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REVIEW

Anti-biofilm activity of bacteriophages and lysins in chronic rhinosinusitis

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Summary. – Chronic rhinosinusitis (CRS) is an otolaryngological disease with a recalcitrant nature, predominantly due to antibiotic resistant bacteria and the biofilm formation. The intracellular residency of *Staphylococcus aureus* bacteria was observed in CRS. The overall prevalence of CRS is estimated between 5–15% in the human population, and biofilms were formed in sinuses in 40–80% of cases. The bacterial species *S. aureus* and *Pseudomonas aeruginosa* are known to form difficult to treat biofilms in CRS. Bacteriophages (phages) or lysins can be alternatives to antibiotics in the biofilm treatment. The application of a *P. aeruginosa* phage cocktail *ex vivo* decreased biofilm biomass of bacterial isolates from the sinuses of CRS patients by a median of 70%. Further, animal studies performed on a sheep sinusitis model demonstrated significant reduction in *S. aureus* and *P. aeruginosa* biofilm biomass by phage cocktails while maintaining safe prolonged topical application (up to 20 days). Staphylococcal lysin P128 used at a concentration of ≥12.5 μg/ml *in vitro* against the biofilm of methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) isolates from the sinuses of CRS patients demonstrated a significant reduction of the biofilm (up to 95.5%). Staphylococcal lysin CHAP(k) applied *in vivo* in mice nasal infection caused a significant 2 log reduction of *S. aureus* suggesting its potential use against bacteria in nasal mucosa. Furthermore, a beneficial effect of phage therapy in the treatment of chronic sinusitis in humans was observed. Here, we summarize the recent, quite scarce data regarding phage application in chronic rhinosinusitis and look further into this phenomenon.

Keywords: bacteriophages; biofilm; chronic rhinosinusitis; lysins; phage therapy

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Abbreviations: AB = Alamar Blue assay; AECRS = exacerbation of chronic rhinosinusitis; ATCC = American Type Culture Collection; CF = cystic fibrosis; CFU = colony forming unit; Cls = clinical strains; CRS = chronic rhinosinusitis; ESS = endoscopic sinus surgery; LFNO = liposomal formulated nitric oxide; MBEC = minimum biofilm eradication concentration assay; MDT = antibiotic tolerant variants; MOI = phage multiplicity of infection; MRSA = methicillin resistant *S. aureus*; MSSA = methicillin sensitive *S. aureus*; NEC = nasal epithelial cells; NO = nitric oxide; PFU = plaque forming unit

Introduction

Chronic rhinosinusitis (CRS) is a common upper respiratory tract disease. The global incidence of CRS is estimated at 5–15% of the population (Szaleniec *et al.,* 2017). The prevalence of CRS was 8.0% in seven Chinese centers, 7.8% in Denmark and 10.9% in 19 European centers (Shi *et al.,* 2015). A decreasing prevalence of CRS was observed in the USA among adults where it dropped from 16% in 1997 to 12% in 2012 (Shi *et al.,* 2015). Symptoms of inflammation of the nasal and paranasal sinuses mucosa can last for at least 12 weeks (Shi *et al.,* 2015). Molecular

diagnostics demonstrated that sinonasal composition of microorganisms differ significantly in health and disease (Lee *et al.*, 2016). Mucosal inflammation in the paranasal sinuses is correlated with decreased microbial diversity and increased bacterial load (Drilling *et al.*, 2016; Sivasubramaniam *et al.*, 2018; Szaleniec *et al.*, 2017). Increasing resistance to available antibiotics (up to 28% of some sinus bacterial isolates from CRS patients) may cause a problem in CRS treatment (Fong *et al.*, 2017; Szaleniec *et al.* 2019). The examined bacterial isolates, that is, *S. aureus, S. epidermidis*, other coagulase-negative staphylococci and *P. aeruginosa* from CRS patients, were the most resistant to antibiotics.

Biofilms were detected in approximately 40-80% of patients with CRS, but were rarely present or were absent in healthy controls (Szaleniec *et al.*, 2017). *P. aeruginosa* 48 h-old biofilm was cultured in studies *ex vivo* from *P. aeruginosa* isolates from sinuses of CRS patients (Fong *et al.*, 2017). *P. aeruginosa* and *S. aureus* 7-day biofilms were created in an *in vivo* sheep model of rhinosinusitis (Drilling *et al.*, 2014; Fong *et al.*, 2019). Biofilms consist of microorganisms surrounded by a matrix of extracellular polymeric substances adhered to biological and abiotic surfaces. It was shown that biofilm increases the resistance to antibiotics, which is associated with a reduced metabolic state of bacteria, slower growth and greater possibility to exchange antibiotic resistance genes (Akanda *et al.*, 2018; Łusiak-Szelachowska *et al.*, 2020; Pires *et al.*, 2017b). Bacteria forming biofilm in comparison to planktonic bacteria are up to 1000-times more resistant to antibiotics and more resistant to host defenses (Szaleniec *et al.*, 2017). Planktonic bacteria are bacteria suspended in a liquid medium, as opposed to adhered to surfaces. Biofilm cultures are bacterial cells, in the matrix of polymeric substances adhered to surfaces, grown usually for 24–48 h in *in vitro* studies or for 1 week in *in vivo* studies. Currently, researchers are examining new anti-biofilm factors, e.g., phages (bacterial viruses) or phage-derived endolysins (peptidoglycan hydrolases).

Reduced metabolic activity of bacteria in biofilm have an impact on increased resistance to antibiotics. Such biofilm features are also challenging for phage therapy as reduced bacterial activity is inevitably correlated with reduced phage replication and development of phage resistance (Łusiak-Szelachowska *et al.*, 2020; Pires *et al.*, 2017b; Sillankorva and Azeredo, 2014; Szaleniec *et al.*, 2017). To increase phage activity and reduce phage resistance in the treatment of biofilm, phage cocktails should be applied (Łusiak-Szelachowska *et al.*, 2020; Pires *et al.*, 2017b; Szaleniec *et al.*, 2017). It is believed that lysins also cause a rather low possibility of inducing bacterial resistance (Borysowski *et al.*, 2011). The application of phages or lysins as a strategy to prevent and reduce biofilm in bacterial infections has been reported in numerous recent studies *in vitro, ex vivo* as well as *in vivo* (Łusiak-Szelachowska *et al.*, 2020; Melo *et al.*, 2019; Pires *et al.*, 2017b; Poonacha *et al.*, 2017; Schuch *et al.* 2017). However, it is believed that the most efficient strategy for reducing biofilm are phages or lysins in combination with antibiotics (Chopra *et al.*, 2015; Łusiak-Szelachowska *et al.*, 2020; Melo *et al.*, 2019; Pires *et al.*, 2017b).

The role of biofilms in the pathogenesis of CRS requires further explanation (Szaleniec *et al.*, 2017). Biofilm may be the cause or consequence of CRS. Some authors believe that bacterial biofilms cause a failure in the treatment of CRS patients (Fong *et al.*, 2017; Szaleniec *et al.*, 2017). Others, on the contrary, emphasize the importance of the beneficial role of some biofilms in sinus physiology, especially the role of commensals in the sinus microbiome (Szaleniec *et al.*, 2017). More severe sinus diseases were related to biofilms formed by polymicrobial rather than by a single species. The presence of *S. aureus* and *P. aeruginosa* was detected in severe stages of sinus infection, whereas *Haemophilus influenzae* biofilms were related to mild symptoms or beneficial outcomes after surgery (Szaleniec *et al.*, 2017).

Despite all of the aforementioned challenges, phages (rather a phage cocktail than a single phage) or lysins can be considered an alternative to antibiotics in treating CRS infections accompanied by biofilm formation (Drilling *et al.*, 2016; Fong *et al.*, 2017, 2019; Szaleniec *et al.*, 2017). The aim of this review is to analyze studies and reports on the application of phages or their lysins in the elimination of biofilm formed in chronic rhinosinusitis (Table 1).

Microbiome of the sinuses

The microbiome from healthy adults and CRS patients' sinuses differs significantly. The most prevalent (100%) and abundant phyla in healthy sinuses were *Firmicutes, Proteobacteria* and *Actinobacteria* with 48, 25 and 23%, respectively (Ramakrishnan *et al.*, 2013). *Bacteroidetes* were identified in 83% of samples with an abundance of 2.5%. *Staphylococcus epidermidis, Propionibacterium acnes* and *S. aureus* were present in 86, 92 and 68% of samples with an abundance of 11, 15 and 8%, respectively. *Corynebacteria* had a prevalence rate of 93%. In healthy sinuses opportunistic pathogens were also detected (*Streptococcus pneumoniae, H. influenzae* and *Moraxella catarrhalis*) and those often detected in CRS (*Stenotrophomonas maltophilia, Enterobacter sp.*, anaerobes), but at lower abundances. It should be emphasized that relative abundance rather than prevalence may have a higher impact on disease pathogenesis (Ramakrishnan *et al.*, 2013). Molecular studies performed on sinus mu-

Table 1. Influence of phages and lysins on the elimination of bacteria from CRS or from nasal cells. Safety of phages applied to sheep sinuses

Phages or lysins	Methods and model	Results	Reference
S. aureus Sa87 phage	*in vitro* The soft agar overlay technique was used for a phage sensitivity test.	71.1% of antibiotic resistant *S. aureus* strains from CRS patients were sensitive to phage.	Zhang *et al.*, 2018
S. aureus Sa83 phage	*in vitro* The soft agar overlay technique was used for a phage sensitivity test.	69.4% of antibiotic resistant *S. aureus* strains from CRS patients were sensitive to phages.	Zhang *et al.*, 2018
S. aureus phage cocktail	*in vitro* The spot test was used for determination of bacterial sensitivity to the *S. aureus* phage cocktail.	63% of *S. aureus* isolates from AECRS patients were sensitive to the *S. aureus* phage cocktail.	Szaleniec *et al.*, 2019
P. aeruginosa phage cocktail	*in vitro* The spot test was used for determination of bacterial sensitivity to the *P. aeruginosa* phage cocktail.	40% of *P. aeruginosa* isolates from AECRS were sensitive to the *P. aeruginosa* phage cocktail.	Szaleniec *et al.*, 2019
S. aureus MR-10 phage	*ex vivo* The murine nasal epithelial cells were cultured at 37°C in the presence of 5% CO_2.	Phages at MOI of 1 and 10 significantly reduced the adherence, invasion and cytotoxicity of the MRSA strain. Effective decolonization of the MRSA nasal carriage by MR-10 with or without mupirocin was observed.	Chhibber *et al.*, 2014
P. aeruginosa CT-PA phage cocktail	*ex vivo* The soft agar overlay small drop assay in duplicate was used for sensitivity of bacterial stains to the CT-PA phage cocktail. 48 h-biofilm biomass was examined by a crystal violet assay.	89% of isolates from CRS patients were sensitive to CT-PA. Single phages lysed 53%–73% of strains from CRS patients. 67% reduction of 48 h -biofilm biomass for all 40 CRS isolates by phage cocktail (titer 10^7PFU/ml and 10^8PFU/ml) after 24 h of treatment. 70% and 64% reduction of 48 h-biofilm biomass of 40 CRS isolates by phage cocktail (titer 10^7PFU/ml and 10^8PFU/ml) after 48 h of treatment.	Fong *et al.*, 2017
P. aeruginosa CT-PA phage cocktail	*in vivo* sheep model of rhinosinusitis Efficacy study. 7 day-biofilm treated twice daily with CT-PA (10^8-10^{10}PFU/ml) for 1 week in frontal trephine flushes. Biofilm was analyzed by LIVE/DEAD BacLight staining and confocal microscopy.	Significant reduction of biofilm biomass after 10^8–10^{10} PFU/ml CT-PA phage administration. Phages were detected in sinuses for at least 16 hours after the final flush.	Fong *et al.*, 2019
P. aeruginosa CT-PA phage cocktail	*in vivo* sheep model Safety study. Twice daily flushes with CT-PA (10^{10} PFU/ml) to frontal trephine for 21 days. Histopathology of sinuses and organs. Scanning electron microscopy (SEM) for ciliary integrity of sinuses. Phages were enumerated in feces, blood, sinuses and organs.	Histology and SEM of tissues revealed no damage. Phages were detected in feces and occasionally in blood, sinuses and organ samples.	Fong *et al.*, 2019
S. aureus CTSA phage cocktail	*in vivo* sheep model of rhinosinusitis Efficacy study. 7 day-biofilm treated once daily with CTSA (10^6PFU/ml) or EDTA for 5 days in frontal sinuses. Biofilm was analyzed by LIVE/DEAD BacLight staining and confocal scanning laser microscopy.	Single EDTA and CTSA (10^6PFU/ml), similar to CTSA-EDTA, were effective in biofilm removal, but no synergy effect on biofilm reduction was found.	Drilling *et al.*, 2014
S. aureus CTSA phage cocktail	*in vivo* sheep model Safety study. Once daily treated with CTSA (10^6PFU/ml) to frontal sinuses for 3 days. Safety was assessed. by histology of sinuses and scanning electron microscopy of sinus mucosal cilia (SEM). Phages were enumerated in feces, sinuses and organs.	Sinus mucosal tissue showed no significant signs of inflammation. SEM analysis indicated no damage to sinus mucosal cilia. No phages were detected in any of the fecal samples. No phage presence in organ samples was detected. The sinuses of 2 CTSA-EDTA–treated sheep were examined for phage titer immediately after harvest. Three out of 4 sinuses had low levels of phages. The low number of phages is likely due to most of the phages being present in the mucus rather than inside the tissue.	Drilling *et al.*, 2014

Table 1. Continued

Phages or lysins	Methods and model	Results	Reference
S. aureus NOVO12 phage cocktail	*in vivo* sheep model Safety study. Twice daily treated sheep with NOVO12 (10^6PFU/ml) into sheep sinuses for 20 days. Safety was assessed by histology of sheep sinus mucosa and scanning electron microscopy (SEM) of sinus mucosa cilia. Phages were enumerated in sera. Host range was examined by the agar spot test.	The study indicated long-term safety of the *S. aureus* phage cocktail on sinus mucosa. No inflammatory infiltration or tissue damage of sinus mucosa were observed. No phages in sera were detected. 59% and 74% of strains from CRS patients were sensitive to monovalent *S. aureus* phages K710 and P68 and 85% to the NOVO12 phage cocktail.	Drilling *et al.*, 2017
S. aureus chimeric lysin P128	*in vitro* 48 h-biofilm biomass was examined by the Alamar Blue assay (AB) after 2 h treatment of P128 and by the minimum biofilm eradication concentration assay (MBEC) after 24 h treatment of P128.	P128 after 2 h and 24 h significantly reduced the *S. aureus* biofilm of clinical CRS isolates (up to 95.5%) at concentrations of P128 ≥12.5 μg/ml.	Drilling *et al.*, 2016
S. aureus chimeric lysin CHAP(k)	*in vitro* The microplate assay.	5 μg/ml of CHAP(k) reduced the *S. aureus* Xen29 strain by 70% in 5 min.	Fenton *et al.*, 2010
S. aureus chimeric lysin CHAP(k)	*in vivo* Mice were inoculated intranasally with 10^{10}CFU/ml of *S. aureus* Xen29. Single treatment (intranasally and orally) with 925μg/60μl CHAP(k).	Single treatment with 925 μg/60 μl CHAP(k) within 1 h caused a significant 2-log reduction of *S. aureus* in the nasal mucosa.	Fenton *et al.*, 2010

AB = Alamar Blue assay; AECRS = exacerbation of chronic rhinosinusitis; CRS = chronic rhinosinusitis; EDTA = ethylenediaminetetraacetic acid; MBEC = minimum biofilm eradication concentration assay; MOI = phage multiplicity of infection; SEM = scanning electron microscopy.

cosa of CRS patients demonstrated the presence of *S. aureus*, Gram-positive and Gram-negative organisms and anaerobes (Paju *et al.*, 2003). Other studies identified *S. pneumoniae* and *S. aureus* as the most commonly grown bacteria in CRS patients and streptococci were the most frequently detected bacteria with PCR (Power *et al.*, 2005). Stephenson *et al.* (2010) detected, with molecular techniques, anaerobes as the predominant microbes in CRS. *S. aureus* was observed in 50% of the CRS samples. In the control, the most frequent bacteria were *S. aureus, Corynebacterium* and *Propionibacterium* (Stephenson *et al.*, 2010). Notably, *Corynebacterium* and *Lactobacillus sakei* may prevent *Staphylococcus* colonization in the nose and sinuses (Drilling *et al.*, 2016). According to the latest report, in 50 patients with acute exacerbation of chronic rhinosinusitis disease (AECRS), *S. aureus*, *S. epidermidis* and other coagulase-negative staphylococci were the most frequently occurring bacteria followed by *H. influenzae, P. aeruginosa* and *Enterobacteriaceae* (Szaleniec *et al.*, 2019). Studies performed on pediatric patients revealed that the most abundant organism associated with chronic rhinosinusitis was *Moraxella* (Stapleton *et al.*, 2021). In pediatric individuals no significant difference in composition or diversity of microbiota were found in CRS in comparison with the control. Pediatric patients undergoing adenoidectomy-only for nasal obstructive symptoms and sleep-disordered breathing were enrolled as the control group. These results indicated that the diversity of bacteria in pediatric patients and healthy control patients is reduced compared to adult patients with CRS and healthy adults. Such contradictory data constitute the first obstacle when considering possible phage application in sinus infections. Developing bacteriophage (phage) preparations, known for their rather narrow host range, might be challenging and time consuming to cover such a broad range of bacterial species associated with the disease.

The difficulties in treating CRS with commercially available antibiotics

Antibiotic resistance creates a problem in the treatment of CRS. Antibiotic resistance of 40 *P. aeruginosa* strains from CRS patients with cystic fibrosis (CF) (n = 21) and non-cystic fibrosis (non-CF) (n = 19) were examined by minimum inhibitory concentration (MIC) (Fong *et al.*, 2017). Gentamicin, amikacin, ciprofloxacin, ceftazidime and piperacillin were tested. The 28% of all clinical isolates and most of the CF isolates were multidrug resistant to antibiotics. Eleven CF-isolates were multidrug resistant. CF-isolates were resistant to gentamicin (n = 9), amikacin (n = 10), ciprofloxacin (n = 13), ceftazidime (n = 12) and piperacillin (n = 3). Non-CF isolates were resistant to gentamicin (n = 1), amikacin (n = 1), ciprofloxacin (n = 2),

ceftazidime (n = 1) (Fong *et al.,* 2017). The data show a greater degree of antibiotic resistance in CRS isolates from CF. Other studies indicated antibiotic resistance in AECRS patients (Szaleniec *et al.,* 2019). Antibiotic resistance was identified in 28% of the isolates. The isolates that were most resistant to antibiotics were *S. aureus* (resistant to methicillin, macrolide-lincosamide-streptogramin), *S. epidermidis* (resistant to erythromycin, macrolide-lincosamide-streptogramin, methicillin), other coagulase-negative staphylococci (resistant to macrolide-lincosamide-streptogramin, methicillin), and *P. aeruginosa* (reduced sensitivity to imipenem). *Corynebacterium sp.*, *Streptococcus viridans, S. pneumoniae, Klebsiella oxytoca, Streptococcus agalactiae, Enterobacter cloacae* and *Serratia marcescens* were antibiotic sensitive, but the number of examined isolates was low (n = 1–4).

Bacterial elimination by phages from chronic rhinosinusitis and from nasal cells in *in vitro* studies

Phages are bacterial viruses that only multiply in bacterial cells. They are highly specific to Gram-positive and Gram-negative bacteria (Weber-Dąbrowska *et al.,* 2016). Phages may undergo two alternative cycles of life: lytic or lysogenic (Sharma *et al.,* 2017). In the lytic cycle, phages infect bacteria which leads to the multiplication of new phages within the host, the rupture of the host cell and the release of new phage particles. In one growth cycle, approx. 50-100 phage particles are released. In the lytic cycle, bacteria are lysed by phages. In the lysogenic cycle the genetic material of a phage integrates into the bacterial genome and does not lead to lysis of the bacteria (Sharma *et al.,* 2017). Phages can be used as a single phage or in a phage cocktail to enhance their spectrum of lytic activity.

Recent *in vitro* studies have demonstrated the efficacy of applying monovalent *S. aureus* phage preparations against *S. aureus* planktonic and biofilm cultures (Zhang *et al.,* 2018). In one such study, Sa83 and Sa87 phage activity against 70% of clinical *S. aureus* isolates was not correlated with an antibiotic-resistance profile and that the antibiotic tolerance or resistance did not affect the infection ability of phages. No significant difference in phage sensitivity between antibiotic-sensitive and resistant or tolerant *S. aureus* clinical isolates in planktonic and biofilm form was shown by the authors (Zhang *et al.,* 2018).

The activity of *S. aureus* and *P. aeruginosa* phage cocktails against bacterial isolates from AECRS patients was investigated (Szaleniec *et al.,* 2019). The authors showed that 59% of pathogens, including 81% of the antibiotic-resistant pathogens were sensitive to phages from the Biophage Pharma collection. The 63% of the *S. aureus* isolates as well as 40% of the *P. aeruginosa* isolates were sensitive to phage cocktails. The authors pointed out the phage susceptibility of antibiotic-resistant strains from the patients with AERCS as the most important finding of the study (Szaleniec *et al.,* 2019). Moreover, a study on the *in vitro* model of cultured murine nasal epithelial cells on the effect of *S. aureus* phage MR-10 on the adherence, invasion and cytotoxicity of *S. aureus* strains was presented (Chhibber *et al.,* 2014).

Zhang *et al.* (2018) tested the ability of *S. aureus* phage to efficiently kill antibiotic resistant and induced antibiotic-tolerant variants (MDT) of clinical strains isolated from patients with CRS. Two virulent *S. aureus* phages Sa83 and Sa87 from the *Myoviridae* family (AmpliPhi, Australia), which were used in clinical trials, and 65 clinical strains (Cls) of antibiotic resistant MDR (resistant to 3 or more antibiotic classes), isolated from CRS patients were used. The *S. aureus* ATCC 51650 (American Type Culture Collection (ATCC), USA) strain was used as a reference for both planktonic and biofilm cultures as well as an induced antibiotic-tolerant variant. The 71.1% (42 of 59) and 69.4% (41 of 59) of antibiotic resistant *S. aureus* strains were sensitive to phages Sa87 and Sa83, respectively. The sensitivity of antibiotic-sensitive *S. aureus* strains to both phages was noted. The study showed the close similarity between the sensitivity of the *S. aureus* reference strain and Cl3 strain to induce antibiotic tolerance in parent strains (master strains). The antibiotic tolerant/resistant *S. aureus* biofilms, antibiotic-tolerant strains induced from reference strain ATCC 51650 and the Cl3 antibiotic-sensitive *S. aureus* isolate, together with their parent strains, and 6 antibiotic-resistant clinical isolates were used in the study. The authors showed that both the ATCC51650 and CI3 antibiotic-sensitive strains in planktonic and biofilm form demonstrated sensitivity to phages even after post-antibiotic tolerance induction.

Moreover, phages Sa83 and Sa87 were able to reduce the induction of MDT ATCC 51650 and Cl3 strains equally as well as their parent strains in planktonic cultures. A significant reduction ($p < 0.05$) of the viability of the reference strain and Cl3 strain forming biofilms as well as single-drug tolerance (SDT) and MDT strains compared to an untreated control was observed. The authors showed that phage activity was independent from antibiotic-resistance profiles. Both antibiotic sensitive planktonic and biofilm cultures were sensitive to the tested phages even after antibiotic-tolerance induction (Zhang *et al.,* 2018). Interestingly, application of phage cocktails may prevent the appearance of phage-resistant bacteria when compared to monovalent phages (Pires *et al.,* 2017b). Phage-resistant bacteria were formed 6 h after biofilm was infected with a monovalent *P. aeruginosa* phage (Pires *et al.*, 2017a). Furthermore, pretreatment of the *P. aeruginosa*

biofilm with a phage cocktail caused a significant reduction of 48 h-old biofilm accompanied by a reduction of phage-resistant bacteria (Fu *et al.*, 2010).

Interesting data have been presented by Szaleniec's team. The authors showed the results of tests performed on 97 bacterial isolates, from 50 patients with AECRS, taken after endoscopic sinus surgery (ESS) (Szaleniec *et al.*, 2019). The antibiotic resistant *S. aureus*, coagulase-negative staphylococci, *H. influenzae, P. aeruginosa, Escherichia coli, Klebsiella pneumoniae, K. oxytoca* and *Acinetobacter baumannii* strains were the most frequent isolates from CRS patients. The 81% of antibiotic resistant bacteria were found to be sensitive to phages from Biophage Pharma S.A. collection and for the two phage cocktails specific for *S. aureus* and *P. aeruginosa* (each consisting of four selected phages) strains.

It has been shown that 63% of staphylococcal isolates were sensitive to the *S. aureus* phage cocktail (Staph Puk/14/14256, Kr/6/1934, W/5/14256, Kos/10/22119), at the concentration of 10^8 plaque forming unit/ml (PFU/ml) per phage). All staphylococcal phages were representatives of the *Myoviridae* family. *P. aeruginosa* isolated strains were found to be in 40% sensitive to the *P. aeruginosa* cocktail (Kos4/1815, Ku/89/1815, Jar/51/21117, P/53/21117) at the concentration of 10^8 PFU/ml per phage). Except for phage Jar/51/21117 (the *Podoviridae* family), the remaining phages represent the *Myoviridae* family. To determine the phage host range, a spot test was used. Both cocktails indicated the relatively high range of the phage lytic spectrum. The authors pointed out the possibility of using it without earlier sensitivity testing against bacterial strains isolated from patients with CRS (Szaleniec *et al.*, 2019).

Chhibber *et al.* (2014) presented the results of their study on the effective decolonization of the methicillin resistant *S. aureus* (MRSA) nasal carriage in BALB/c mice by the specific *Staphylococcus* phage MR-10. The phage was administered with and without mupirocin. In an *ex vivo* mice model, the effect of phages on the adherence, invasion and cytotoxicity of MRSA strains in cultured murine nasal epithelial cells (NEC) and untreated control was studied. The *Staphylococcal* MRSA 43300 strain was used to establish nasal colonization in BALB/c mice. The nasal epithelial cells were isolated from mouse nasal tissue and cultured at 37°C in the presence of 5% CO_2. For the *in vitro* testing of adherence, invasion and cytotoxic effect, two *S. aureus* MRSA ATCC 43300 and methicillin sensitive *S. aureus* (MSSA) ATCC 2913 strains from ATCC, USA collection were used. The study of the reduction of the nasal carriage of the MRSA 43300 strain in BALB/c mice was performed on four groups (n = 4) with 20 mice (n = 20) per group. The virulent phage MR-10, alone and in combination with mupirocin (2 and 4 μg/ml), was administered. The phage multiplicity of infection (MOI) of 1 and 10 were used. The phage had a broad host range against four standard ATCC MRSA and MSSA strains. The phage MR-10 lytic spectrum was tested on 34 isolated clinical MRSA strains. The authors demonstrated that the *S. aureus* phage MR-10 at MOI of 1 and 10 added to *S. aureus* 43300 culture significantly ($p<0.05$) reduced the adherence, invasion and cytotoxicity in the *in vitro* model of cultured murine nasal epithelial cells as compared to an untreated control. At MOI of 1 only 0.4% of the bacterial cells showed adherence as well as minimal invasion and cytotoxic damage to the NEC cells. No invasion and cytotoxic damage as well as reduction in all three tested parameters at MOI of 10 on NEC were observed. The significant reduction of invasion and cytotoxic damage of five clinical MRSA strains and no detectable invasion or cytotoxicity on NEC by phage at MOI of 1 and 10 was observed. As was shown in the study, the therapeutic potential of *S. aureus* phage MR-10 and mupirocin in eliminating the nasal carriage of MRSA 43300 in healthy BALB/c mice was significant. Complete bacterial eradication was achieved by combined administration of both phages and mupirocin (Chhibber *et al.*, 2014).

The *in vitro* studies suggest a possible use of phages in nasal and sinusitis infection, but *in vivo* studies in animals and further clinical human studies must confirm the efficacy and safety of phages in CRS patients. However, one must remember that *in vitro* studies as well as *in vivo* animal experiments often do not predict the final therapeutic outcome in humans. The following chapters present *in vivo* animal research as well as preliminary experimental phage therapy in patients with CRS.

Phages against biofilm in chronic rhinosinusitis *ex vivo* and *in vivo* studies

Phages cause bacterial destruction thanks to their highly specific activity with the use of phage receptor binding proteins (RBP) that recognize only selected receptors of the host cell (Stone *et al.*, 2019). Recently, researchers investigated the effect and safety of the *P. aeruginosa* phage cocktail of *ex vivo* model biofilm from CRS patients and an *in vivo* sheep model of sinusitis (Fong *et al.*, 2017, 2019) as well as an *in vivo* study of safety and efficacy of *S. aureus* phage cocktails in a sheep sinusitis model (Drilling *et al.*, 2014, 2017). *P. aeruginosa* is a gram-negative bacterium occurring in the sinonasal tract in 9% of CRS patients. Moreover, *P. aeruginosa* infects 49% of cystic fibrosis (CF) patients with CRS. In *ex vivo* studies, 47 strains of *P. aeruginosa* were isolated during an endoscopy from the sinuses of patients with CRS with CF (n = 24) and without CF (n = 23) (Fong *et al.*, 2017). *P. aeruginosa* reference strain ATCC 15692 (PAO1) was

used as a control in phage sensitivity and biofilm assay. Twenty-eight percent of all clinical isolates were multidrug resistant to antibiotics, where most were CF isolates. The *P. aeruginosa* CT-PA phage cocktail used in the study consisted of 4 *P. aeruginosa* phages (Pa193, Pa204, Pa222 and Pa223) with a titer of 10^8 PFU/ml. Pa193 and Pa204 belong to the family *Myoviridae* and Pa222 and Pa223 to *Podoviridae*. The phages are lytic and their genome has been sequenced. The range of lytic activity of individual phages and the CT-PA phage cocktail against 45 strains of *P. aeruginosa* was determined. The 89% of the isolates were sensitive to CT-PA. Single phages lysed 53%–73% of the strains. As expected, the application of a phage cocktail increased the range of lytic activity. Biofilm biomass for all 40 isolates was estimated 24 h and 48 h after treatment with CT-PA using a crystal violet assay (Fong *et al.,* 2017; O`Toole, 2011). The median of the 48 h-old biofilm biomass reduction for all 40 tested isolates was the same (67%) for two CT-PA phage titers (10^7 PFU/ml and 10^8 PFU/ml) after 24 h of treatment and after 48 h of treatment for 10^7 PFU/ml and 10^8 PFU/ml CT-PA in 70% and 64%, respectively. The results of the *ex vivo* study suggest a slightly greater biofilm reduction by a phage cocktail CT-PA with a titer of 10^7 PFU/ml when compared to a titer of 10^8 PFU/ml after 48 h, despite similar biofilm removal of the two phage treatment concentrations at 24 h. Higher titer of a phage is not correlated with more effective elimination of bacterial biofilm. A non-linear relationship for some phages may be due to the self-replicating nature of phages (Abedon, 2011). The increase in phage titers after treatment for almost all clinical isolates suggests that phage replication and bacterial lysis occurred (Fong *et al.,* 2017). In summary, high reductions (up to 70%) in 48 h-biofilm biomass of sinus isolates from CRS patients were obtained in *ex vivo* studies by applying a *P. aeruginosa* phage cocktail at a concentration of 10^7 PFU/ml and 10^8 PFU/ml after 24 h and 48 h.

The efficacy and safety of the CT-PA phage cocktail were determined in a sheep model of rhinosinusitis (Fong *et al.,* 2019). In efficacy studies, a 7-day biofilm of *P. aeruginosa* was treated twice-daily with CT-PA for 1 week in frontal trephine flushes. In efficacy arm protocol each frontal sinus was inoculated with 2 ml of 10^8 colony forming unit/ml (CFU/ml) of *P. aeruginosa* (a clinical CRS strain). Seven days following inoculation, 4 groups of 3 sheep received 7 days` treatment to frontal sinuses with 1 ml of (1) 0.9% saline, (2) $4x10^8$ PFU/ml CT-PA, (3) $4x10^9$ PFU/ml CT-PA, (4) $4x10^{10}$ PFU/ml CT-PA each diluted in 50 ml of 0.9% saline. Biofilm quantification on sinus mucosa was performed by staining with LIVE/DEAD BacLight bacterial viability kit and examined in a confocal microscope (Fong *et al.,* 2019; Singhal *et al.*, 2012). Significant reduction of biofilm biomass was observed *in vivo* after 10^8 - 10^{10} PFU/ml of phage cocktail CT-PA administration in comparison to saline. The reduction of biofilm biomass in the 3 CT-PA groups of sheep did not differ significantly from each other. Significant reduction of biofilm with all CT-PA treatments was observed when only *P. aeruginosa* was present prior to inoculation, but no significant reduction was achieved when other bacterial species were present. Phages were present in the sinuses for at least 16 h after the final flush. In safety arm protocol sheep received twice-daily flushes with CT-PA into each frontal trephine for 21 days. Two groups of 3 sheep were treated with 1 ml $4x10^{10}$ PFU/ml CT-PA and 1 ml PBS+Mg (control) each diluted in 50 ml of 0.9% saline. In safety studies that continued for 3 weeks, phages were detected in feces and occasionally in blood and organ samples collected from sheep receiving CT-PA. Histology of tissues indicated no damage. Phages were not detected in any fecal, blood or organ samples from control sheep. Phages were found in the feces of all CT-PA sheep with average titers of 10^3, 10^4 and 10^4 PFU/g on days 8, 15 and 22, respectively. Phages were detected in 1 blood sample after phage enrichment of CT-PA sheep on day 22. Phages were present after phage enrichment in 1 liver, 1 spleen, 1 kidney, 2 hearts and in 3 of 6 sinus samples from CT-PA sheep. Phages were found in 1 lung sample with titer 10^4 PFU/g from CT-PA sheep. Phages were not detected in any brain samples from CT-PA sheep (Fong *et al.,* 2019) probably due to the highly selective blood-brain barrier (BBB). The *in vivo* study performed in a sheep model of rhinosinusitis indicated that the *P. aeruginosa* phage cocktail can be applied for 1 week to reduce *P. aeruginosa* 7 day-biofilm biomass. The titer of the phage cocktail should be between 10^8 and 10^{10} PFU/ml. Additionally, studies on an *in vivo* sheep model of rhinosinusitis demonstrated the safety of topical, long *P. aeruginosa* phage cocktail administration. The results suggest that the phage cocktail (CT-PA) has the potential to treat *P. aeruginosa* biofilm infections in CRS.

It is known that *S. aureus* is an opportunistic bacterium. *S. aureus* infection is connected with exacerbation of CRS and development of recalcitrance especially with the appearance of biofilm. *In vivo* animal studies may explain the efficacy and safety of *S. aureus* phage cocktails in sinusitis. The studies of Drilling *et al.* (2014, 2017) contribute to such explanations. In the first study from 2014, Drilling *et al.* presented their findings on the safety and efficacy of the *S. aureus* phage with or without ethylenediaminetetraacetic acid (EDTA) topical administration in a sheep rhinosinusitis model of infection. It was shown that phages and EDTA are safe in short-term topical application to the sinuses and that both the EDTA and CTSA phage cocktail (10^6 PFU/ml) used alone for 5 days successfully reduce mucosal 7 day-biofilm load and both may be used in the clinical treatment of *S. aureus* infections and mucosal biofilm removal.

The *S. aureus* ATCC 25923 strain as an infective agent as well as the *S. aureus* phage cocktail (CTSA), active ($2x10^8$ PFU/ml) and heat-inactivated (as a control), from Special Phage Services Pty Ltd (Sydney, Australia) was used in the study. In the frontal sinuses infection of 15 Merino cross weathers sheep, the *S. aureus* ATCC 25923 strain was used as a pathogen for 7 days (a treatment efficacy arm). In the safety study 12 sheep were divided into 4 treatment groups: (1) no treatment control (NT), (2) EDTA (0.075 mg /ml in 0.9% saline), (3) CTSA ($2x10^6$ PFU/ml), (4) CTSA and EDTA in combination. Each group consisted of 3 sheep and was treated once a day for 3 days with 100 ml applied into both frontal sinuses. In the efficacy study, each treatment group consisted of 3 sheep and was treated once a day for 5 days. Histological testing and scanning electron microscopy (SEM) after 3 days of treatment in a safety study was used. A significant decrease in the levels of subepithelial acute inflammatory cells in the EDTA ($p = 0.03$) and CTSA- EDTA ($p = 0.01$) in comparison to the NT group was observed. When sheep from the safety arm were treated alone with EDTA, a significant reduction in chronic inflammatory cell numbers in the subepithelial layer, when compared to both NT and CTSA – EDTA-treated tissue, was observed. No such difference was observed between the CTSA – EDTA group and the NT control and EDTA- treated sheep showed a significantly lower level of the chronic cell infiltrate in the subepithelium compared to the CTSA – EDTA group. The study showed a significant reduction of biofilm mass levels when compared to the NT control after single EDTA and CTSA treatment, similar to CTSA – EDTA treatment. No synergic effect on biofilm removal in CTSA and EDTA treatment was found. No phage presence (less than 1 PFU/ml) in the kidney, spleen, liver, and brain samples was detected. The authors suggest that phages administered topically do not cross the sinus mucosal barrier into the bloodstream at a detectable level as well as the possible presence of an anti-phage immune response, and in consequence their quick elimination from the body (Drilling *et al.*, 2014). Phages were found occasionally in blood and organ samples from sheep receiving the *P. aeruginosa* phage CT-PA in most samples after phage enrichment and after prolonged use of phages in sheep (Fong et al., 2019). In summary, the authors showed that both CTSA (10^6 PFU/ml) and EDTA treatment used separately was effective as anti – *S. aureus* agents without synergy in their combination as well as the safety and efficacy in the use of CTSA and EDTA in short-term topical sinonasal treatment (Drilling *et al.,* 2014). Use of the *S. aureus* phage cocktail (10^6 PFU/ml) *in vivo* in sheep sinusitis significantly reduced biofilm biomass, but did not eradicate it completely, whereas the *P. aeruginosa* phage cocktail at higher concentrations (10^8–10^{10} PFU/ml) *in vivo* against biofilm in sheep sinusitis indicated a significant reduction of biofilm biomass and safety use of this phage preparation (Drilling *et al.,* 2014; Fong *et al.,* 2019).

Drilling *et al.* (2017) studied the long-term safety of *Staphylococcus* phage cocktail topical application to sheep frontal sinuses and its lytic activity against the Australian *S. aureus* strains isolated from patients with CRS. The staphylococcal phage cocktail NOVO12, consisting of two purified, well-characterized phages K710 and P68 (Novolytics Pty Ltd., United Kingdom) at a concentration of $1x10^8$ PFU/ml, active and heat-inactivated (Hip) for both an *in vivo* and *in vitro* study, was used. The phage K710 and P68, as well as the NOVO12 cocktail host range on 61 *S. aureus* isolates from 61 CRS patients, was performed. The 50 ml of NOVO12 was administered to the sheep sinuses twice daily for 20 days with one of three different treatments (n = 7 sinuses per group): (1) 0.9% saline (control), (2) 0.9% saline with $2x10^6$ PFU/ml of heat-inactivated phage cocktail NOVO12 (Hip) or (3) 0.9% saline containing $2x10^6$ PFU/ml of active NOVO12 cocktail. A histological analysis (inflammation, edema, fibrosis) and scanning electron microscopy (SEM) of mucosal tissue were performed. The serum and mucosal samples from all groups were tested for phage presence. The study showed that 59% (36/61) of tested strains were sensitive to phage K710, 74% (45/61) to phage P68, and 85% (52/61) to the NOVO12 cocktail. The significantly higher lytic spectrum ($p = 0.0022$) for NOVO12 in comparison to a single application of K710 and P68 (not statistically significant) was observed. No significant differences in histological evaluation of the samples were found. Similarly, no lytic phage particles were detected. The authors showed in the study the safety of long-term topical phage therapy of sheep sinonasal *S. aureus* infection without sinus mucosal lining damage and altered the profile of the immune cells. The notable effect in the increase of K710 phage lytic spectrum from 59% to 85% of lysed staphylococcal strains after the addition of phage P68 was observed. In conclusion, the staphylococcal NOVO12 phage cocktail demonstrated the broad lytic spectrum (85%) of clinical isolates (including MRSA strains) from CRS patients and that long term (20 days) application (10^6 PFU/ml) to sheep frontal sinuses was safe and did not modify or damage the structure of the sinus mucosa lining. The authors postulate the possibility of using phages as a topical antimicrobial therapy in CRS (Drilling *et al.,* 2017).

In vitro and *in vivo* studies on the elimination of *S. aureus* or *P. aeruginosa* biofilms with the use of phage cocktails have shown promising results in CRS patients. In an osteomyelitis model in rats, *S. aureus* and *P. aeruginosa* biofilm formations were significantly reduced by phages applied along with antibiotics (Yilmaz *et al.,* 2013).

The knowledge about capability of phages to pass through the sinonasal mucosal barrier is poor (Szale-

niec *et al.,* 2017). The nasal cavity is available for local medications, but their passage to the sinuses is limited. Intranasal drug administration in CRS is less effective due to blocking the sinus ostia. Drug administration into the sinuses may be more effective during endoscopic sinus surgery (ESS). The topical antibiotic therapy in sinusitis is more efficient in some patients after ESS when the sinus ostia is open and the microbial infection is recognized. However, evidence-based reviews do not recommend topical antibiotic therapy in CRS and delivery of medications into the sinuses endoscopically. The authors claim that CRS does not only have a bacterial background in humans, but that infections are usually mixed and also fungal. In general, CRS is a chronic inflammation of the sinus mucosa of as yet undetermined etiology. Bacterial infection is not considered as the only factor responsible for mucosal dysfunction. The 'healthy' and 'sick' sinus microbiome is very complex and the use of antibiotics does not eliminate all pathogenic bacterial flora, which determines the outcome of the therapy (Szaleniec *et al.,* 2017).

Still, eradication of polymicrobial biofilms and multidrug-resistant bacteria occurring in CRS remains a challenge (Miyake and Bleier, 2019). Oral antibiotics often mitigate symptoms in acute CRS exacerbations, but they also often fail to eradicate biofilm. Topical treatment may potentially deliver higher drug concentrations without causing systemic side effects and with reducing antibiotic resistance. Phages have the ability to penetrate bacterial biofilms and they may be potentially applied in the topical therapy of CRS. *In vivo* sheep studies indicated the safety and efficacy in short- and long-term topical phage cocktail administration in a frontal sinusitis model against *S. aureus* or *P. aeruginosa* infection (Drilling *et al.,* 2014, 2017; Fong *et al.,* 2019). Further evaluation of phage cocktails in CRS therapy is required. The studies on application of phage cocktails *ex vivo* and *in vivo* against bacterial biofilm formed in CRS suggest a potential use of these preparations to eliminate biofilm forming bacteria.

Lysins against biofilm in chronic rhinosinusitis *in vitro* and against nasal colonization in *in vivo* studies

Phage-derived lysins are phage enzymes that cleave peptidoglycan in the cell wall of Gram-positive or Gram-negative bacteria causing lysis of the bacterial cell (Borysowski *et al*., 2011; Fischetti 2018). The cell wall of Gram-positive bacteria has teichoic acids and several layers of peptidoglycan, whereas the cell wall of gram-negative bacteria has a single layer of peptidoglycan surrounded by an outer membrane with the component of lipopolysaccharide (LPS). The occurrence of the outer membrane protects the bacteria against exogenously added lysins (Fischetti, 2018; Lood *et al.,* 2015). Lysins are produced in bacterial cells infected with phages at the final stage of the lytic cycle of phages (Borysowski *et al*., 2006). Lysins have a narrow antibacterial range (Fischetti, 2017), similar to phages.

Another alternative to combat bacterial biofilms is the use of lysins or chimeric lysins with anti-bacterial and anti-biofilm activity (Łusiak-Szelachowska *et al.,* 2020; Poonacha *et al.,* 2017; Schuch *et al.,* 2017). *S. aureus* biofilms tend to occur in CRS patients. Biofilm resistance constitutes a serious problem and the possibility of effectively removing the biofilm by using antibacterial agents, e.g., phages and lysins, is being investigated (Drilling *et al.,* 2016; Fong *et al.,* 2017; Łusiak-Szelachowska *et al.,* 2020; Pires *et al.,* 2017b). *S. aureus* resistance to antibiotics, intracellular residency and forming biofilm makes it dangerous and difficult to eradicate pathogens.

Two chimeric staphylococcal lysins were investigated *in vitro* (lysin P128 and lysin CHAP(k)) and *in vivo* (lysin CHAP(k)) against nasal staphylococcal isolates (Drilling *et al.,* 2016; Fenton *et al.,* 2010). Recently, the *S. aureus* chimeric lysin P128 was constructed (Drilling *et al.,* 2016). This P128 protein is a combination of a modified murein hydrolase domain derived from a K phage and a cell wall binding domain derived from lysostaphin. Lysostaphin is a metallo-endopeptidase produced from *Staphylococcus simulans* with antibacterial activity (Sadeghi *et al.,* 2019). P128 specifically breaks down the peptidoglycan of staphylococcal species. Researchers described the activity of P128 *in vitro* against the *S. aureus* biofilm of the ATCC 25923 reference strain and 2 clinical MSSA isolates Cl1 and Cl2, and 1 clinical MRSA isolate from sinuses in CRS patients (Drilling *et al*., 2016). A 48 h-old biofilm was obtained and treated with various concentrations of P128 (0–100 μg/ ml) for 2 h (AB-Alamar Blue assay) (Drilling *et al.,* 2016) and 24 h (MBEC-minimum biofilm eradication concentration assay) (Drilling *et al.,* 2016). The presence of planktonic bacteria in the MBEC test was also examined. The biofilm present in MBEC was stained with the fluorescent BacLight stain and imaged in a confocal microscope. Cell viability was estimated in the AB test. Biofilms of Cl1 and Cl2 isolates observed in MBEC assay were significantly reduced by P128 ≥25 μg/ml for 24 h treatment. MRSA biofilm was reduced by P128 ≥12.5 μg/ml and the reference strain decreased by P128 ≥6.25 μg/ml. Biofilms of all clinical isolates examined in AB assay were reduced by P128 ≥12.5 μg/ml within 2 h treatment. The reference strain was more resistant, because a larger P128 dose of 25 μg/ml was required to reduce biofilm. Planktonic bacteria tested in MBEC assay were decreased by P128 ≥50 μg/ml for Cl1 and Cl2 isolates and by P128 ≥12.5 μg/ml for MRSA and the reference strain. In conclusion,

the studies indicated the effectiveness of P128 for 2 h and 24 h against 48-h *S. aureus* biofilms causing significant reductions (up to 95.5%) of biofilm at concentrations of P128 ≥12.5 μg/ml for the tested strains. The high biofilm reduction through the use of staphylococcal lysin P128 was observed for both MSSA and MRSA strains isolated from CRS patient sinuses. Future research is needed to optimize the delivery method of P128 at the site of infection in the nose. The requirement for a minimum concentration protein administration suggests using nasal spray or gel rather than rinsing (Drilling *et al.,* 2016).

The activity of other chimeric lysin CHAP(k) was investigated in *in vitro* studies (Fenton *et al.,* 2010). CHAP(k) was derived from a staphylococcal K phage. CHAP(k) (1.0–50 μg/ml) *in vitro* activity was tested against *S. aureus* Xen29 in a microplate assay during 5–15 min incubation. Five μg/ml of CHAP(k) reduced the OD_{590} 0.5 *S. aureus* Xen29 by 70% in 5 min. Also, in *in vivo* studies in mice, chimeric lysin CHAP(k) was investigated against *S. aureus* Xen29 nasal colonization (Fenton *et al.,* 2010). One-hour post-infection mice inoculated intranasally by 10^{10} CFU/ml indicated successful nasal colonization by *S. aureus*. Two groups of 7 mice were administered with (1) 60 μl of 925 μg/60 μl CHAP(k) in enzyme buffer or (2) 60 μl of buffer as a control. Single treatment (intranasally and orally) with 925 μg/60 μl CHAP(k) within 1 h caused a significant 2-log reduction of *S. aureus* in the nasal mucosa of mice. The mean log of the CHAP(k) treated group was 5.29 ± 0.26 CFU/g in comparison to a mean log of 7.35 ± 0.10 CFU/g for the buffer treated control. Lysin demonstrates good solubility, high lytic activity and high specificity against staphylococci. This enzyme had a low possibility of inducing bacterial resistance and may be considered as an alternative therapeutic option (Fenton *et al.,* 2010). Studies *in vivo* indicated that the staphylococcal lysin CHAP(k) at a concentration of 925 μg/60 μl applied in mice nasal infection for 1 h caused a significant 2 log reduction of *S. aureus*. This observation can be used to potentially apply lysin against staphylococcal bacteria in nasal mucosa. The studies *in vitro* and *in vivo* suggest a potential implementation of phage-derived lysins to remove bacteria from nasal and sinus mucosa.

Nitric oxide against biofilm in chronic rhinosinusitis *in vitro* study

Nitric oxide (NO) is a soluble gas that is synthesized by the enzyme nitric oxide synthase (NOS) from its precursor L-arginine (Garcia and Stein, 2006; Shinde *et al.,* 2000). NO is produced by the endothelial cells, macrophages and specific neurons in the brain. There are three types of NOS: endothelial, neuronal and cytokine-inducible. NO plays an essential role in vasodilation, neurotransmission, regulation of wound healing, nonspecific immunity and host defense. Small quantities of NO mediate these physiological processes. A large quantity of NO is implicated in cytotoxic effects. NO is involved in the host`s response to infection. NO antimicrobial activity can involve two mechanisms: 1) reactive species derived from NO synthase possess antimicrobial activity and 2) interactions between NO and reactive oxygen species lead to the formation of multiple antimicrobial metabolites (peroxy nitrate, 5-nitrosothiols and nitrogen dioxide), which may damage microbial DNA, proteins and lipids. NO also exerts immune-modulation effects and cytotoxic activity. The cytotoxic effect of NO concerns not only microorganisms, but also tissue damage (Garcia and Stein, 2006; Shinde *et al.,* 2000). Other report also confirms that NO plays a major role in inflammation and immune regulation (Guzik *el al.,* 2003).

Furthermore, there are some associations between phage biology and function and NO. For example, NO may be associated with prophage induction as well as with synthesis and propagation of phage particles. Thus, NO may inhibit phage-dependent Shiga-toxin synthesis (Vareille *et al*., 2007). What is more, phages engineered to induce NO production by macrophages have adjuvant properties (Namdee *et al*., 2018). Interestingly, engineered phages have recently been successfully used in the treatment of mycobacterial infection in a lung transplant recipients (Dedrick *et al*., 2019).

Further, NO that has antimicrobial properties is endogenously produced in the sinuses. Patients with CRS have significantly lower levels of sinonasal NO, whereas high levels of NO were found in the sinuses of healthy subjects. Jardeleza *et al.* (2011) demonstrated the results of *in vitro* studies on the nitric oxide (NO) effect on the *S. aureus* biofilm formation and its potential role in CRS patients for whom a significantly lower sinonasal NO level was observed. The *S. aureus* ATCC 25923 as biofilm-forming strain, and 7 *S. aureus* strains, cultured on cerebrospinal fluid (CSF) broth (Oxoid, Australia), isolated from the sinuses of CRS patients were used in the study. Different NO levels (1–1000 μM) were used in the study. The Live/Dead BacLight stain and confocal scanning laser microscopy were used for biofilm visualization. The Calgary Biofilm or MBEC media (Innotech, Inc., Canada) was used depending on the current authors' preferences for bacterial biofilm growth which was identified as irreversibly attached, immotile coccal structures of appropriate size (0.5–2 μm diameter) and morphology, existing in characteristic clusters. Biofilm biomass was computed by the biofilm quantification software Comstat2 and was provided with all 7 *S. aureus* isolates. Bacterial quantification in planktonic culture for the assessment of NO's effect on *S.*

aureus isolates was performed in duplicates. The ATCC 25923 and three clinical isolates demonstrated a stronger capacity for biofilm formation. The authors showed that anti-biofilm and pro-biofilm mass formation depends on NO concentration. The biofilm growth pattern and trend to respond to NO were similar in all tested isolates. At a higher NO concentration (125–1000 μM) biofilm biomass decreased from 0.105 to 0.057 $\mu m^3/\mu m^2$, but increased to 0.470 $\mu m^3/\mu m^2$ at a lower NO concentration (0.9–2.0 μM). The average biomass at a high vs. low concentration was statistically significant ($p < 0.001$). The study shows a lack of NO effect on both planktonic growth and distinct pattern among these isolates. Considering the fact that CRS patients have significantly lower levels of sinonasal NO and its *S. aureus* anti-biofilm effect during *in vitro* study, the authors suggested NO in the potential antimicrobial use in CRS patients who suffer from *S. aureus* infections (Jardeleza *et al.,* 2011). The authors pointed out the possible use of NO at a higher concentration as an antimicrobial agent on *S. aureus* biofilms in the treatment of CRS. The challenges of this consist of attaining and sustaining the antibiofilm concentration of NO and effective delivery into the sinuses.

The *in vivo* study in a sheep model of *S. aureus* biofilm rhinosinusitis with the topical application of a liposomal formulation of an NO donor (LFNO) using isosorbide mononitrate (ISMN) was performed (Jardeleza *et al.,* 2015). LFNO-treated sheep had less inflammation and comparable ciliary preservation compared to the control group. LFNO (liposomal-formulated ISMN at dose 60 mg/ml) treated sheep had a significantly lower biofilm biomass compared to the control group. Further studies are required to explain the possibility of applying this agent in CRS therapy.

Phase 1 clinical trial in recalcitrant CRS

Recently, a phase 1 clinical trial of a phage cocktail ABSA01 against *S. aureus* in recalcitrant CRS patients was conducted in Australia (Ooi *et al.,* 2019). Trial registration: http://anzctr.org.au identifier: ACTRN12616000002482. The phage cocktail was administered to nine post-endoscopic sinus surgery patients showing resistance to medical therapies. The reduction of recalcitrant symptoms of CRS is difficult to obtain. Bacteria susceptible to the phage cocktail were present in 9 patients. Three cohorts (3 patients/cohort) received twice daily intranasal irrigations with phage cocktail $3x10^8$ PFU/ml for 7 days, $3x10^8$ PFU/ml for 14 days and $3x10^9$ PFU/ml for 14 days. The treatment was well tolerated and no serious side effects were observed. It resulted in clinical and microbiological eradication of the infection in 2 of 9 patients. Application of a phage cocktail of doses to $3x10^9$ PFU/ml for 14 days was safe and well tolerated and preliminary observations of the effectiveness were promising (Ooi *et al.,* 2019).

Discussion

All of the aforementioned studies on the use of phages in sinusitis present a rather promising picture for the future, at least on an experimental level. Obviously, despite such an assumption, further determination of therapeutic usefulness of phage preparations needs to be carefully investigated. Such confirmation, involving clinical trials, is still to be determined and might bring new insights into the potential application of such a treatment. We need to remember that *P. aeruginosa* and *S. aureus* are pathogens that tend to create mixed biofilm, significantly more resistant to known antimicrobials (Tkhilaishvili *et al.,* 2020). This phenomenon involves the necessity to use higher antibiotic doses, too high to be reached in clinical practice (Tkhilaishvili *et al.,* 2020). In such cases, a combination of both antimicrobials, phages and antibiotics could be the best possible option to treat chronic sinusitis.

The complex nature of interactions between phages and their bacterial hosts cannot be underestimated as well. Wettstadt (2020) described a protective role of *Pseudomonas* Pf4 phages that lead to forming crystalline structures that serve as a physical wall keeping antibiotics away from the bacteria in biofilm.

Another aspect that is worth investigating concerns the form of phage application (time, dose, route of administration). As our group already showed, topical phage application might be involved in the higher induction of antiphage antibodies that could neutralize phage particles. Considering the harsh therapeutic niche in sinuses caused by biofilm formation, such an additional obstacle certainly would not support phage activity at the site of infection. On the other hand, induction of phage antibodies is not directly correlated with the clinical outcome of phage treatment. The final effect also depends on the immune status of patients, which might be impaired by prolonged use of antibiotics and other factors (Górski *et al.,* 2012; Łusiak-Szelachowska *et al.,* 2014, 2017; Żaczek *et al.,* 2016, 2019). Recent studies by Guła *et al.* (2020) suggest that the time of application is crucial in combating biofilm formation. The application of phages at an early stage (six hours) and on a three-day biofilm formed by *P. auruginosa* PAO1 caused a significant slowdown in biofilm dynamics, whereas the two-day biofilm turned out to be insensitive to phage infection. A 48 h-old biofilm formed by bacterial strains isolated from CRS patients was efficiently reduced by *P. aeruginosa* phage cocktail after 24 h and 48 h treatment as well as by staphylococcal lysin for 2 h and 24 h

treatment (Drilling *et al.,* 2016; Fong *et al.,* 2017). Of note, rhinosinusitis is a chronic infection accompanied by the presence of a well-developed biofilm and the level of biofilm development should always be considered before implementing phages in the treatment.

Historical data from a Polish group (Weber-Dąbrowska *et al.,* 1996) describe a group of 32 patients with chronic sinusitis aged 5–65 years treated with phage preparations developed at the Hirszfeld Institute in Wrocław, Poland. Considering the average time of patients' disease (6 years), one could consider them as difficult patients with impaired immunity and significant biofilm formations. Despite the above characteristics, 24 patients out of 32 finished phage treatment with full recovery confirmed by complete bacteria eradication in microbiological tests. Notably, among patients suffering from multibacterial infections (*S. aureus* with *Escherichia* or *S. aureus* with *Pseudomonas*), only 1 patient out of 4 achieved complete bacteria eradication. This observation is in line with conclusions made by Tkhilaishvili *et al.* (2020) 25 years later regarding difficulties in reducing mixed biofilm. Similar favorable clinical results were described in another work (Weber-Dąbrowska *et al.,* 2000) where 83% of patients (38 out of 46) with suppurative sinusitis had confirmed elimination of bacteria from the infection site and only 5 of them (11%) finished treatment with no clinical effect. These days, such data have mostly a historical value but can be an important indication for clinicians trying to find alternatives for increasingly less effective antibiotics.

Conclusions

Research and report analysis concerning the application of phages *in vitro*, *ex vivo* and *in vivo* against *S. aureus* or *P. aeruginosa* biofilms in sinus disease has shown a potential use for this antimicrobial factor in CRS. Topical application of phage cocktails was effective and safe in *in vivo* studies conducted in a sheep sinusitis model. A preliminary yet promising effect of experimental phage therapy in humans was achieved. Additionally, *in vitro* and *in vivo* studies on the application of phage-derived lysins in sinus and nasal infections indicated an effectiveness in eliminating *S. aureus* bacteria. Considering the nature and complexity of sinus infections, further studies are necessary to prove phage usefulness in a possible therapeutic application of phage-based therapeutics, including lysins, in CRS.

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References

Abedon S., In Allen I, Laskin SS, Geoffrey MG (Eds): Advances in Applied Microbiology. Vol. 77. San Diego, CA: Academic Press, pp. 1–40, 2011. https://doi.org/10.1016/B978-0-12-387044-5.00001-7

Akanda ZZ, Taha M, Abdelbary H., J. Orthop. Res. 36, 1051–1060, 2018.

Borysowski J, Lobocka M, Międzybrodzki R, Weber-Dąbrowska B, Górski A., BioDrugs 25, 347–355, 2011. https://doi.org/10.2165/11595610-000000000-00000

Borysowski J, Weber-Dąbrowska B, Górski A., Exp. Biol. Med. (Maywood) 231, 366–377, 2006. https://doi.org/10.1177/153537020623100402

Chhibber S, Gupta P, Kaur S., BMC Microbiol 14, 212, 2014.

Chopra S, Harjai K, Chhibber S., Appl. Microbiol. Biotechnol. 99, 3201–3210, 2015. https://doi.org/10.1007/s00253-015-6460-1

Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russel DA, Ford K, Harris K, Gilmour KC, Soothill J, Jacobs-Sera D, Schooley RT, Hatfull GF, Helen Spencer H., Nat. Med. 25, 730–733, 2019. https://doi.org/10.1038/s41591-019-0437-z

Drilling AJ, Cooksley C, Chan C, Wormald PJ, Vreugde S., Int. Forum Allergy Rhinol. 6, 349–355, 2016 https://doi.org/10.1002/alr.21680

Drilling AJ, Ooi ML, Milijkovic D, James C, Speck P, Vreugde S, Clark J, Wormald PJ., Front. Cell Infect. Microb. 7, 49, 2017. https://doi.org/10.3389/fcimb.2017.00049

Drilling A, Morales S, Boase S, Jervis-Bardy J, James C, Jardeleza C, Tan NC, Cleland E, Speck P, Vreugde S, Wormald PJ., Int. Forum Allergy Rhinol. 4, 176–186, 2014. https://doi.org/10.1002/alr.21270

Fenton M, Casey PG, Hill C, Gahan CG, Ross RP, McAuliffe O, O'Mahony J, Maher F, Coffey A., Bioeng. Bugs 1, 404–407, 2010. https://doi.org/10.4161/bbug.1.6.13422

Fischetti VA., Curr. Top. Microbiol. Immunol. 409, 529–540, 2017.

Fischetti VA., Viruses 10, pii: E310, 2018. https://doi.org/10.3390/v10060310

Fong SA, Drilling A, Morales S, Cornet ME, Woodworth BA, Fokkens WJ, Psaltis AJ, Vreugde S, Wormald PJ., Front. Cell. Infect. Microbiol. 7, 418, 2017. https://doi.org/10.3389/fcimb.2017.00418

Fong SA, Drilling AJ, Ooi ML, Paramasivan S, Finnie JW, Morales S, Psaltis AJ, Vreugde S, Wormald PJ., Transl. Res. 206, 41–56, 2019. https://doi.org/10.1016/j.trsl.2018.12.002

Fu W, Forster T, Mayer O, Curtin JJ, Lehman SM, Donlan RM., Antimicrob. Agents Chemother. 54, 397–404, 2010. https://doi.org/10.1128/AAC.00669-09

Garcia X, Stein F., Semin. Pediatr. Infect. Dis. 17, 55–57, 2006. https://doi.org/10.1053/j.spid.2006.04.002

Górski A, Międzybrodzki R, Borysowski J, Dąbrowska K, Wierzbicki P, Ohams M, Korczak-Kowalska G, Olszowska-Zaremba N, Łusiak-Szelachowska M, Kłak M, Jończyk E, Kaniuga E, Gołaś A, Purchla S, Weber-Dąbrowska B, Letkiewicz S, Fortuna W, Szufnarowski K, Pawełczyk

Z, Rogóż P, Kłosowska D., Adv. Virus Res. 83, 41–71, 2012. https://doi.org/10.1016/B978-0-12-394438-2.00002-5

Guła G, Szymanowska P, Piasecki T, Góras S, Gotszal T, Drulis-Kawa Z., Viruses 12, pii: E407, 2020. https://doi.org/10.3390/v12040407

Guzik TJ, Korbut R, Adamek-Guzik T., J. Physiol. Pharmacol. 54, 469–487, 2003.

Jardeleza C, Foreman A, Baker L, Paramasivan S, Field J, Tan LW, Wormald PJ., Int. Forum Allergy Rhinol. 1, 438–444, 2011. https://doi.org/10.1002/alr.20083

Jardeleza C, Thierry B, Rao S, Rajiv S, Drilling A, Miljkovic D, Paramasivan S, James C, Dong D, Thomas N, Vreugde S, Prestidge CA, Wormald PJ., Transl. Res. 166, 683–692, 2015. https://doi.org/10.1016/j.trsl.2015.06.009

Lee JT, Frank DN, Ramakrishnan V., Am. J. Rhinol. Allergy 30, 3–16, 2016. https://doi.org/10.2500/ajra.2016.30.4255

Lood R, Winer BY, Pelzek AJ, Diez-Martinez R, Thandar M, Euler CW, Schuch R, Fischetti VA., Antimicrob. Agents Chemother. 59, 1983–1991, 2015. https://doi.org/10.1128/AAC.04641-14

Łusiak-Szelachowska M, Weber-Dąbrowska B, Górski A., Virol. Sin. 35, 125–133, 2020. https://doi.org/10.1007/s12250-019-00192-3

Łusiak-Szelachowska M, Żaczek M., Weber-Dąbrowska B, Międzybrodzki R, Kłak M, Fortuna W, Letkiewicz S, Rogóż P, Szufnarowski K, Jończyk-Matysiak E, Owczarek B, Górski A., Viral Immunol. 27, 295–304, 2014. https://doi.org/10.1089/vim.2013.0128

Łusiak-Szelachowska M, Żaczek M, Weber-Dąbrowska B, Międzybrodzki R, Letkiewicz S, Fortuna W, Rogóż P, Szufnarowski K, Jończyk-Matysiak E, Olchawa E, Walaszek KM, Górski A., Future Microbiol. 12, 109–117, 2017. https://doi.org/10.2217/fmb-2016-0156

Melo LDR, Pires DP, Monteiro R, Azeredo J., In Górski A, Międzybrodzki R, Borysowski J (Eds): Phage Therapy: A Practical Approach. Springer Nature Switzerland, pp. 295–313, 2019. https://doi.org/10.1007/978-3-030-26736-0_11

Miyake MM, Bleier BS., Int. Forum Allergy Rhinol. 9, S32–S46, 2019. https://doi.org/10.1002/alr.22341

Namdee K, Khongkow M, Boonrungsiman S, Nittayasut N, Asavarut P, Temisak S, Saengkrit N, Puttipipatkhachorn S, Hajitou A, Ruxrungtham K, Yata T., Mol. Ther. Nucleic Acids 12, 33–44, 2018. https://doi.org/10.1016/j.omtn.2018.04.012

Ooi ML, Drilling AJ, Morales S, Fong S, Moraitis S, Macias-Valle L, Vreugde S, Psaltis AJ, Wormald PJ., JAMA Otolaryngol. Head Neck Surg. 145, 723–729, 2019. https://doi.org/10.1001/jamaoto.2019.1191

O'Toole GA., J. Vis. Exp e2437, 2011. https://doi.org/ 10.3791/2437

Paju S, Bernstein JM, Haase EM, Scannapieco FA., J. Med. Microbiol. 52, 591–597, 2003. https://doi.org/10.1099/jmm.0.05062-0

Pires DP, Dötsch A, Anderson EM, Hao Y, Khursigara CM, Lam JS, Sillankorva S, Azeredo J., Front. Microbiol. 8. 1229, 2017a. https://doi.org/10.3389/fmicb.2017.01229

Pires DP, Melo LDR, Vilas Boas D, Sillankorva S, Azeredo J., Curr. Opin. Microbiol. 39, 48–56, 2017b. https://doi.org/10.1016/j.mib.2017.09.004

Poonacha N, Nair S, Desai S, Tuppad D, Hiremath D, Mohan T, Vipra A, Sharma U., Antimicrob. Agents Chemother 61, pii: e00457–17, 2017. https://doi.org/10.1128/AAC.00457-17

Power DA, Burton JP, Chilcott CN, Tagg JR, Dawes PJ., J. Clin. Microbiol. 43, 5822–5824, 2005.

Ramakrishnan VR, Feazel LM, Gitomer SA, Ir D, Robertson CE, Frank DN., PLoS One 8, e85507, 2013. https://doi.org/10.1371/journal.pone.0085507

Sadeghi S, Bakhshandeh H, Ahangari Cohan R, Peirovi A, Ehsani P, Norouzian D., Int. J. Nanomedicine 14, 9777–9792, 2019. https://doi.org/10.2147/IJN.S230269

Schuch R, Khan BK, Raz A, Rotolo JA, Wittekind M., Antimicrob. Agents Chemother. 61, pii: e02666–16, 2017. https://doi.org/10.1128/AAC.02666-16

Sharma S, Chatterjee S, Datta S, Prasad R, Dubey D, Prasad RK, Vairale MG., Folia Microbiol. 62, 17–55, 2017. https://doi.org/10.1007/s12223-016-0471-x

Shi JB, Fu QL, Zhang H, Cheng L, Wang YJ, Zhu DD, Lv W, Liu SX, Li PZ, Ou CQ, Xu G., Allergy 70, 533–539, 2015. https://doi.org/10.1111/all.12577

Shinde UA, Mehta AA, Goyal RK., Indian J. Exp. Biol. 38, 201–210, 2000.

Sillankorva S, Azeredo J., In Borysowski J, Międzybrodzki R, Górski A (Eds): Phage Therapy: Current Research and Application. Caister Academic Press, chapter 13, 2014.

Singhal D, Jekle A, Debabov D, Wang L, Khosrovi B, Anderson M, Foreman A, Wormald PJ., Int. Forum Allergy Rhinol. 2, 309–315, 2012. https://doi.org/10.1002/alr.21038

Sivasubramaniam R, Douglas R., World J. Otorhinolaryngol. Head Neck Surg. 4, 216–221, 2018. https://doi.org/10.1016/j.wjorl.2018.08.004

Stapleton AL, Shaffer AD, Morris A, Li K, Fitch A, Methe BA., Int. Forum Allergy Rhinol. 11, 31–39, 2021. https://doi.org/10.1002/alr.22597

Stephenson MF, Mfuna L, Dowd SE, Wolcott RD, Barbeau J, Poisson M, James G, Desrosiers M., J. Otolaryngol. Head Neck Surg. 39, 182–187, 2010.

Stone E, Campbell K, Grant I, McAuliffe O., Viruses 11, 567, 2019. https://doi.org/10.3390/v11060567

Szaleniec J, Gibała A, Pobiega M, Parasion S, Składzień J, Stręk P, Gosiewski T, Szaleniec M., Antibiotics (Basel) 8, pii: E175, 2019. https://doi.org/10.3390/antibiotics8040175

Szaleniec J, Górski A, Szaleniec M, Międzybrodzki R, Weber-Dąbrowska B, Stręk P, Składzień J., Future Microbiol. 12, 1427–1442, 2017. https://doi.org/10.2217/fmb-2017-0073

Tkhilaishvili T, Wang L, Perka C, Trampuz A. Gonzalez Moreno M., Front. Microbiol. 11, 695, 2020. https://doi.org/10.3389/fmicb.2020.00695

Vareille M, de Sablet T, Hindre T, Martin C, Gobert AP., Proc. Natl. Acad. Sci. USA 104, 10199–10204, 2007. https://doi.org/10.1073/pnas.0702589104

Weber-Dąbrowska B, Jończyk-Matysiak E, Żaczek M, Łobocka M, Łusiak-Szelachowska M, Górski A., Front. Microbiol. 7, 1177, 2016. https://doi.org/10.3389/fmicb.2016.01813

Weber-Dąbrowska B, Koźmińska J, Mulczyk M, Kaczkowski H., Post. Med. Klin. Dośw. 5, 291–293, 1996. [article in Polish]

Weber-Dąbrowska B., Mulczyk M., Górski A., Arch. Immunol. Ther. Exp. 48, 547–551, 2000.

Wettstadt S., Environ. Microbiol. 22, 2461–2462, 2020. https://doi.org/10.1111/1462-2920.15046

Yilmaz C, Colak M, Yilmaz BC, Ersoz G, Kutatelidze M, Gozlugol M., J. Bone Joint Surg. Am. 95, 117–125, 2013. https://doi.org/10.2106/JBJS.K.01135

Zhang G, Zhao Y, Paramasivan S, Richter K, Morales S, Wormald PJ, Vreugde S., Int. Forum Allergy Rhinol. 8, 406–414, 2018. https://doi.org/10.1002/alr.22046

Żaczek M, Łusiak-Szelachowska M, Jończyk-Matysiak E, Weber-Dąbrowska B, Międzybrodzki, R., Owczarek, B., Kopciuch A, Fortuna W, Rogóż P, Górski A., Front. Microbiol. 7, 1681, 2016. https://doi.org/10.3389/fmicb.2016.01681

Żaczek M, Łusiak-Szelachowska M, Weber-Dąbrowska B, Międzybrodzki R, Fortuna W, Rogóż P, Letkiewicz S, Górski A., In Górski A, Międzybrodzki R, Borysowski J (Eds): Phage Therapy: A Practical Approach. Cham, Switzerland: Springer Nature Switzerland AG, pp. 123–143, 2019. https://doi.org/10.1007/978-3-030-26736-0_5