coevolutionary dynamics between a bacterial pathogen and its bacteriophages. Proceedings of the National Academy of Sciences. 2014;111(30):11109-14.
554. Pal C, Maciá MD, Oliver A, Schachar I, Buckling A. Coevolution with viruses drives the evolution of bacterial mutation rates. Nature. 2007;450(7172):1079-81.
555. Morona R, Klose M, Henning U. Escherichia coli K-12 outer membrane protein (OmpA) as a bacteriophage receptor: analysis of mutant genes expressing altered proteins. J Bacteriol. 1984;159(2):570-8.
556. Lim WS, Phang KKS, Tan AH-M, Li SF-Y, Ow DS-W. Small Colony Variants and Single Nucleotide Variations in Pf1 Region of PB1 Phage-Resistant Pseudomonas aeruginosa. Frontiers in Microbiology. 2016;7(282).
557. Guttman BS. Lysogeny. In: Brenner S, Miller JH, editors. Encyclopedia of Genetics. New York: Academic Press; 2001. p. 1130-1.
558. Bondy-Denomy J, Qian J, Westra ER, Buckling A, Guttman DS, Davidson AR, et al. Prophages mediate defense against phage infection through diverse mechanisms. The ISME Journal. 2016;10(12):2854-66.
559. Tsao Y-F, Taylor VL, Kala S, Bondy-Denomy J, Khan AN, Bona D, et al. Phage Morons Play an Important Role in Pseudomonas aeruginosa Phenotypes. Journal of Bacteriology. 2018;200(22):e00189-18.
560. Hill DF, Short NJ, Perham RN, Petersen GB. DNA sequence of the filamentous bacteriophage Pf1. Journal of molecular biology. 1991;218(2):349-64.
561. Rakonjac J, Bennett NJ, Spagnuolo J, Gagic D, Russel M. Filamentous bacteriophage: biology, phage display and nanotechnology applications. Current issues in molecular biology. 2011;13(2):51-76.
562. Ilyina TS. Filamentous bacteriophages and their role in the virulence and evolution of pathogenic bacteria. Molecular Genetics, Microbiology and Virology. [journal article]. 2015;30(1):1-9.
563. Hagens S, Habel A, Bläsi U. Augmentation of the Antimicrobial Efficacy of Antibiotics by Filamentous Phage. Microbial Drug Resistance. 2006;12(3):164-8.
564. Platt MD, Schurr MJ, Sauer K, Vazquez G, Kukavica-Ibrulj I, Potvin E, et al. Proteomic, Microarray, and Signature-Tagged Mutagenesis Analyses of Anaerobic Pseudomonas aeruginosa at pH 6.5, Likely Representing Chronic, Late-Stage Cystic Fibrosis Airway Conditions. Journal of Bacteriology. 2008;190(8):2739-58.

565. Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, et al. Gene expression in Pseudomonas aeruginosa biofilms. Nature. 2001;413(6858):860-4.
566. Fasano A, Baudry B, Pumplin DW, Wasserman SS, Tall BD, Ketley JM, et al. Vibrio cholerae produces a second enterotoxin, which affects intestinal tight junctions. Proc Natl Acad Sci U S A. 1991;88(12):5242-6.
567. Rice SA, Tan CH, Mikkelsen PJ, Kung V, Woo J, Tay M, et al. The biofilm life cycle and virulence of Pseudomonas aeruginosa are dependent on a filamentous prophage. ISME J. 2009;3(3):271-82.
568. Waldor MK, Mekalanos JJ. Lysogenic Conversion by a Filamentous Phage Encoding Cholera Toxin. Science. 1996;272(5270):1910-4.
569. Koonin EV. The second cholera toxin, Zot, and its plasmid-encoded and phage-encoded homologues constitute a group of putative ATPases with an altered purine NTP-binding motif. FEBS Letters. 1992;312(1):3-6.
570. Russel M. Filamentous phage assembly. Molecular Microbiology. 1991;5(7):1607-13.
571. Morrisette T, Kebriaei R, Lev KL, Morales S, Rybak MJ. Bacteriophage Therapeutics: A Primer for Clinicians on Phage-Antibiotic Combinations. Pharmacotherapy. [Article in Press]. 2019.

# Appendix

## Chapter 2 – Supplementary Table 1

**Supplementary Table 1: MLST profiles of *P. aeruginosa* strains used in this study**

| Isolate | Country of origin | ST | acsA | aroE | guaA | mutL | nuoD | ppsA | trpE |
|---|---|---|---|---|---|---|---|---|---|
| PA01 | Lab reference | 549 [a f] | 7 | 5 | 12 | 3 | 4 | 1 | 7 |
| P1 | Australia | 348 [b] | 22 | 20 | 11 | 3 | 3 | 3 | 7 |
| P2 | Australia | 446 | 18 | 4 | 5 | 13 | 1 | 17 | 13 |
| P3 | Australia | 910 | 34 | 5 | 20 | 31 | 1 | 15 | 10 |
| P4 | Australia | 155 | 28 | 5 | 36 | 3 | 3 | 13 | 7 |
| P5 | Australia | 242 | 28 | 5 | 5 | 11 | 3 | 15 | 44 |
| P6 | Australia | 1517 | 11 | 5 | 30 | 3 | 3 | 38 | 7 |
| P7 | Australia | 274 [c] | 23 | 5 | 11 | 7 | 1 | 12 | 7 |
| P8 | Australia | 17 [g] | 11 | 5 | 1 | 7 | 9 | 4 | 7 |
| P9 | Australia | 348 [b] | 22 | 20 | 11 | 3 | 3 | 3 | 7 |
| P10 | Australia | 395 | 6 | 5 | 1 | 1 | 1 | 12 | 1 |
| P11 | Australia | 643 [f] | 28 | 5 | 12 | 3 | 4 | 1 | 18 |
| P12 | Australia | 499 | 11 | 5 | 7 | 27 | 2 | 7 | 33 |
| P13 | Australia | 642 | 125 | 105 | 36 | 3 | 3 | 15 | 2 |
| P14 | Australia | 988 | 16 | 5 | 36 | 3 | 4 | 7 | 37 |
| P15 | Australia | 399 | 11 | 5 | 1 | 2 | 2 | 15 | 2 |
| P16 | Australia | 266 | 16 | 5 | 11 | 72 | 44 | 7 | 52 |
| P17 | Australia | 386 | 17 | 5 | 11 | 18 | 4 | 10 | 3 |
| P18 | Australia | 270 | 22 | 3 | 17 | 5 | 2 | 10 | 7 |
| P19 | Australia | 1637 | 11 | 5 | 3 | 3 | 8 | 1 | 9 |
| P20 | Australia | 1399 | 28 | 10 | 1 | 3 | 27 | 4 | 7 |
| P21 | Australia | 527 | 16 | 52 | 11 | 85 | 59 | 15 | 10 |
| A01 | Netherlands | 575 | 11 | 5 | 83 | 2 | 4 | 13 | 7 |
| A03 | Netherlands | 1221 | 89 | 30 | 64 | 90 | 48 | 24 | 32 |
| A10 | Netherlands | 274 [c] | 23 | 5 | 11 | 7 | 1 | 12 | 7 |
| A14 | Netherlands | 1684 [e] | 39 | 6 | 9 | 11 | 3 | 3 | 2 |
| A17 | Netherlands | 511 | 6 | 85 | 1 | 5 | 1 | 4 | 68 |
| A23 | Netherlands | 549 [a f] | 7 | 5 | 12 | 3 | 4 | 1 | 7 |
| B08 | Netherlands | 584 | 17 | 5 | 5 | 18 | 4 | 12 | 83 |
| B12 | Netherlands | 559 | 11 | 5 | 77 | 5 | 3 | 6 | 68 |
| B14 | Netherlands | 406 | 40 | 5 | 11 | 3 | 4 | 13 | 7 |
| B18 | Netherlands | 164 | 1 | 5 | 1 | 11 | 4 | 10 | 10 |
| B20 | Netherlands | 1225 | 118 | 106 | 85 | 92 | 60 | 54 | 72 |
| B22 | Netherlands | 245 [e] | 39 | 6 | 12 | 11 | 3 | 15 | 2 |
| B24 | Netherlands | 497 [g] | 11 | 5 | 1 | 7 | 3 | 56 | 7 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| C2 | Netherlands | 581 | 39 | 5 | 20 | 5 | 1 | 67 | 31 |
| C4 | Netherlands | new | 17 | 1 | 11 | 11 | 4 | 4 | 7 |
| C5 | Netherlands | new | 124 | 5 | 6 | 3 | 4 | 4 | 189 |
| C7 | Netherlands | 558 | 40 | 22 | 1 | 3 | 2 | 6 | 7 |
| C10 | Netherlands | 485 | 11 | 76 | 5 | 3 | 61 | 14 | 3 |
| C12 | Netherlands | 492 | 40 | 5 | 30 | 61 | 1 | 7 | 14 |
| UAB 1-1 | USA | new [d] | 164 | 3 | 7 | new | 2 | 4 | 19 |
| UAB 1-2 | USA | new [d] | 164 | 3 | 7 | new | 2 | 12 | 19 |
| UAB 2 | USA | 274 [c] | 23 | 5 | 11 | 7 | 1 | 12 | 7 |
| UAB 3 | USA | 27 | 6 | 5 | 6 | 7 | 4 | 6 | 7 |
| UAB 4-2 | USA | new | 15 | 5 | 11 | 3 | 4 | 42 | 9 |
| UAB 5 | USA | 179 | 36 | 27 | 28 | 3 | 4 | 13 | 7 |
| UAB 6 | USA | new | 99 | 5 | 30 | 67 | 3 | 33 | 14 |

ST: MLST sequence type

a, b, c: Clonal groups (identical sequence type)

d: Clonal complex (6 alleles in common)

e, f, g: BURST group isolates (5 alleles in common)

## Chapter 3 – Supplementary Table 1

**Supplementary Table 1: Efficacy arm treatment groups**

| Treatment | Sheep # |
|---|---|
| 0.9% saline | 17<br>18<br>19 |
| 4 x $10^8$ PFU/mL CT-PA | 21<br>22<br>23 |
| 4 x $10^9$ PFU/mL CT-PA | 13<br>14<br>15 |
| 4 x $10^{10}$ PFU/mL CT-PA | 10<br>11<br>12 |

## Chapter 3 – Supplementary Table 2

**Supplementary Table 2: Safety arm treatment groups**

| Treatment | Sheep # |
|---|---|
| Vehicle control (PBS+Mg) | 31<br>32<br>33 |
| 4 x $10^{10}$ PFU/mL CT-PA | 28<br>29<br>30 |

**Supplementary Table 3: Sinus histopathological parameters**

- Degree of sinus mucosal inflammation
    - Grade 0 - no inflammation
    - Grade 1 - mild: <30% of superficial half of the lamina propria occupied by inflammatory cells at a density of 50+ cells/high power field*
    - Grade 2 - moderate: 30% - 60% of superficial half of the lamina propria occupied by inflammatory cells at a density of 50+ cells/high power field*
    - Grade 3 - severe: >60% of superficial half of the lamina propria occupied by inflammatory cells at a density of 50+ cells/high power field*
- Composition of inflammatory cell population: % neutrophilic, lymphocytic, plasmacytic, lymphoplasmacytic
- Epithelial hyperplasia
    - Grade 0: absent / minimal
    - Grade 1: mild
    - Grade 2: mild-moderate
    - Grade 3: moderate
    - Grade 4: moderate-severe
    - Grade 5: severe
- Fibrosis
    - Grade 0: no fibrosis
    - Grade 1: mild fibrosis
    - Grade 2: moderate fibrosis
    - Grade 3: severe fibrosis

* 40x magnification