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Rīga Stradiņš University

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Antimicrobial Activity of Bacteriophages in Multidrug-Resistant and Biofilm Associated Infections

Summary of the Doctoral Thesis for obtaining the scientific degree “Doctor of Science (*PhD*)”

Sector Group – Medical and Health Sciences
Sector – Clinical Medicine
Sub-Sector – Internal Medicine

Riga, 2023

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The Doctoral Thesis is available in RSU Library and on RSU website: https://www.rsu.lv/en/dissertations

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Abbreviations used in the Thesis

ADNP	Autosomal dominant polycystic kidney disease
AMC	Amoxicillin/clavulanate
AMK	Amikacin
AMP	Ampicillin
BPC	Biofilm prevention concentration
CAZ	Ceftazidime
CAZ-AVI	Ceftazidime/avibactam
CD	Diabetes mellitus
CIP	Ciprofloxacin
CL	Confluent lysis
CLI	Clindamycin
CMV	Cytomegalovirus
CST	Colistin
CTX	Cefotaxime
DN	Diabetic nephropathy
EDTA	Ethylenediaminetetraacetic acid
EOP	Efficiency of Plating
ERY	Erythromycin
ESBL	Extended-spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FEP	Cefepime
FOF	Fosfomycin
FOX	Cefoxitin
GEN	Gentamicin
GN	Glomerulonephritis
HIN	Chronic interstitial nephritis

HN	Hypertensive nephropathy
HNS	Chronic kidney disease
HOPS	Chronic obstructive pulmonary disease
HSM	Congestive heart failure
I	Susceptible, increased exposure
IMP	Imipenem
IP	Individual plaques
CFU	Colony-forming unit
LB	Luria-Bertani medium
LVAD	Left Ventricular Assist Device
MBEC	Minimum biofilm eradication concentration
MDR	Multidrug-resistant
MEM	Meropenem
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant S. aureus
MSSA	Methicillin-susceptible S. aureus
N/A	Not applicable
N/D	Not determined
NIT	Nitrofurantoin
NOR	Norfloxacin
OD	Optical density
ODc	Optical density cut-off value
ODs	Optical density of the bacterial isolate
PD	Peritoneal dialysis
PFU	Plaque-forming unit
PL	Partial lysis
PSCUH	Pauls Stradiņš Clinical University Hospital
QAMH	Queen Astrid Military Hospital

R	Resistant
RECUH	Riga East Clinical University hospital
RIF	Rifampicin
RR	Relative risk
RSU	Rīga Stradiņš University
S	Susceptible
SCL	Semi-confluent lysis
SD	Standard deviation
SXT	Trimethoprim/sulfamethoxazole
TET	Tetracycline
TIC	Ticarcillin
TIM	Ticarcillin/clavulanate
TOB	Tobramycin
TSA	Trypticase soy agar
TSB	Trypticase soy broth
TZP	Piperacillin-tazobactam
UTI	Urinary tract infection
VRE	Vancomycin-resistant Enterococcus faecium

Introduction

Multi-drug resistant infections are on the rise, while effective antimicrobial agents are becoming scarce (1). The European Centre for Disease Prevention and Control reported in 2022 that antimicrobial resistance is responsible for an average of 35 000 deaths in the European Union and the European Economic Area, and 1.27 million deaths worldwide, leading the European Commission to identify antimicrobial resistance as one of the three most important health problems in July 2022 (2). The European Union has an action plan to tackle microbial resistance, which has been revised several times and was last adopted on 1 June 2023, which includes the development of new antimicrobials and alternative methods to limit the spread of multi-drug resistant microorganisms (3). One of these methods is bacteriophage therapy, the use of which has been increasingly investigated in recent years, although the number of studies is still insufficient.

Bacteriophages were first described in 1915 by William Twort and in 1917 by Félix d'Hérelle. Soon after, they were used to treat infections, but with the discovery and widespread use of antibiotics, they were forgotten because their use is relatively complex and more complicated than that of antibiotics for effective therapy. Bacteriophages or phages are bacterial viruses, which, translated directly from Greek, mean "bacterial eaters". Phages have the unique ability to infect bacteria and, by forming new viral particles in the bacterium or host cell, to lyse the bacterium. This property makes bacteriophages a potential tool in the fight against pathogenic bacteria, especially those that have developed resistance to antimicrobial agents (4). Phages are mainly specific to host species and strains (5, 6), so it is important to identify the bacterial pathogen and select the appropriate bacteriophage for successful therapy. To date, there are no publicly available data on this type of studies in the Baltic region. Seeing the promise of such a personalised therapeutic approach against multidrug-resistant

bacterial strains and the infections they cause, regional phage centres are being established in some countries, e.g. Belgium, Poland and Germany. There is now renewed interest and acceptance of phage therapy in clinical practise.

Some of the most important clinically relevant microorganisms are *S. aurues*, *E. coli,* and *P. aeruginosa*. They are capable of causing community-acquired and nosocomial infections, urinary tract infections, endocarditis, peritonitis, wound, and other infections. *S. aureus* is the most frequent causative agent of peritoneal dialysis (PD) catheter exit-site infections and also causes PD-associated peritonitis in 6–14 % of cases (7-9). Peritonitis caused by *S. aureus* has a higher risk of recurrence, catheter replacement, and death (10-12), where various virulence factors of the bacterium play an important role, one of them being its ability to form biofilms (13, 14). In certain clinical situations, such as urinary tract infection (UTI), bacterial biofilms are involved in the development of disease in up to 60 % of cases (15). The most common causative agent for UTI is *E. coli* (16, 17), which has a broad spectrum of antimicrobial resistance and biofilm formation capacity. In biofilms formed by antibiotic susceptible bacteria, the concentration of antibiotics is often subinhibitory, this leads to an insufficient antibacterial effect and may encourage bacteria to form more pronounced biofilms (18, 19). However, in chronic wound infections, bacterial biofilms play a role in up to 78.2 % of cases (20). *P. aeruginosa* is a challenging agent of complicated wounds with a high likelihood of disease recurrence. This is due to the rapid and extensive development of antimicrobial resistance, multiple virulence factors, and a strong capacity for biofilm formation, which plays a crucial role in the development of chronic bacterial colonisation (21, 22). The treatment of such infections is complex and includes both antibacterial therapy and surgical treatment with debridement (23).

Bacteriophages can degrade the structure of the biofilm by producing substances that can break down the biofilm, such as polysaccharide depolymerases and lysins, after which phages can reach the deeper layers of the biofilm (24–26). These properties make phages potential antimicrobial agents that can be used to control multidrug-resistant and biofilm-associated infections. The available data on the effect of bacteriophages in the treatment of biofilm-associated infections are inconsistent, attributable to multiple variables such as different bacterial agents and different bacteriophages that have been used with or without antibiotics.

Currently, there are no comprehensive clinical trial data available on the effect of phages in clinical situations, but the existing data are reasonably convincing that phage therapy is safe. There is a lack of scientifically robust and systematic data on phage administration routes, doses, pharmacokinetics and pharmacodynamics, the development of resistance of phages, and interactions with antibiotics (27, 28). Phage therapy is frequently most used in combination with antibiotics to achieve the desired effect. However, the interaction between phages and antibiotics is not always predictable and can be synergistic, additive, and sometimes even antagonistic, mainly determined by the life cycle of the phage, the mechanism of action of the antibiotic, and the duration and sequence of use of the two agents (4, 29). Comprehensive and systemic studies are needed to define these interactions precisely, as phage therapy uses a wide variety of viruses that differ from each other.

No less important is the process of developing resistance to bacteriophages and its role in bacteriophage therapy. The development of phage-resistant bacterial clones is known to occur even during treatment, which is why phage therapy usually involves the use of several phages in a cocktail and the addition of antibiotics (30, 31). The genetic variability of the bacterium during phage therapy is important and several studies have shown that, in combination

with phage resistance, changes in the expression of bacterial virulence factors and the disappearance of existing antibiotic resistance mechanisms can occur, contributing to the cure of the infection (32, 33).

These considerations make bacteriophage therapy a promising option for the treatment of multidrug-resistant and biofilm-associated infections, but in the absence of data, mainly from clinical trials, the use of phages remains an experimental therapy. Research is needed on several aspects of the use of bacteriophages, especially in clinical applications. Given the diversity of phages and their possible different combinations with antibiotics, it is important to evaluate the effects of phages in biofilms with and without antibiotics *in vitro*. In addition to efficacy, bacterial variability, such as the development of bacterial resistance to phages and the variability of bacterial antimicrobial resistance due to phage treatment, should be evaluated.

Aim of the Thesis

To evaluate the antimicrobial effect of bacteriophages and their interaction with antibiotics *in vitro* of selected multidrug-resistant and biofilm-forming bacteria and in the treatment of life-threatening infections.

Tasks of the Thesis

1. To assess the frequency of S. aureus colonisation in peritoneal dialysis patients, their biofilm-forming capacity and bacteriophage efficiency, and to analyse the impact of colonisation on PD outcomes.
2. To determine and evaluate the antibacterial and biofilm eradication effects of bacteriophages and their combinations with antibiotics in cultures of uropathogenic *E. coli*.

3. To assess bacteriophage resistance and its frequency in isolated bacteria, and to perform bacteriophage adaptation to overcome resistance.
4. To evaluate the effect of the phage cocktail BFC 1.10 in combination with ceftazidime-avibactam in the treatment of multidrug-resistant *P. aeruginosa* osteomyelitis and under laboratory conditions.
5. To evaluate the lytic effect of bacteriophages PNM and PT07 and their combination with antibiotics in the treatment of multidrug-resistant *P. aeruginosa* LVAD infection and in laboratory models.

Hypotheses of the Thesis

Bacteriophages and their combination with antibiotics have the ability to eradicate biofilms of multidrug-resistant bacteria.

Novelty of the Thesis

The research work includes an in-depth evaluation of the therapeutic effect of bacteriophages in the treatment of biofilm-associated as well as multidrug-resistant infections. The study evaluates various factors such as phage resistance, bacterial capacity to form biofilms, phage interaction with antibiotics, which determine the differences in the lytic effect of phages in planktonic cells and bacterial biofilms.

The lytic activity of bacteriophages and their combination with antibiotics in antibiotic-susceptible and multidrug-resistant bacterial cultures was evaluated, and the biofilm eradication effect was assessed using a dynamic biofilm model. The interaction of bacteriophages with antibiotics was determined and the optimal order of administration was assessed.

The emergence of bacteriophage resistance and its frequency in biofilm inhibition models were assessed. Adaptation of bacteriophages was performed to overcome bacteriophage resistance.

This is one of the few studies using phages in patients in difficult clinical situations and is the first study worldwide (to the author's knowledge) to consider their application in peritoneal dialysis patients. To date, there are no publicly available data in Latvia on the use of bacteriophage therapy and the evaluation of its effect in biofilm-associated multidrug-resistant infections.

Personal contribution

The author planned, organised and participated in all stages of the scientific and clinical work. He collected biological material (bacterial cultures), collected patient data and developed the treatment plan. He has carried out bacterial characterisation, antimicrobial susceptibility, bacteriophage susceptibility, bacteriophage adaptation and evaluation of bacteriophage effect in biofilm models. The author has introduced and modified the methods used in the work, which are necessary for the study of phages and biofilms. He has compiled, processed and analysed the data, including statistical methods. Prepared scientific publications and wrote this thesis.

Ethical aspects

The study was conducted in accordance with the ethical aspects of the Helsinki Declaration. The study protocols were agreed and approved by the Research Ethics Committee of Riga Stradiņš University, document No 32/28.01.2016 and No 8/08.09.2016. Treatment of patients with bacteriophages was performed in accordance with paragraph 37 of the Declaration of Helsinki and written informed consent was obtained prior to patient involvement.

1 Material and methods

1.1 Time, place and design of the study

The dissertation research was carried out in the Department of Biology and Microbiology at RSU, where the work with isolated bacterial cultures and bacteriophages was carried out. The study material was obtained from patients in collaboration with the Pauls Stradins Clinical University Hospital (PSCUH). An experimental part of the study was conducted at Riga East Clinical University Hospital (RECUH) and PSCUH, treating patients with bacteriophages. The study activities took place from September 2016 to December 2022. Analysis of patient clinical and demographic data was performed for all patients from whom bacterial cultures were obtained and used, or who underwent bacterial carriage screening. The study design and the methods used in each study are shown in Figure 1.1. The dissertation study is designed as a set of four consecutive publications.

The study consisted of three sections:

1. Determination of biofilm formation and evaluation of bacteriophage effect in *S. aureus* isolates from PD patients.
2. Evaluation of bacteriophage efficacy in biofilm-forming uropathogenic *E. coli* isolates.
3. Implementation and evaluation of experimental phage therapy in patients with multidrug-resistant *P. aeruginosa* infections. Two patients with multidrug-resistant *P. aeruginosa* infection were included, one with femoral osteomyelitis and the other with LVAD cable infection.

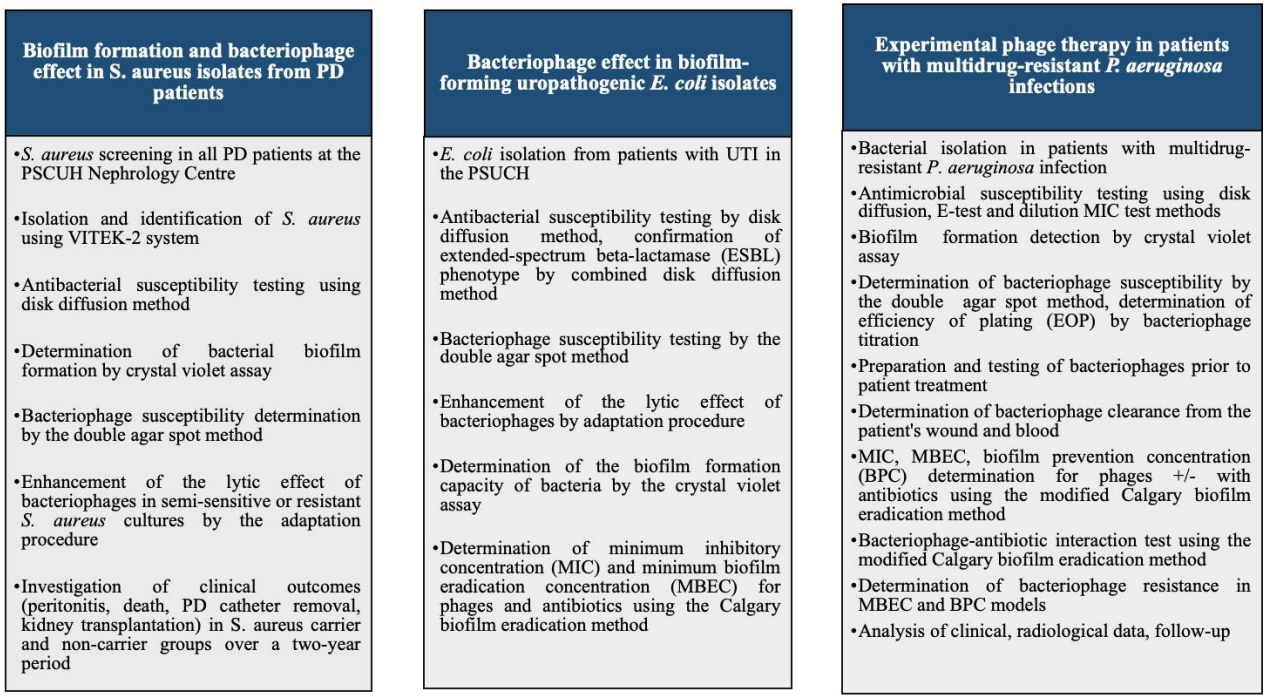


Figure 1.1 **Study design with 3 sections, main methods used in each section**

1.2 Materials

1.2.1 Bacterial cultures

The study used cultures of *S. aureus*, *E. coli* and *P. aeruginosa* isolated from patients. *E. coli* and *P. aeruginosa* cultures were obtained from the PSUCH or RACUH laboratories. The following reference cultures were used to assess and compare the biofilm-forming capacity of the bacteria in the patients and to perform bacteriophage propagation*: S. aureus* ATCC 4336 and ATCC 15923, *E. coli* ATCC 29522, *P. aeruginosa* ATCC 14209, ATCC 27853 and CN573.

1.2.2 Bacteriophages

Commercially available lytic bacteriophage preparations with known composition and spectrum of activity were used in the *S. aureus* and *E. coli* experiments. Six bacteriophage preparations were obtained from Eliava BioPreparations, Tblisi, Georgia: Staphylococcus bacteriophage, Pyo, Ses, Fersisi, Enko and Intesti bacteriophage. As well as bacteriophage Pyobacteriophage from Microgen, Perm, Russia.

For the treatment of patients and further studies with *P. aeruginosa*, phages were obtained from the Queen Astrid Military Hospital in Brussels, Belgium. These phages are produced according to safety and quality standards for bacteriophage therapy (34, 35). These phages have previously been used to treat patients (36–38) and are subject to independent quality assessment by the Belgian Health Institute Sciensano prior to use.

In a patient with osteomyelitis, the lytic bacteriophage BFC 1.10 was used. In a patient with LVAD cord infection, the Podoviridae bacteriophage PNM and the Myoviridae bacteriophage PT07 were used.

1.3 Methods

1.3.1 Microbiological investigation of *S. aureus*

Patient swabs were taken using the AMIES universal transport medium. They were transported to the laboratory within 2 h and cultured using selective media. Bacterial identification was performed using the VITEK-2 system (bioMerieux, France).

1.3.2 Antimicrobial susceptibility detection

Antimicrobial susceptibility was determined for all bacterial cultures using the disk diffusion method according to the current standard of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

1.3.3 Determination of bacteriophage titre (concentration)

The method of plaque (negative bacterial colonies) assay was used to determine the concentration or titre of bacteriophages. Several 10-fold dilutions of bacteriophage preparations were made, 50 µl of each dilution was mixed with 100 µl of bacterial suspension in semi-liquid TSA poured onto TSA Petri plates. The plates were incubated for 16–18 h. The following day, each plate was assessed and the concentration of bacteriophages was determined. In the experimental part of the study, the bacteriophage titre was determined in a blood sample from the patient. In this case, the initial sample was 4–5 ml of the patient's blood taken using an Ethylenediaminetetraacetic acid (EDTA) blood tube.

1.3.4 Efficiency of plating (EOP) detection

Initially, the concentration of phages in the host culture or reference culture and the concentration of phages in the patient culture are determined. It is assumed that the host or reference culture has a higher phage efficiency. The results obtained are compared with each other using Formula 1.1. The higher efficiency is observed when the EOP number for a given bacterial isolate is closer to 1.

$$EOP = F_{CT} / F_{CR} \quad (1.1)$$

EOP – efficiency of phage plating in the patient isolate

F_{CT} – phage concentration in patient isolate

F_{CR} – phage concentration in the host bacterium or reference strain

1.3.5 Double-layer agar spot assay for or the detection of the lytic effect of bacteriophages

The double-layer agar spot test was used to detect the lytic effect of bacteriophages. 100 µl of bacterial suspension was mixed with 4–5 ml of melted 0,7 % TSA and gently mixed in a 15 ml tube, which was then poured as a top layer onto a TSA Petri dish. Drops of 10 µl of the bacteriophage preparation are then applied to the plate with a micropipette and incubated at 35 °C for 16–18 h. The phage results are scored and read visually. Lysis zones of *E. coli* and S. aureus are scored as complete lysis (CL or +++), partial lysis (SCL or ++), weak lysis or individual plaques (PL/IP or +), absence of lysis zone (R or –). P. aeruginosa is scored as full lysis (CL or ++++), partial lysis (SCL or +++), weak lysis (PL or ++), individual plaques (IP or +), absence of lysis zone (R or –).

1.3.6 Bacteriophage propagation using host or reference culture

S. aureus ATCC 4336, *E. coli* ATCC 29522 and *P. aeruginosa* ATCC 27853 and CN 573 strains were used for propagation. The propagation process is carried out using phage titration plates, which show a large number of plaques, visually identified as a sieve-like structure in the bacterial lawn. The upper part of the medium is collected by pouring TSB on it, followed by cell lysis with chloroform and centrifugation, after which the sample is filtered through a 0.2 μm filter (Filtropur S, Sarstedt, Germany). If the phage concentration was insufficient, the resulting filtrate was filtered again and concentrated by centrifugation at 4000 × g for 20 min at 4 °C in an Amicon® Ultra-15 filter (Merck Millipore, Ireland).

1.3.7 Bacteriophage adaptation

A modified Appelmans method was used (39). The adaptation procedure consisted of several cycles of repeated steps. For each cycle, several 10-fold dilutions of bacteriophage preparations were made, bacteria were added and incubated for 48 h at 37 °C. After the incubation period, the optical density of each tube was measured. The tube with the highest dilution, having an optical density equivalent to that of the negative control, is used for further adaptation. The bacteria are lysed with chloroform in the tube and the sample is centrifuged and then filtered. The resulting preparation is re-diluted and incubated as described above, thus performing subsequent cycles of sample adaptation. The phage is adapted for at least 3 cycles before being tested for lytic effect.

1.3.8 Detection of bacterial biofilm formation capacity using the crystal violet assay

The biofilm formation capacity of the bacteria was determined using 96-well microplates. Initially, a pure culture of bacteria was isolated, then 3–5 morphologically similar bacterial colonies were cultured in liquid medium for 16–18 hours. After cultivation, a bacterial suspension was made in liquid broth medium at a dilution of 1:100 to obtain a final concentration of $1–3 \times 10^7$ CFU/ml. Using a multichannel pipette, the cultures were transferred to a 96-well plate by adding 200 µl of the previously prepared suspension to each well. A minimum of 8 wells per bacterium were used. A negative control, sterile appropriate broth medium (minimum 8 wells), was included in each microplate. For biofilm production, the microplates were incubated at 37 °C (*E. coli* and *S. aureus*) and 35 °C (*P. aeruginosa*) for 2 to 48 hours according to the selected purpose. After the incubation period, the planktonic cells that had grown in the microplates were discarded and the wells of the microplates were rinsed with a multichannel pipette 2 times with 250 µl saline. After rinsing, the biofilm was stained with 200 µl of 0.1 % crystal violet solution for 15–20 minutes. Then the microplate rinsing was repeated by filling each well with 250 µl distilled water 3 times. A 96 % ethanol solution was used as a biofilm decolouriser and was topped up (200 µl). To evaluate the result, the optical density of the stained biofilms was measured using a TECAN INFINITE F50 optical densitometer at the appropriate wavelength for the bacterium. An OD value greater than 0.1 was used as the bacterial growth threshold.

1.3.9 Interpretation of the biofilm optical density results

Biofilm production was assessed quantitatively as well as to varying degrees compared with negative controls as defined by *Stepanovic et al* (40). The biofilm production capacity of the bacteria was evaluated by averaging the

calculated optical density values (ODs) of the bacterial isolate. ODc is the optical density cut-off value calculated as the mean of all measurements of the negative control in one microplate + 3 standard deviations (SD) of the negative control.

Table 1.1

Biofilm production degrees

Average bacterial OD value	Biofilm production degree
ODs ≤ ODc	None
ODc < ODs ≤ 2 × ODc	Weak
2 × ODc < ODs ≤ 4 × ODc	Moderate
4 × ODc < ODs	Strong

OD, optical density; ODc, optical density cut-off value; ODs, optical density of the bacterial isolate.

1.3.10 Determination of bacterial growth inhibition, minimum inhibitory concentration (MIC), minimum biofilm eradication concentration (MBEC) and biofilm preventive concentration (BPC) in biofilm models

A modified Calgary method was used to assess the effects of bacteriophages and antibiotics in bacterial biofilms (41–43). Bacterial cultures were incubated for 16–18 h in TSA plates, colonies were mixed in liquid broth and dilutions were prepared to achieve an inoculum concentration of 1.0 × 107 CFU/ml. The prepared bacterial inoculum was added to a sterile 96-well flat-bottom microplate (Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate, Thermo Fisher Scientific, Roskilde, Denmark) at 150 µl per well. The 96-well microplate was then closed with a 96-well lid with pegs (Nunc™ Immuno TSP Lids) and incubated for 24 h in a rotating table incubator (InforsTM HT Ecotron, Basel, Switzerland) at 35 °C, 150 rpm.

After biofilm formation, the microplate lid with pegs was transferred to a new 96-well microplate containing 200 µl of broth with the desired antibiotic, phage or combination of antibiotics in each well. After the selected incubation

period, the optical density of the microplate wells was measured. The value obtained represented the MIC for antibiotics and the bacterial growth inhibition for phages and antibiotic-phage combinations, also denoted as MIC in the results for comparison purposes. To increase the phage exposure in *P. aeurginosa*, the lid with pegs was transferred to a new 96-well microplate with the same phages and/or antibiotics and their concentrations for a further 12 h incubation. The pegged-lid was then placed in a fresh 96-well microplate containing 200 µl of sterile broth per well. To remove the biofilm from the pegs, the microplates with the lid were placed in a sonicator for 25–30 min at 44 Hz using an ultrasonic bath (model 08855-02, Cole-Parmer, Vernon Hills, IL, USA). For the growth of surviving bacteria, the microplate was covered with a sterile lid without pegs and incubated for 22 h stationary at 35 °C. The minimum biofilm eradication concentration (MBEC) was then determined by measuring the optical density of the microplate. The biofilm prevention concentration (BPC) was determined by simultaneous inoculation of bacteria, phages and antibiotics at their respective concentrations immediately placed in the microplate with pegged-lid.

1.3.11 Detection of susceptibility changes and resistance of bacteria to bacteriophages in biofilm models

To investigate changes in phage susceptibility, bacterial cultures from biofilm models in 96-well microplates were grown on solid media. Twenty-two bacterial cultures were randomly taken using the patient isolate P. aeruginosa PAP01. The cultured bacteria were tested for bacteriophage susceptibility using the double-layer agar spot assay (see 1.3.5).

The cut-off value for phage resistance was calculated using the positive control (bacteria only) according to Formula 1.2.

$$OD_R = OD_{PC} - (3 \times SD_{PC}) \quad (1.2.)$$

OD_R – calculated threshold value defining the presence of bacteriophage resistance

OD_{PC} – mean value of the optical density of the positive control

SD_{PC} – calculated standard deviation of positive control

A bacterial clone (OD_{well}) was considered resistant to a bacteriophage if its measured optical density was greater than or equal to the calculated resistance threshold (OD_R). Formula 1.3 was used to reflect the relationship between the measured mean optical density of the isolate clone in the microplate well (OD_{well}) compared to the calculated optical density threshold (OD_R) for resistance.

$$OD_{ratio} = OD_{well} / OD_R \quad (1.3.)$$

OD_{well} – the optical density value of the clone of the cultivated bacteria

OD_R – calculated threshold value defining the presence of bacteriophage resistance.

If the value was less than 1, the bacteria were not considered resistant, but if the value was greater than or equal to 1, the bacteria were considered resistant to the phage.

1.3.12 Statistical analysis of data

Statistical analysis of the data was performed using Microsoft Excel 2016, IBM SPSS Statistics version 27, and Graph Pad Prsim version 9.

For all quantitative measurements, the mean was calculated and presented, and the standard deviation (SD) was calculated with a 95 % dispersion range of the data. The normality of the continuous data was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests. The t-test was used for parametric data analysis comparing two samples, and the one-way ANOVA test for comparing three or more samples. The Mann-Whitney U test was used for non-parametric data analysis comparing two samples, the Kruskal-Wallis test for three or more samples. If the null hypothesis was rejected and significant differences between groups were found, multiple pairwise comparisons were made using post-hoc analysis with the Tukey test. Results were considered statistically significant if the p value was less than 0.05.

To calculate the risk of clinical outcomes in peritoneal dialysis patients, the relative risk (RR) was calculated with a confidence interval of 95 %.

2 Results

2.1 Bacteriophage effect in *S. aureus* isolated from peritoneal dialysis patients

Part of this research is described and published in the publication by Kārlis Rācenis, Juta Kroiča, Dace Rezevska, Lauris Avotiņš, Edgars Šķudītis, Anna Popova, Ilze Puide, Viktorija Kuzema and Aivars Pētersons *S. aureus Colonization, Biofilm Production, and Phage Susceptibility in Peritoneal Dialysis Patients*. Antibiotics (Basel). 2020 Sep 7;9(9):582. doi: 10.3390/antibiotics9090582.

2.1.1 Study patient characteristics

Seventy-one PD patients were screened for *S. aureus* carriage, but seventy patients were included in the study, as one patient with PD catheter had not initiated dialysis; 51 % (n = 36) were male and 49 % (n = 34) were female, with an average age 59.96 years (SD 15.9).

Causes for end-stage renal disease vary in the study group: glomerulonephritis (40 %, n = 28), diabetic nephropathy (14.3 %, n = 10), chronic interstitial nephritis (20 %, n = 14), ADPKD (10 %, n = 7), hypertensive nephropathy (12.9 %, n = 9); and unknown (2.9 %, n = 2).

S. aureus carriers were 30 % (n = 21). 71.4 % of carriers were male (n = 15) and 28.6 % female (n = 6). No statistically significant correlation was noted between S. aureus carriage and comorbidities such as diabetes mellitus (p = 0.05), chronic heart failure, viral hepatitis, gout, and chronic obstructive pulmonary disease. Of the carrier group patients, 28.6 % had diabetes mellitus (n = 6), compared to only 10.2 % in the noncarrier group (n = 5). Prior to and during the study, none of the patients used topical antimicrobial for S. aureus

decolonization. The mean length of patient participation in the study in the noncarrier group was 16.61 months, and 13.95 months in the carrier group.

2.1.2 Clinical outcomes in *S. aureus* carrier and noncarrier groups

In total 32 cases of peritonitis during the study (78.1 %, n = 25 in noncarrier group; 21.9 %, n = 7 in carrier group) were noted. Causative agents in the carrier group were mixed culture of (MSSA)/*Pseudomonas spp*. (14.3 %, n = 1), *Streptococcus spp.* (42.9 %, n = 3), and culture-negative (42.9 %, n = 3). In the noncarrier group, causative agents were *Streptococcus spp.* (36 %, n = 9), culture-negative (28 %, n = 7), MSSA (8 %, n = 2), *Aerococcus spp.* together with *Pseudomonas spp., Bacillus spp., Candida spp., Enterococcus spp., Aeromonas spp.*, methicillin-sensitive coagulase-negative *Staphylococcus* and methicillin-resistant coagulase-negative *Staphylococcus*, each 4 %, n = 1.

The overall incidence of peritonitis was 0.35 episodes per patient year. In the noncarrier group, there were 0.37 episodes per patient year compared to 0.29 per patient year in carrier group.

Death as an outcome was detected in 31.1 % (n = 8) of carriers and 16.3 % (n = 8) in the noncarrier group. Data showed the statistical tendency that risk of death in the carrier group was 2.33 times greater than that in the noncarrier group, the clinical outcomes are shown in Table 2.1.

Table 2.1

Two-year clinical outcome of *S. aureus* carriers and noncarriers

Clinical Outcomes	*S. aureus* Carriers	*S. aureus* Noncarriers	Total	RR	CI 95 %
Number of patients	30.0 % (n = 21)	70.0 % (n = 49)	100 % (n = 70)	–	–
Death	31.1 % (n = 8)	16.3 % (n = 8)	22.9 % (n = 16)	2.33	1.01–5.38
Transplantation	28.6 % (n = 6)	18.4 % (n = 9)	21.4 % (n = 15)	1.56	0.63–3.81
Removal of PD catheter	0 % (n = 0)	20.4 % (n = 10)	14.3 % (n = 10)	–	–
Peritonitis	19.1 % (n = 4)	34.7 % (n = 17)	30.0 % (n = 21)	0.55	0.21–1.44

2.1.3 Isolated *S. aureus* antimicrobial susceptibility and biofilm production

In total, 34 S. aureus strains were obtained from 213 patient samples, all sensitive to commonly used antibiotics (cefoxitin, ciprofloxacin, trimethoprim/sulfamethoxazole, clindamycin, gentamycin, tetracycline, rifampicin), two strains were resistant to erythromycin. None of the isolated strains were methicillin resistant. Most commonly *S. aureus* colonization was detected in nose (53 %, n 18), see Table 2.2.

Biofilm production was observed among all isolated strains. Most commonly, strains produced strong biofilm (21, 61.8 %), moderate (10, 29.4 %), and weak (3, 8.8 %) biofilm. Strong biofilm production of *S. aureus* isolates was detected in 15 out of 21 individual who did carry *S. aureus* in at least one of isolation sites (see Table 2.2). The biofilm production capacity of *S. aureus* isolates significantly varied ($p <$ in 8 out of 10 patients when strains were compared among different patient *S. aureus* isolation sites (see Figure 2.1).

Table 2.2

Isolation sites of *S. aureus* and their biofilm production degree

		Carriage site, code, and biofilm production degree of isolate		
		Nose	**Groin**	**PDC**
		18 (53 %)	**11 (32 %)**	**5 (15 %)**
Biofilm Production degree, n	Weak	2	1	–
	Moderate	6	2	2
	Strong	10	8	3

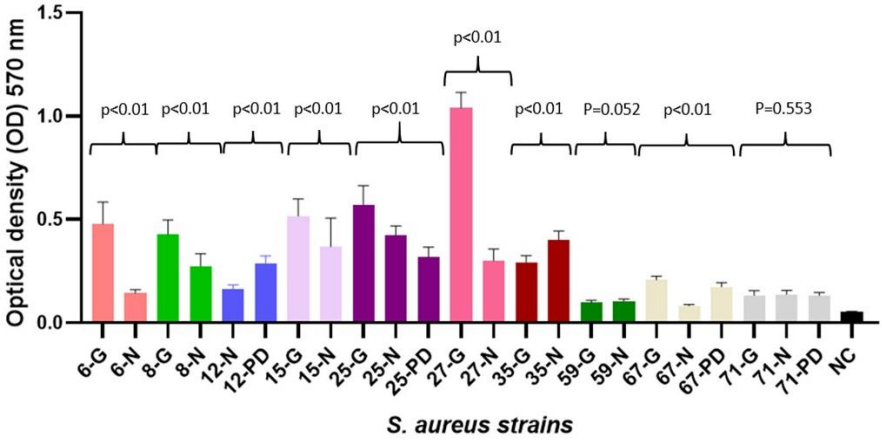


Figure 2.1 **Biofilm production capability on microtitre plates of the 23 clinical isolates of *S. aureus****

* Bars represent mean values of OD (measured at wavelength of 570 nm), NC represents negative control or broth only

2.1.4 *S. aureus* bacteriophage susceptibility and phage adaptation

From all seven commercial bacteriophage cocktails, the Staphylococcal Bacteriophage (Eliava) had an original titre of 10^4 PFU/mL, while Pyo, Enko, Intesti Bacteriophages (Eliava) and Pyobacteriophage (Microgen) had 10^5 PFU/mL on *S. aureus* ATCC 4336. Only the Ses and Fersisi Bacteriophages

(Eliava) demonstrated a titre of 10^6 PFU/mL on *S. aureus* ATCC 4336. After bacteriophage cocktail propagation on *S. aureus* ATCC 4336, of seven phage stocks all but one presented a titre of 10^9 PFU/mL, namely Pyo, Ses, Fersisi, Intesti and Pyobacteriophage. Conversely, an estimated titre of Staphylococcal Bacteriophage was 10^7 PFU/mL. All phage titres were equalised to 10^7 PFU/mL before phage lytic activity testing against *S. aureus* strains.

The evaluation results of bacteriophage lysate lytic activity obtained in the spot assay are shown in Table 3.3. When tested against all 34 *S. aureus* isolates, 6 bacteriophage stocks except Staphylococcal Bacteriophage (Eliava) revealed positive lytic results in all cases. Bacterial resistance to bacteriophage represented in the spot test was determined in 9 (26 %) out of 34 *Staphylococcus aureus* isolates to Staphylococcal bacteriophage (Eliava), see Table 2.3.

Table 2.3

Bacteriophage lytic spectrum in *S. aureus* isolates

Phage lytic activity	Staphylococcal phage (Eliava)		Pyo, n (%)	Ses, n (%)	Fersisi, n (%)	Enko, n (%)	Intesti, n (%)	Pyobacteriophage, n (%)
	Not adapted, n (%)	Adapted, n (%)						
CL (+++)	4 (12)	0 (0)	2 (6)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)
SCL (++)	19 (56)	11 (100)	31 (91)	32 (94)	33 (97)	32 (94)	34 (100)	31 (91)
IP or PL (+)	2 (6)	0 (0)	1 (3)	2 (6)	1 (3)	1 (3)	0 (0)	3 (9)
R (−)	9 (26)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Staphylococcal Bacteriophage (Eliava) against 11 chosen bacterial strains (9 with resistance and 2 with individual plaques) were taken for phage adaptation. In all cases, the adaptation procedure resulted in overcoming bacterial resistance; all 11 bacterial strains (100 %) showed a positive lytic effect after adaptation, see Table 2.3.

2.2 Bacteriophage effect in uropathogenic *E. coli* planktonic cells and biofilms

Part of this research is described and published in the publication by Laima Mukāne, Kārlis Rācenis, Dace Rezevska, Aivars Pētersons and Juta Kroiča *Anti-Biofilm Effect of Bacteriophages and Antibiotics against Uropathogenic Escherichia coli.* Antibiotics (Basel). 2022 Nov 26;11(12):1706. doi: 10.3390/antibiotics11121706.

2.2.1 Biofilm formation of *E. coli*

Five strains showed significantly different biofilm formation capabilities when compared to negative control ($p < 0.001$). Two isolates 021UR being moderate biofilm producer and 01206UR being strong biofilm producer with the highest optical density values, these isolates were selected for further MIC and MBEC testing. Other isolates (01032UR, 01081UR, 01168UR) were weak biofilm producers expect for 01108UR that did not produce biofilm.

2.2.2 Antibacterial and phage susceptibility of *E. coli*

Bacterial isolates showed different antimicrobial susceptibilities, see Table 2.4. From the six bacterial isolates, 01081UR and 01108UR were susceptible to all tested antimicrobials, 01032UR showed the broadest antimicrobial resistance being resistant to six antimicrobials and being ESBL

producer, 021UR and 01206UR were resistant to three antimicrobials, 01168UR was resistant to two antimicrobials.

The original titre of the bacteriophage cocktail Ses was 4×10^5 PFU/mL, Intesti 7×10^5 PFU/mL and Pyobacteriophage 3×10^6 PFU/mL using *E. coli* ATCC 29522 reference strain. After propagation of the phage cocktail on the same reference strain, the titre increased to 4×10^6, 1×10^8, and 4×10^6 PFU/mL for Ses, Intesti, and Pyobacteriophage, respectively. Data on bacterial strains' susceptibility to phages are summarised in Table 2.4.

E. coli isolate 021UR was more sensitive to all phage cocktails having semi-confluent lysis in case of Ses and Pyobacteriophage and confluent lysis when Intesti phage cocktail was applied. The least sensitivity of phage cocktails was observed for 01108UR and 01206UR. Isolate 01108UR showed resistance to Intesti and Pyobacteriophage, partial lysis to Ses. The isolate 01206UR showed resistance to Ses and Intesti phage cocktails and partial lysis to Pyobacteriophage. Phage adaptation was performed to improve the efficiency of the Pyobacteriophage cocktail with 01206UR, as this strain was chosen for further phage and biofilm interaction testing. After adaptation Pyobacteriophage titre increased to 7×10^7 PFU/mL. Titre of phage was equalised to 106 PFU/mL to perform spot assay for phage susceptibility testing. Improvement of Pyobacteriophage effect in isolate 01206 from individual plaques (+) to confluent lysis (+++) was assessed after adaptation, see Table 2.4 .

Table 2.4

Results of antimicrobial and phage susceptibility testing of *E. coli*.

Antibiotic, phage	**021UR**	**01032UR**	**01081UR**	**01108UR**	**01168UR**	**01206UR**
AMP	R	R	S	S	R	R
AMC	R	R	S	S	S	S
TZP	S	I	S	S	S	S
CTX	S	R	S	S	S	S

Table 2.4 continued

Antibiotic, phage	021UR	01032UR	01081UR	01108UR	01168UR	01206UR
CAZ	S	R	S	S	S	S
IMP	S	S	S	S	S	S
MEM	S	S	S	S	S	S
CIP	S	R	S	S	S	R
NOR	S	N/A	N/A	N/A	S	R
GEN	S	R	S	S	S	S
SXT	R	S	S	S	R	S
NIT	S	S	S	S	S	S
ESBL	R	+	–	–	–	–
Ses	++	+	++	+	++	–
Intesti	+++	+	++	–	+	–
Pyobacterio-phage	++	+	+	–	+	+
Pyobacterio-phage*	N/A	N/A	N/A	N/A	N/A	+++

S – sensitive; R – resistant, I –intermediate; +++ CL; ++ SCL; + IP or PL; Ampicillin (AMP); Amoxicillin-clavulanic acid (AMC); Piperacillin-tazobactam (TZP); Cefotaxime (CTX); Ceftazidime (CAZ); Imipenem (IMP); Meropenem (MEM); Ciprofloxacin (CIP); Norfloxacin (NOR); Gentamicin (GEN); Sulfamethoxazole – trimethoprim (SXT); Nitrofurantoin (NIT); extended spectrum beta-lactamase (ESBL); * Pyobacteriophage phage cocktail after adaptation; not applicable (N/A)

2.2.4 The effect of ciprofloxacin and amoxicillin-clavulanic acid in planktonic cells and biofilms

According to EUCAST standard, the MIC values of amoxicillin-clavulanic acid and ciprofloxacin are 8 mg/L and 0.25 mg/L, respectively. Isolate 021UR was resistant to amoxicillin-clavulanic acid, and the MIC value was reached only at a concentration of 256 mg/L, but MBEC was not reached even at the highest concentration (1024 mg/L). Ciprofloxacin MIC value was achieved at 0.25 mg/L but showed resistance in the biofilm: MBEC was 64 mg/L. Although strain 01206UR was susceptible to amoxicillin-clavulanic acid and the MIC value was reached at 8mg/mL, this antibiotic failed to eradicate the bacterial biofilm at concentration of 1024 mg/L. Isolate 01206UR was also resistant to

ciprofloxacin: MIC value was 128 mg/L, but MBEC was not reached by 1024 mg/L. The strain 01206UR, determined to be resistant to ciprofloxacin, showed a high MIC value at 128 mg/L and did not destroy the biofilm even at 1024 mg/L. This shows that ciprofloxacin was also unable to kill the bacteria in the biofilm. Regardless of the previously determined (disk diffusion test, results shown in Table 2.4) antimicrobial susceptibility, both antibiotics were not able to kill *E. coli* in the biofilm, whereas the MIC values obtained were in agreement with the antimicrobial susceptibility data obtained by the disk diffusion method.

2.2.5 The effect of bacteriophages in planktonic cells and biofilms in *E. coli*

The mean values of the MIC and MBEC tests of the phages are represented in Figures 2.2, 2.3. We conclude that bacteria was sensitive to phage if the OD values were < 0.1 measured at 650 nm or if the result was close to the negative control. By using phages, the growth of bacterial isolate 021UR was reduced in all cases, including planktonic cells (MIC) and biofilms (MBEC), compared to the positive control, but not in all cases the effect is adequate to declare that the strain is completely lysed by phage cocktail and the growth of the bacteria is not observed. In all cases, a statistically significant bacteriophage lytic effect ($p < 0.01$) was observed, regardless of the bacteriophage concentration used. The lytic effect was not sufficient to conclude that the growth of cells in the biofilm was completely inhibited by Ses and Intesti phages. Complete killing of *E. coli* cells was observed with Pyobacteriophage at all concentrations. The other isolate, 01206UR, showed resistance to all three phage cocktails (Pyobacteriophage, Ses, and Intesti). Resistance to Ses and Intesti phage stocks was already determined in the phage susceptibility test. Still, for Pyobacteriophage, we observed low lytic effect, so the adaptation of the Pyobacteriophage was performed. After that, efficiency improved: bacterial

growth was markedly reduced in both planktonic (MIC) and biofilm-forming cells (MBEC), $p < 0.01$. However, the OD values were slightly above 0.1 for the MIC which is to be expected as the MIC value is used in the standard for substances rather than living beings. The results showed that after adaptation the phage could efficiently eradicate the biofilm, see Figure 2.2.

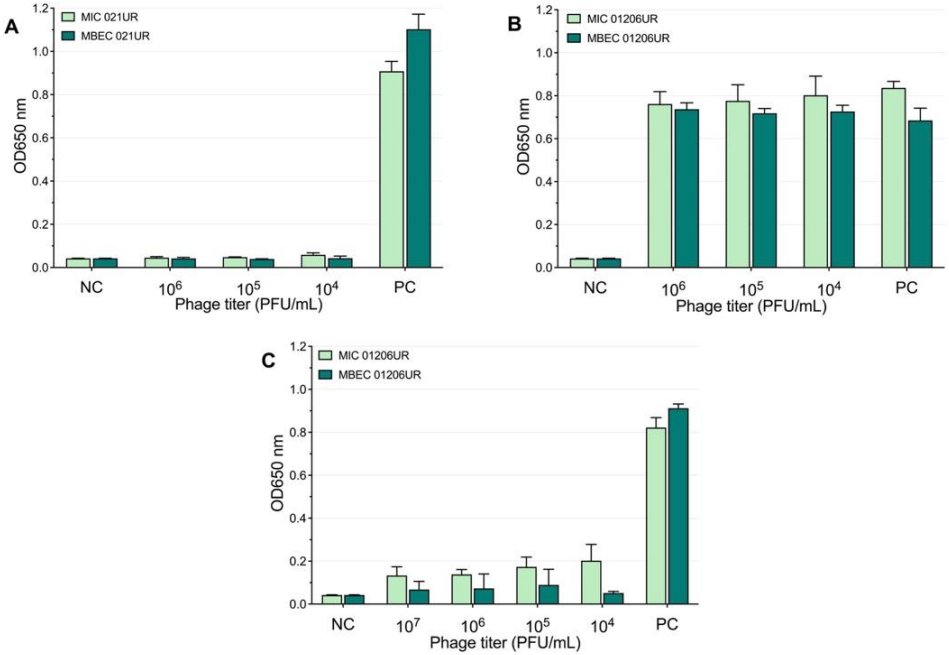


Figure 2.2 **Pyobacteriophage MIC and MBEC values for 021UR (A), 01206UR before adaptation (B) and after adaptation(C) ***

* Bars represent mean values of bacterial OD (measured at wavelength of 570 nm) using different concentrations of Pyobacteriophage, NC represents negative control or broth only, PC represents positive control or bacteria only.

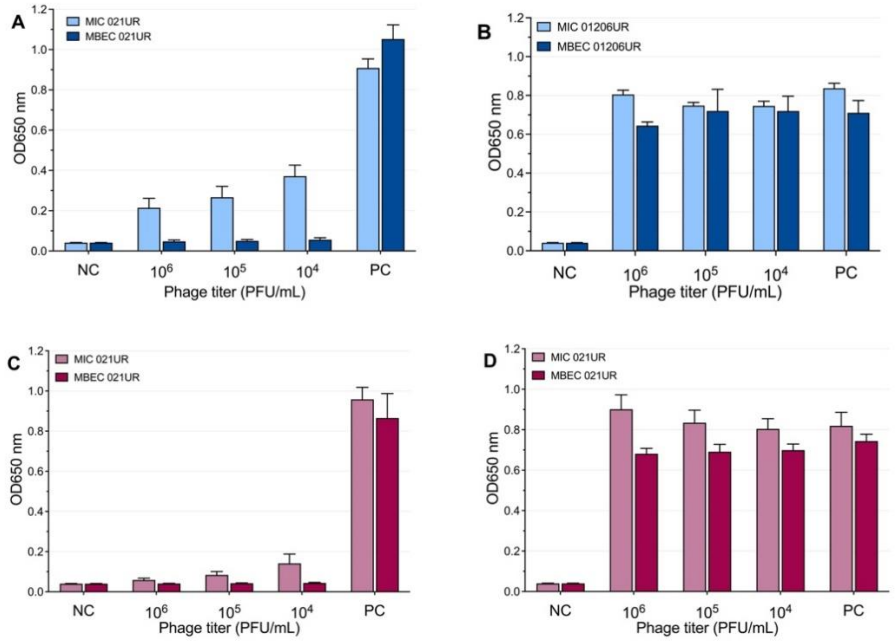


Figure 2.3 **Ses (A, B) and Intesti (C, D) MIC and MBEC for 021UR (A, C) and for 01206UR (B, D) ***

* Bars represent mean values of bacterial OD (measured at wavelength of 570 nm) using different concentrations of Pyobacteriophage, NC represents negative control or broth only, PC represents positive control or bacteria only.

2.3 Experimental phage treatment and effect in multidrug-resistant *Pseudomonas aeruginosa* femur osteomyelitis

Part of this research is described and published in the publication by Kārlis Rācenis, Dace Rezevska, Monta Madelāne, Ervīns Lavrinovičs, Sarah Djebara, Aivars Pētersons and Juta Kroiča *Use of Phage Cocktail BFC 1.10 in Combination With Ceftazidime-Avibactam in the Treatment of Multidrug-Resistant Pseudomonas aeruginosa Femur Osteomyelitis-A Case Report*. Front Med (Lausanne). 2022 Apr 25;9:851310. doi: 10.3389/fmed.2022.851310.

2.3.1 Patient description

In July 2018, a 21-year-old man was hospitalised after a road accident with open comminuted proximal right femoral and acetabular fractures, laceration of the right lower hand, and haemorrhagic shock. Wound debridement, fasciotomy, femur fracture stabilization with gamma nail, and tissue reconstruction were performed on the 14th of July. On consecutive days, the patient developed secondary MDR *P. aeruginosa*, carbapenem-resistant *Acinetobacter baumannii*, and vancomycin-resistant *Enterococcus faecium* (VRE) wound infections and multiple organ dysfunction syndrome. The patient underwent five debridement procedures and therapy using a wound vacuum system. Broad-spectrum IV antimicrobial treatment with meropenem, colistin, piperacillin-tazobactam, linezolid, and fluconazole was administered, and renal replacement therapy was initiated. Regardless of the treatment, the patient developed an osteosynthesis-associated infection and osteomyelitis, and repeatedly positive wound cultures grew with VRE, MDR *P. aeruginosa*, and MDR *A. baumannii*. On the 13th of August, the gamma nail was removed, and proximal femoral segment resection was performed, followed by tissue reconstruction and lower leg external fixation. Based on the antibiogram, the antimicrobial regimen was changed to intravenous fosfomycin, meropenem, and

colistin. On the 13th of September, the right thigh wound was closed using a scapular flap. The patient's condition improved gradually, and there were no signs of systemic or local inflammation. Repeated cultures of the wound were negative. The patient was discharged on the 15th of October with IV meropenem and colistin treatment, which was discontinued after two weeks due to acute kidney injury, presumably colistin-induced nephrotoxicity. In November, purulent discharge from the right upper thigh appeared. Computed tomography with contrast injection in the cutaneous wound opening revealed a fistula that connects femoral head and skin on the right upper third of the lateral femur surface. The patient underwent fistulotomy, and MDR *P. aeruginosa* and VRE were isolated from the wound. With a presumptive diagnosis of recurrent femoral osteomyelitis, two-stage surgery was planned to preserve hip replacement surgery in the future. Local bacteriophage therapy was planned using the bacteriophage cocktail BFC 1.10 produced at Queen Astrid Military Hospital in Brussels, Belgium, consisting of phages active against *P. aeruginosa* and *S. aureus*

2.3.2 Diagnostic assessment, therapeutic intervention, follow-up, and outcomes

The treatment was performed according to Paragraph 37 of the Declaration of Helsinki (44). The patient provided written informed consent for the use of the bacteriophages. On the 5th of December, a right femoral head excision was performed and replaced with colistin-impregnated cement spacer. The proximal femoral culture was positive for MDR *P. aeruginosa*, VRE, and *Staphylococcus epidermidis*. The pathology and intraoperative findings confirmed femoral head osteomyelitis with fistula. After surgery, the patient was treated with IV colistin for 7 days and linezolid for 23 days. On the 7th of December, 2000 mL of BFC 1.10 cocktail with 10^7 plaque-forming units (PFU)

per mL of each phage were shipped to Latvia. Prefilled sterile containers containing 30, 40 or 50 mL of phage solution were prepared under sterile conditions. Three days before the procedure, the patient was treated with IV ceftazidime-avibactam, which was continued for 15 days. On the 13th of December, bone cement was removed; wound and acetabular cultures were taken, and were positive for MDR *P. aeruginosa*. Next, wound rinsing with 50 mL BFC 1.10 bacteriophage suspension was performed intraoperatively, tissue damage was replaced with a serratus muscle flap, and an irrigation system for local bacteriophage application was installed. For the first 7 days, the patient was treated with 40 mL (1ml/min) of BFC 1.10 three times daily and then with 30 mL (1ml/min) of BFC 1.10 two times daily via an irrigation catheter for another 7 days. The wound was rinsed with 50 ml of 4.2 % sodium bicarbonate solution before the phage application using syringe. Together with the local phage treatment, linezolid and ceftazidime-avibactam were continued. During and after phage treatment on days 1, 3, 4, 7, 10, and 15, no bacterial cultures from the wound grew. Phages were isolated from the wound in the morning buffer sample before phage administration on days 1, 3, 4, 7, 10, and 15. At the end of treatment, the wound healed with no local or systemic signs of infection. When the irrigation catheter was removed, the tip of the catheter was positive for *Candida tropicalis*, which was not treated (Figure 2.4). No adverse effects, such as fever, local rash, itchiness, or other symptoms, were noted during phage therapy. Patient was discharged with lower leg external fixation until hip replacement surgery.

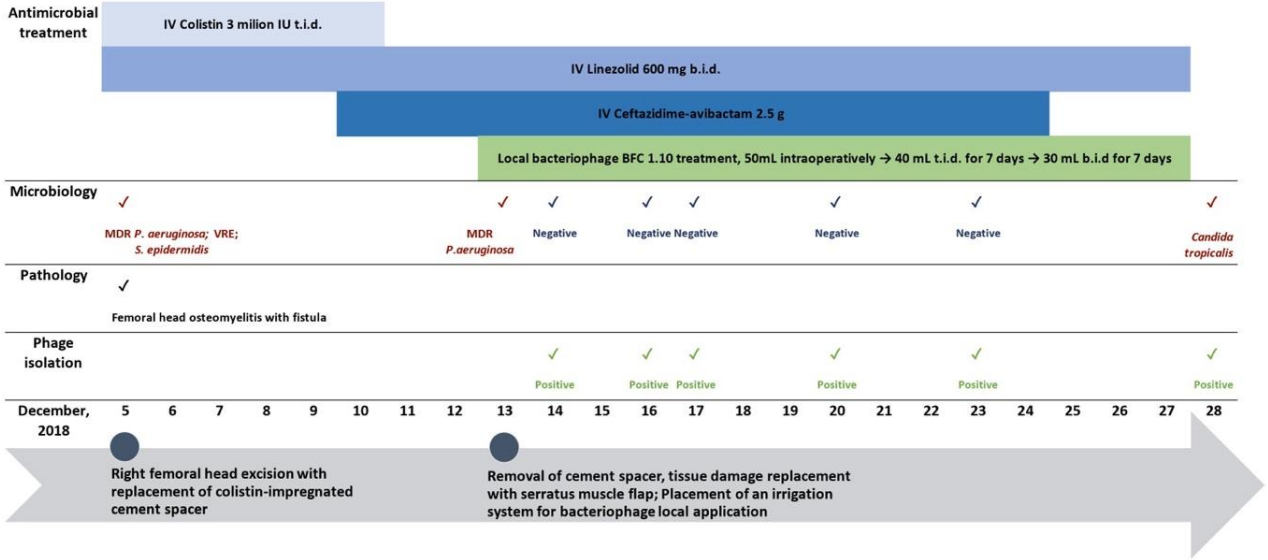


Figure 2.4 **Timeline showing surgical interventions, relevant antimicrobial therapy, and microbiological and pathology findings**

Two months later, the wound healed and there were no signs of inflammation that was reassured with magnetic resonance imaging (MRI) of the right hip. Three months after phage treatment, computed tomography of the right hip and femur revealed no fluid collection or signs of osteomyelitis. In the following six months, two punctures from the right femur were performed and were culture negative, three months before hip replacement lower leg external fixation was removed. On the 3rd of September 2019, a hip replacement with a silver-coated implant was performed. During the surgery bacterial cultures were taken, the distal part of the femur was positive for MDR *P. aeruginosa* and VRE (fosfomycin susceptible), but acetabular bone and proximal part muscular tissue were culture negative. Patient received one dose of IV vancomycin for perioperative prophylaxis and IV colistin that was continued until microbiology results. Once the cultures came back positive patient was kept on IV colistin and IV fosfomycin. Sixteen days later DAIR (debridement, antibiotics and implant retention) was performed because of hematoma development and possible prosthesis infection, swabs taken during the surgery from periprosthetic tissue in distal segment were positive for MDR *P. aeruginosa*. On 4th of October punctures from periprosthetic tissue were performed and were culture negative. Three days later patient was discharged and continued antimicrobial therapy in outpatient setting with colistin and fosfomycin. For this episode patient received colistin for six weeks and fosfomycin for five months. During the follow-up period a year later, there were no local signs of infection, and the patient noted limited mobility in the right leg; however, he could continue to play basketball. Radiography of the right hip and femur fifteen months later did not reveal any signs of inflammation.

2.3.3 Bacteriophage and antibiotic susceptibility of *P. aeruginosa* isolates form patient with osteomyelitis

From all the samples obtained, 7 *P. aeruginsoa* isolates were used in the study. Isolate PA (4) isolated on 23.11.2018 from a wound was used for bacteriophage susceptibility, MIC and BPC testing. Phage spot testing of MDR *P. aeruginosa* (isolate PA (4)) isolated from the patient before the phage application showed positive lytic effect with partial lysis that is observed on agar as incomplete lysis of bacteria. Using phage propagation, a high-titre phage stock was obtained, resulting in a significant increase in the phage titre from 1.6×10^7 PFU/mL to 2.5×10^9 PFU/mL that was used for biofilm formation detection, MIC and BPC detection.

2.3.4 The effect of bacteriophages and antibiotic in planktonic cells and biofilm formation prevention in *P. aeruginosa* PA (4) from patient with femur osteomyelitis

The MIC and BPC values of ceftazidime-avibactam were 8 mg/L and 16 mg/L, respectively. Thus, according to the EUCAST standard, acquired values showed susceptibility of planktonic cells but failed to prevent biofilm formation. The phage cocktail BFC 1.10 demonstrated no bacterial growth with a titre of 5×10^7 PFU/mL and prevented biofilm formation only when applied with the highest titre of 1.6×10^9 PFU/mL. An additive antimicrobial effect of ceftazidime-avibactam and BFC 1.10 was observed in the planktonic state of *P. aeruginosa* isolate (when detecting MIC) and on prevention of biofilm formation (BPC). When ceftazidime-avibactam was used in combination with BFC 1.10, the MIC and BPC values of ceftazidime-avibactam reduced from 8 to 4 mg/L ($p = 0.03$) and from 16 to 8 mg/L ($p = 0.023$), respectively, compared to those

obtained using ceftazidime-avibactam alone. The antibiotic concentration required for biofilm prevention decreased to the MIC cut-off value (≤ 8 mg/L) according to the EUCAST standard, making the strain susceptible to ceftazidime-avibactam (Figure 2.5).

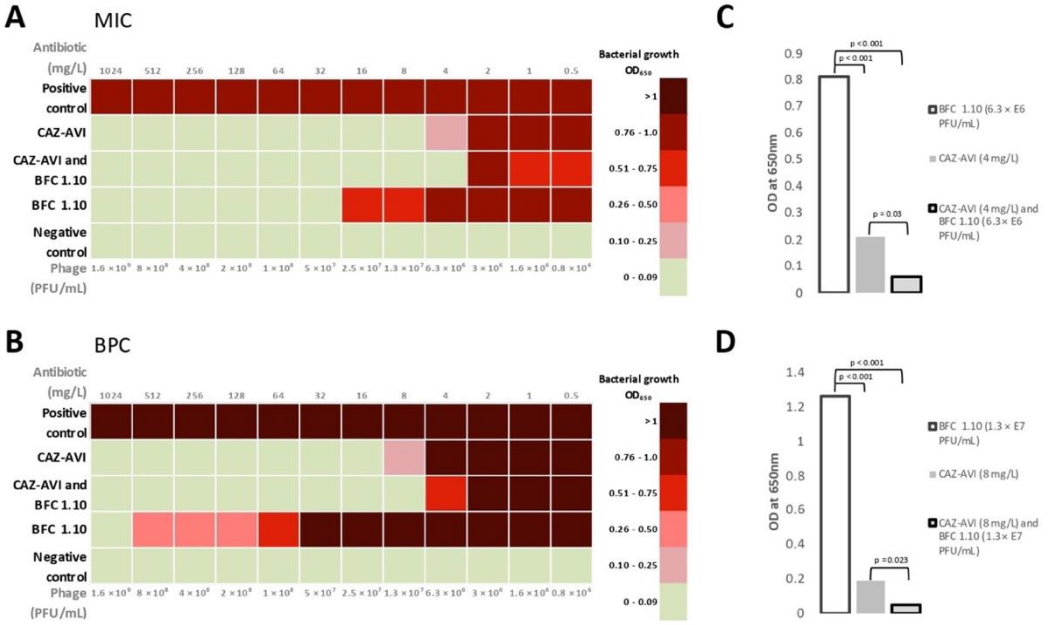


Figure 2.5 **MIC and BPC values of phage and antibiotic in *P. aeruginosa* from patient with osteomyelitis ***

*Mean values of MIC (A) and BPC (B) for BFC 1.10 and/or ceftazidime–avibactam.
The graph shows the differences between MIC (C) values using 4 mg/L ceftazidime–avibactam and/or BFC 1.10 6.3×10^6 PFU/mL and mean BPC (D) values using 8 mg/l ceftazidime–avibactam and/or BFC 1.10 3.3×10^7 PFU/mL.

2.4 Experimental phage treatment and effect in multidrug-resistant *Pseudomonas aeruginosa* LVAD driveline infection

Part of this research is described and published in the publication by Kārlis Rācenis, Jānis Lācis, Dace Rezevska, Laima Mukāne, Aija Vilde, Ints Putniņs, Sarah Djebara, Maya Merabishvili, Jean-Paul Pirnay, Marika Kalniņa Aivars Pētersons, Pēteris Stradiņš, Sandis Mauriņš and Juta Kroiča *Successful Bacteriophage-Antibiotic Combination Therapy against Multidrug-Resistant Pseudomonas aeruginosa Left Ventricular Assist Device Driveline Infection. Viruses. 2023 May 20;15(5):1210. doi: 10.3390/v15051210.*

2.4.4 Patient description and diagnostic assessment

In October 2020, a 54-year-old male was admitted to PSCUH with purulent discharge from the LVAD HeartMate 3 (HM3) driveline exit site, inflammation of the exit site, febrile temperature, and elevated inflammatory markers (C-reactive protein (CRP) 44 mg/l).

In November 2016, an LVAD HM3 device was implanted as a bridge to heart transplantation candidacy due to dilatation cardiomyopathy with severe end-stage heart failure (INTERMACS profile 1). In 2017, the patient was placed on the heart transplant waiting list.

The symptoms mentioned first appeared 46 months after LVAD HM3 implantation. Immediate antibacterial therapy with piperacillin/tazobactam was started for 2 weeks. The exit site wound swabs were positive for *P. aeruginosa*. After wound improvement, antibacterial treatment was changed to prolonged suppressive ciprofloxacin therapy, and the patient was discharged at the end of October 2020.

Nineteen weeks later, in March 2021, the patient was hospitalised again due to increased purulent discharge and fistula formation along the driveline. The results of the wound swab showed the presence of MDR *P. aeruginosa* with no

alternative oral antibiotics available. Intravenous antibiotic therapy with colistin was initiated with loading dose 9 million and then 3 million IU 3 times a day and continued until surgical intervention. A decision was made to prepare the patient for driveline repositioning. Fluorine-18-fluorodeoxyglucose positron emission tomography integrated with computed tomography (18F-FDG PET/CT) showed an active metabolic process along the driveline up to the level of the abdominal muscle, with a slight infiltration of the rectus abdominis muscle, representing infection.

To enhance the likelihood of a successful treatment outcome, phages were applied locally and intravenously during the intraoperative and postoperative phases. This decision was made based on the patient's inability to respond to previous treatments. Additionally, no effective antibiotic options were available, with ceftazidime/avibactam and amikacin being the only remaining intravenous alternatives. Two lytic phages, PNM and PT07, were shipped from the Queen Astrid Military Hospital (QAMH, Brussels, Belgium) to Riga (Latvia), and the phage treatment modality was discussed with the local treatment team and QAMH specialists. The treatment was conducted in accordance with Article 37 of the Declaration of Helsinki (44), and written informed consent was obtained from the patient prior to the procedure.

2.4.2 Therapeutic intervention

On June 16, the operation began with extensive tissue debridement along the course of the driveline, including partial removal of the rectus abdominis muscle. During debridement, multiple wound swabs were cultured to rule out undetected microorganisms and to understand the depth of the infectious process roughly and retrospectively. To increase adhesion in subcutaneous tissue, the outer layer of the LVAD driveline is covered with velour, which complicates the chance of eradication of microorganisms. Therefore, the driveline’s outer layer

was removed and sent for microbiological investigation. The operation was continued with wound irrigation and local treatment with Prontosan® solution (B. Braun, Germany) with betaine surfactant and 0.1 % Polyaminipropyl Biguanide (Polihexanide) as active substances. Then a new subcutaneous canal was prepared in the anterior abdominal wall to reposition the driveline. To reduce the chance that fluid or tissue material might enter the new modular cable connector and ensure sterility during repositioning, a sterile ultrasound probe cover was used to cover the sides of the subcutaneous tunnel. The new subcutaneous canal and the previous canal infected with *P. aeruginosa* were irrigated with 250 ml 0.9 % NaCl and then with 250 ml 4.2 % NaHCO3, to make the surrounding environment more alkaline. Five min later, 50 ml of phage suspension consisting of PNM and PT07, each at a concentration of 10^7 PFU/ml, was applied to each wound. A new modular cable already connected to the LVAD controller was guided through the subcutaneous tunnel. The driveline was temporarily disconnected from the old modular cable and connected to the new one. After ensuring that the haemodynamics of the patient were stable and the LVAD was running, the driveline was repositioned through the subcutaneous tunnel. An 8-FR catheter was inserted along the driveline to administer the phage solution. The wound was left open for secondary healing.

Intravenous application of phages PNM and PT07, with a titre of 10^7 PFU/ml each, started 2 h before surgery using an infusion pump at a rate of 13 ml/h for 6 h through a central venous catheter with a total volume of 80 ml, and this for 8 days. On the next day after surgery, the wound was rinsed using an 8-Fr catheter with 50 ml of PNM and PT07, with a titre of 10^7 PFU/ml each, and was continued daily for 3 days. Before the local application of phages, the wound was rinsed through the catheter with 250 ml 0.9 % NaCl, and then with 250 ml 4.2 % NaHCO3. Intravenous antibiotic therapy consisting of ceftazidime/avibactam 2.5 g 3 times a day and amikacin 750 mg 2 times a day

was started 2 h before the operation. Amikacin was continued for 4 weeks, and ceftazidime/avibactam for 6 weeks (Figure 2.6). Intraoperative wound samples showed the presence of *P. aeruginosa* at all wound levels. Changes in wound dressing and swabbing were performed daily and did not show the presence of *P. aeruginosa*. Six days after repositioning the driveline, the secondary healing wound did not reveal the presence of infection and was closed. During phage treatment, no adverse events were observed. The phage titre in the patient's blood was stable for 7 consecutive treatment days with a concentration of 10^2 PFU/ml. Phages were no longer detected from the first day after cessation of phage administration (Figure 2.6).

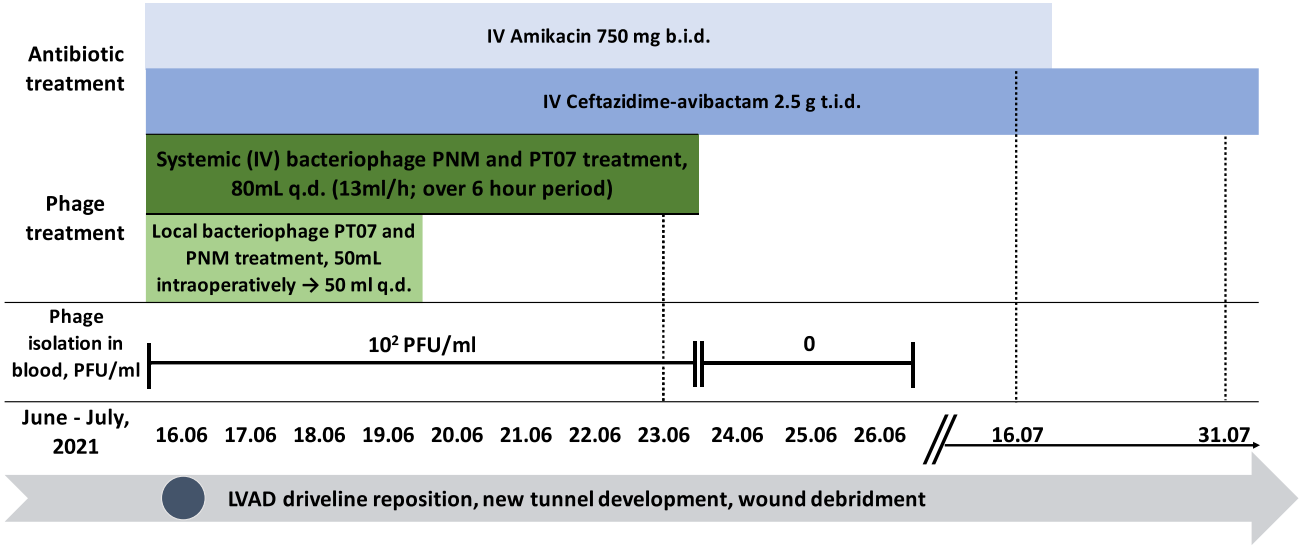


Figure 2.6 **Timeline showing antibiotic and phage treatment modalities, surgical interventions, and phage concentrations detected in the patient's blood**

2.4.3 Follow-up and outcomes

Six weeks after the driveline repositioning control 18F-FDG PET/CT scan was per-formed, showing slight residual metabolic activity in the most proximal part of the driveline, but to a lesser extent than before the driveline repositioning. Considering that PET/CT was performed early after repositioning and most likely represented reactive changes, the wound showed no signs of inflammation, with inflammatory markers lying within normal limits, the patient was discharged on postoperative day 45. Thirty-four weeks after the operation, another control PET/CT scan was performed, showing no signs of significant metabolic activity. The patient is regularly checked and shows no signs of recurrence 21 months after treatment.

2.4.4 Bacteriophage and antibiotic susceptibility of *P. aeruginosa* isolates form patient with LVAD driveline infection

In total, 5 bacterial isolates were used, all identified as *P. aeruginosa*. Their antimicrobial susceptibility varied over time, with widespread antimicrobial resistance. Before bacteriophage therapy was used, their susceptibility was determined for isolate PA03, which was obtained from LVAD driveline exit-site. For further biofilm modelling and phage testing, the PAP01 isolate obtained from the driveline velour removed during surgery was used. The results show that the phage susceptibility using the double agar spot method was moderate for PT07 and partial for PNM. However, the EOP was 0.1 for PT07 using both isolates and 0.001 for PNM using PA03 and 0.0005 for PAP01. The results show that the lytic effect of the PNM phage was more pronounced and the phage was more virulent against the patient's P. aeruginosa isolates, whereas the PNM phage was less virulent and its lytic effect decreased over time against the newer isolate (PAP01) when the EOP results were assessed, see Table 2.5.

Table 2.5

Results of antimicrobial, phage susceptibility and EOP of *P. aeruginosa* isolates from patient with LVAD driveline infection

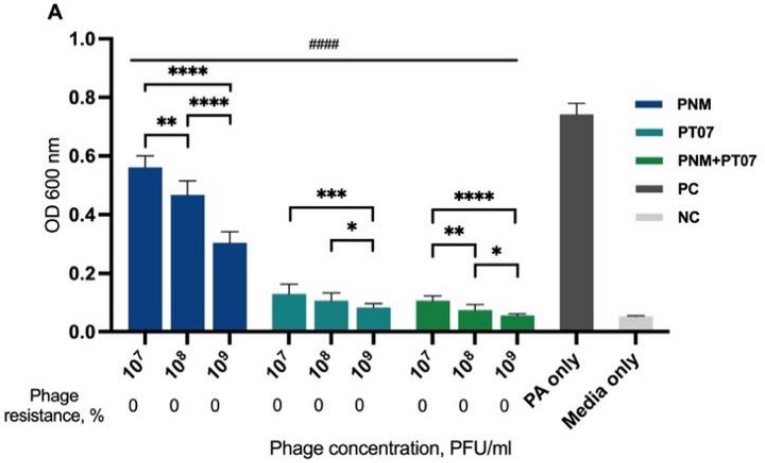
	CN573	**PA01**	**PA02**	**PA03**	**PAP01**	**PAP02**
Type of the isolate	**Phage host strain**	**Discharge from LVAD driveline exit-site**	**Discharge from LVAD driveline exit-site**	**Discharge from LVAD driveline exit-site**	**Velour from the driveline**	**Velour from the driveline**
Isolation time	–	04/10/20	05/03/21	19/04/21	16/06/21	16/06/21
Antibiotics	–	–	–	–	–	–
AMK	S (MIC ≤ 4)	S	S	S	S (MIC ≤ 4)	S (MIC ≤ 4)
FEP	I (MIC = 4)	N/A	N/A	N/A	I (MIC = 2)	I (MIC = 4)
CAZ	I (MIC = 2)	I	R	I	I (MIC = 2)	I (MIC = 2)
CAZ/AVI	S (MIC ≤ 1)	N/A	N/A	N/A	S (MIC ≤ 1)	S (MIC ≤ 1)
CIP	I (MIC = 0.25)	I	R	R	I (MIC = 0.25)	I (MIC = 0.25)
CST	S (MIC = 2)	N/A	S	S	S (MIC ≤ 1)	S (MIC = 2)
FOF	R (MIC > 128)	R (MIC > 128)	R (MIC > 128)	N/A	R (MIC > 128)	R (MIC > 128)
IPM	I (MIC ≤ 1)	I	R	R	I (MIC ≤ 1)	I (MIC ≤ 1)

Table 2.5 continued

	CN573	PA01	PA02	PA03	PAP01	PAP02
Type of the isolate	**Phage host strain**	**Discharge from LVAD driveline exit-site**	**Discharge from LVAD driveline exit-site**	**Discharge from LVAD driveline exit-site**	**Velour from the driveline**	**Velour from the driveline**
MEM	S (MIC = 1)	S	R	I	S (MIC ≤ 0.125)	S (MIC = 1)
TOB	S (MIC = 0.5)	N/A	R	R	S (MIC = 0.5)	S (MIC = 0.5)
TZP	I (MIC = 8)	I	R	I	I (MIC = 4)	I (MIC = 8)
Phage PNM	++++	N/A	N/A	++ EOP=0.001	++ EOP=0.0005	N/A
Phage PT07	++++	N/A	N/A	+++ EOP=0.1	+++ EOP=0.1	N/A

2.4.5 Phage effect against planktonic cells, biofilm eradication and bacterial resistance to phages in PAP01 using biofilm eradication model

The results of the biofilm eradication test in PAP01 showed that only phage PNM alone at concentration 10^9 PFU/ml could decrease biofilm formation to some extent (Figure 2.7). Other phage concentrations of PNM and PT07, the combination of PT07 and PNM, at any concentration, did not have a biofilm eradication effect. Planktonic cell growth after 12 h decreased for all phages and concentrations tested; however, after 24 h, this effect persisted only for PNM at concentrations 10^7–10^8 PFU/ml and for a combination of phages at the tested concentrations. In all cases, except for PT07 concentrations 10^7 versus 10^8 PFU/ml, the lytic effect after 12 h was better when higher phage concentrations were used. This was not observed 24 h later and was even the opposite for PNM at concentrations 10^7 versus 10^9, and 10^8 versus 10^9 PFU/ml. Phage resistance developed in all cases after 24 h; however, it was less common in phage combination and reached 100 % in bacterial cells recovered from biofilms for PT07 at all concentrations tested and for PNM at concentration 10^7 PFU/ml.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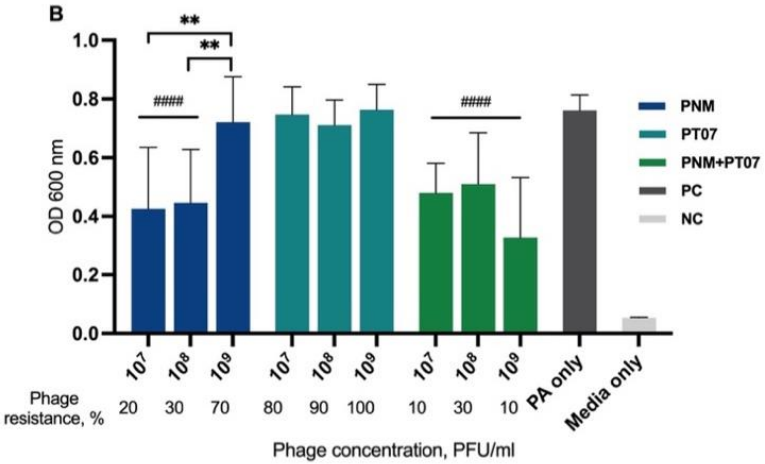
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Phage concentration, PFU/ml

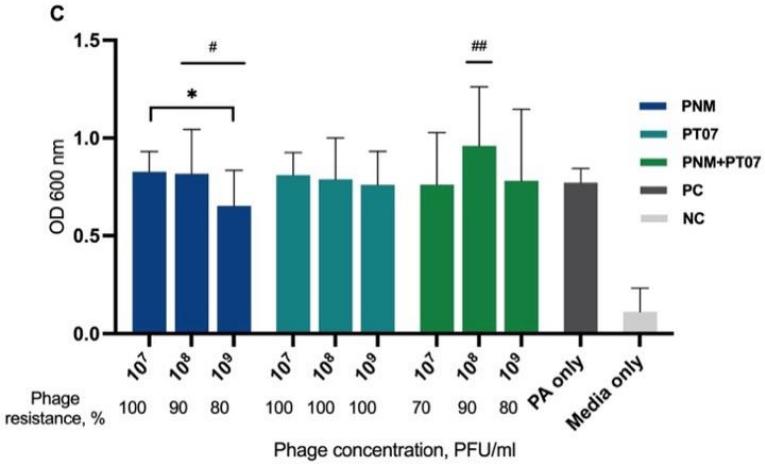


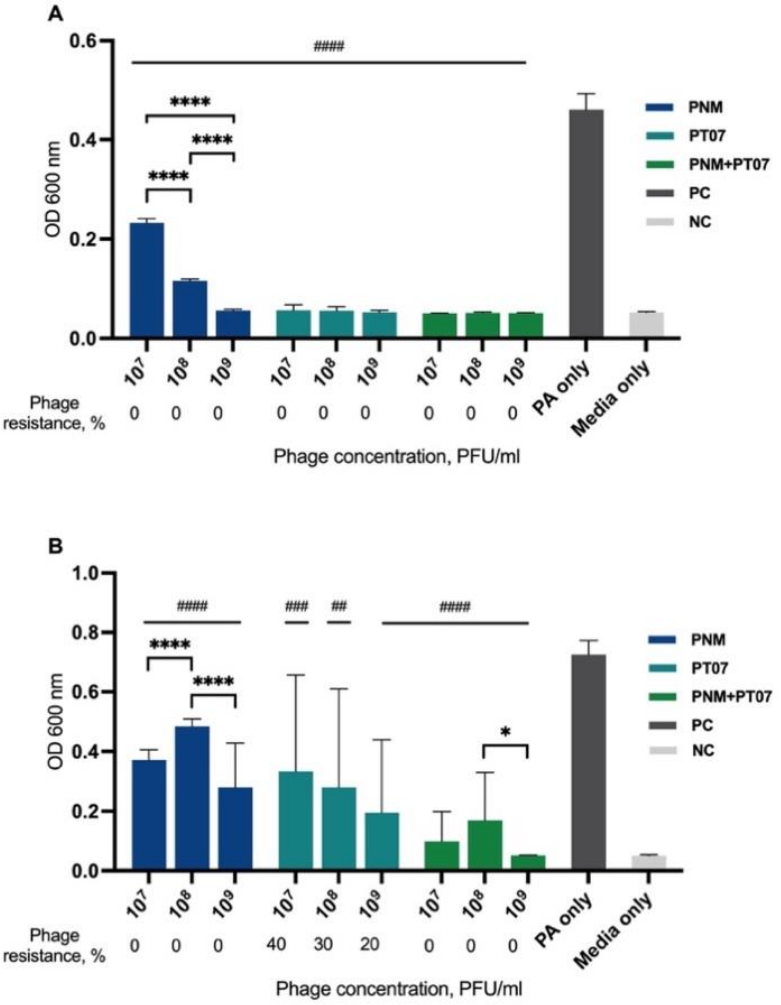
Figure 2.7 **MIC and MBEC mean values + standard deviations of PNM, PT07 or both combined against PAP01 in a biofilm eradication model ***

* The number of phage-resistant strains in % is represented below the bars. (A) MIC12; (B) MIC24; (C) MBEC; PC – positive control, untreated PAP01; NC – negative control, media only; Bars with a hash sign (#) are statistically different from the positive control, and bars with an asterisk (*) represent the statistical difference between concentrations of the same phage; */# p value < 0.05, **/## p value < 0.01, ***/### p value < 0.001 and ****/#### p value < 0.0001.

2.4.6 Phage effect against planktonic cells, biofilm prevention and bacterial resistance to phages in PAP01 using biofilm prevention model

The biofilm prevention capacity in PAP01 for PT07 and the combination of PNM with PT07 was determined at all tested concentrations (the same concentrations were used in biofilm eradication model). PNM alone did not prevent biofilm formation; on the contrary, the use of 10^7 and 10^8 PFU/ml concentrations led to a higher bacterial count compared to untreated PAP01. Planktonic cell death was observed for all phages and their tested concentrations 12 and 24 h after incubation. Phage resistance developed for all phages tested in bacteria recovered from biofilms, it reached 100 % for PT07 at all tested

concentrations. Planktonic cell resistance was observed only after 24 h of incubation for PNM phage, but the resistance rate to PNM differed in cells recovered from the biofilm (Figure 2.8).



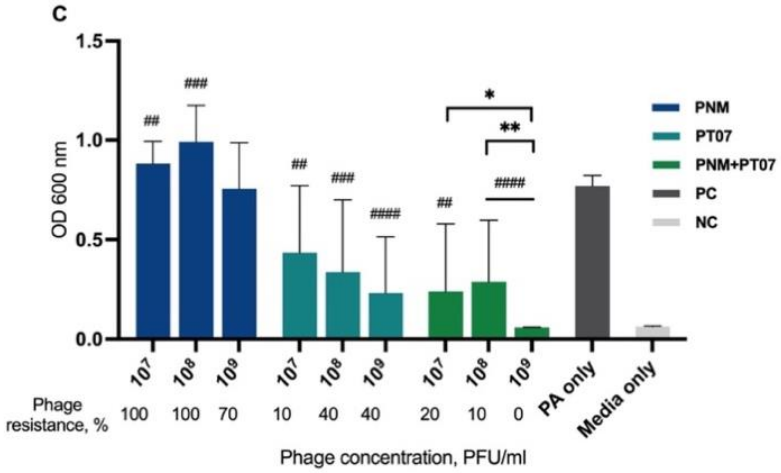


Figure 2.8 **MIC and BPC mean values + standard deviations of PNM, PT07 or both combined against PAP01 in a biofilm prevention model ***

* The number of phage-resistant strains in % is represented below the bars. (A) MIC12; (B) MIC24; (C) BPC; PC – positive control, untreated PAP01; NC – negative control, media only; Bars with a hash sign (#) are statistically different from the positive control, and bars with an asterisk (*) represent the statistical difference between concentrations of the same phage; */# p value < 0.05, **/## p value < 0.01, ***/### p value < 0.001 and ****/#### p value < 0.0001.

2.4.7 Differences in planktonic cell growth and antibiofilm effect using phages first and then antibiotics in CN573

Phage effect using phages PNM, PT07 or both combination in concentration 10^7 PFU/ml first for 12 hours and then ceftazidime-avibactam for 12 h in biofilm eradication model showed that statistically significant differences were detected ($p < 0.05$).

In planktonic cells ceftazidime-avibactam only at concentration 2 mg/l failed to kill bacteria. Using phages for the first treatment plate for 12 h instead of ceftazidime-avibactam and then ceftazidime-avibactam for 12 h showed that both phages and their combination caused additive effect and resulted in bacterial killing.

In biofilm eradication model synergistic effect of phages and ceftazidime-avibactam was observed using phages first (for 12 h) and then antibiotic in all tested ceftazidime-avibactam concentrations where antibiotic alone failed to eradicate biofilm.

3 Discussion

The dissertation is devoted to the possible use of bacteriophages in the control of multidrug-resistant biofilm-associated infections in various clinical situations, where these problems are particularly relevant as infections of implanted devices (PD catheters, LVADs), in severe bone and soft tissue damage, and in patients with risk factors (immunosuppression, structural and/or physiological tissue changes).

Bacteriophages or phages are bacterial viruses that have the unique ability to infect and kill bacteria. Lytic phages are widespread in the environment and are unable to infect either human or animal cells. These specific properties make bacteriophages a potential tool in the fight against bacterial infections, especially those that cannot be treated with antibiotics. Bacteriophage therapy as an alternative treatment for infections is included in the European Union's Antimicrobial Resistance Plan.

Studies conducted on the effect of bacteriophages in multidrug-resistant infections indicate their potential, but there is no clear answer on the phage interactions in the biofilms formed by these bacteria. There is also a lack of clinical data to safely and effectively use bacteriophages in the treatment of patients.

Bacteriophage effect in *S. aureus* isolated from peritoneal dialysis patients

A positive or lytic bacteriophage effect was detected in all 71 S. aureus strains using commercially available bacteriophage cocktails from Eliava (Pyo, Ses, Fersisi, Enko, Intesti) and Microgen (Pyobacteriophage), except Eliava Staphylococcal Bacteriophage, where the incidence of resistance or no lytic effect was 26 % (n = 9). The most commonly observed degree of positive phage lytic effect was partial lysis, ranging 56 %–100 % among isolates and could

depend on phage concentration within the cocktail, time of incubation, and phage resistance. Our results of wide phage lytic activity of 100 % for all except Eliava Staphylococcal Bacteriophage could be also associated with possible low strain genetic diversity, as in various parts of the world, we can find genetically and biologically distinct strains of the same bacterial species (45). In cases of bacterial resistance or a weak lytic effect towards Eliava Staphylococcal Bacteriophage, an adaptation procedure or so-called host range expansion was performed. The outcome after adaptation was indicative of persistent enhancement in bactericidal action, leading to 100 % lytic activity. In nine resistant strains, adaptation led to overcoming bacterial resistance; in two strains, improvement of positive lytic activity from individual plaques (+) to partial lysis (++) was achieved. The principle of phage adaptation is mainly due to spontaneous mutations and possible gene recombination between genetically diverse bacteriophages within the phage cocktail. Such changes lead to altered structural gene products, for example, encoding phage tail fibre assembly proteins that are necessary for phage attachment to the host cell or adsorption, which is the first step of phage infection. Adaptation can also reduce lysis time and increase phage burst size (46, 47). Recent findings on the Eliava Staphylococcal Bacteriophage cocktail that consist of Twort-like phage Sb-1 showed that after the adaptation process, newly formed phage clones were found in phage stock, and such a process increased phage lytic activity from 87 % to 96 % on globally diverse S. aureus strains. Interestingly, genetic differences between the mutant and parental Sb-1 phages were found in the phage genome hypervariable complex repeat structure, but the adsorption rate between parental and mutant phage was similar, with the conclusion that the process of host range expansion in the Sb-1 phage is still unclear (48). Another study from Switzerland showed similar results in that after phage–bacterium adaptation of Eliava Pyo

Bacteriophage with *E. coli* isolates from urinary tract infection, the lytic spectrum increased from 65.9 % to 92.7 % (49).

Results revealed clinically relevant (strong and moderate) biofilm production in 91.2 % (n = 31) of strains, an observation similar to another study from Brazil where *S. aureus* strains isolated from PD peritonitis patients produced biofilm in 88.7 % (n = 55) of cases, even though a different biofilm detection method was used (50), indicating that microtitre plate assay can be used as a cost-effective screening method for biofilm detection in clinically relevant *S. aureus* strains. Phage lytic activity was not affected by different degrees of *S. aureus* biofilm production, showing that their biofilm production capability does not interfere with *in vitro* testing of phage on planktonic bacteria.

Results about biofilm production and phage susceptibility support *S. aureus* phenotypic variability, even within one patient, $p < 0.01$, Figure 2.1; however, such phenomena did not interfere with the bacteriophage positive lytic effect that was detected in the majority of strains.

Bacteriophage effect in uropathogenic E. coli planktonic and biofilm-producing cells

Our data indicate that *E. coli* isolated from patients with UTI can often form biofilms. The results show that although amoxicillin-clavulanic acid and ciprofloxacin can successfully deal with planktonic cells, they cannot destroy biofilms. In contrast to antibiotics, our research demonstrates that if bacteriophages affect planktonic cells, they can also destroy biofilms. In situations where the bacteriophages initially showed a weak effect against the strain, phages could be adapted, thus achieving a lytic effect in both planktonic and biofilm-forming cells.

E. coli is often associated with biofilm formation (51-54), although our research shows that the ability of bacteria to form a biofilm is highly variable depending on each individual strain. The ability of bacteria to form biofilms should be considered as an important factor in the treatment of infection. The use of antibiotics is commonly based on their effect on planktonic bacterial cells without paying enough attention to the risk of biofilm formation. Although antibiotics can successfully destroy planktonic bacterial cells, they are often unable to destroy biofilms. Bacteria in the biofilm often survive treatment and can cause reactivation of the infection (55).

Occasionally, to achieve MIC and MBEC values in biofilm models, it is necessary to use high concentrations of antibiotics (19). Such high doses cannot be used for patients because they can be harmful or even fatal to the patient; therefore, such antibiotics will not provide an effect in therapeutic doses and are not recommended for treatment. Our results show that phages can achieve a better effect on biofilms (MBEC values) than on planktonic cells (MIC values). This can be observed most clearly when analyzing the effect of the SES phage against the 021UR strain (see Figure 2.3) and could be explained by the fact that phages begin active replication when bacteria are released from the biofilm and become metabolically active (56). The three phage cocktails could disrupt bacterial biofilms for the 021UR strain. However, this strain was resistant to amoxicillin–clavulanic acid and, by ciprofloxacin, the MBEC value was reached only at a concentration of 64 mg /L. The results indicate that phages have a stronger *in vitro* antibiofilm effect in this case compared to the tested antibiotics.

Interestingly, the results show that although both strains, 021UR and 01206UR, are strong biofilm formers and express resistance to several antibiotics, they are still susceptible to phages. This leads to the conclusion of phenomena described in the literature that the development of antimicrobial

resistance is not equally linked to bacteriophage resistance and that phage-antibiotic cross-resistance does not occur (57). As a limitation, it should be mentioned that our study did not use individual phages but commercial phage cocktails. Using commercial phage cocktails, it is difficult to multiply them because we do not have access to the original host strains on which these phages were grown and propagated.

Unlike antibiotics, phages are living viruses and their activity can be affected by various factors, such as the amount of nutrients, temperature, and pH. Therefore, it is critical to provide appropriate growth conditions during research. The Calgary biofilm device model provides fast and reproducible results in determining the minimum biofilm eradication concentration (41). It is possible to supply dynamic conditions and add fresh nutrients to the biofilms formed, and after their destruction, it is possible to detect viable cell regrowth, which gives more reliability to the results of the study.

Each bacteriophage has its spectrum of activity. Therefore, the effect against some bacterial strains may be weak, or even bacteria may be completely resistant to them. One possibility to expand the range of phage activity is to use multi-phage cocktails, as was also done in this study. The second way to improve phage activity is to provide adaptation of phages, which significantly improves their efficiency (58, 59), similar to the previous part of the study with *S. aureus* isolates. Pyobacteriophage was initially unable to kill the 01206UR strain, but after adaptation, a significant improvement in the effect was observed in both planktonic cells and biofilms (see Figure 2.2). In complicated cases, when antibiotics do not have the desired effect and the efficiency of phages is low, adaptation can be the solution and cure the patient.

Experimental use of bacteriophages in the treatment of multidrug-resistant *Pseudomonas aeruginosa* femur osteomyelitis

Infections are frequent complications of severe high-energy trauma, and posttraumatic osteomyelitis can develop in up to 19 % of cases (60). Antimicrobial resistance has become an emerging major public health problem during the last few decades. Factors such as trauma-related severe bone fractures (Grade III, Gustilo-Anderson classification) and extensive soft tissue damage are associated with a remarkably high risk for developing surgical site infection (SSI) (4–52 %) (61, 62). Importantly, a considerable increase in the proportion of infections caused by gram-negative bacilli and polymicrobial flora has been observed, notably in grade III injuries, predicting a poorer prognosis (63). Infections caused by *P. aeruginosa* are difficult to treat and have a high recurrence rate (64, 65). Multiple factors determine the high virulence and subsequent persistence of infection of *P. aeruginosa*, such as potential adaptation to various environmental factors, production of exotoxins leading to possible severe tissue damage, the rapid development of antimicrobial resistance, and the ability to produce highly structured biofilms. *Pseudomonas* infections require a prolonged treatment course and a combination of two or more different classes of antimicrobials (21, 22, 66, 67).

Similarly, for the patient in our clinical case, infection was caused by the polymicrobial flora of MDR *P. aeruginosa*, carbapenem-resistant *A. baumannii*, and VRE. In addition to the recurrent infection, femur osteomyelitis, predominantly caused by MDR *P. aeruginosa*, developed despite several debridement procedures and extensive antimicrobial treatment. Our study results demonstrate a strong biofilm formation capability of the isolated MDR *P. aeruginosa* that affected conventional treatment causing failure. The isolated MDR *P. aeruginosa* strain was susceptible to ceftazidime-avibactam. However, the risk of resistance development towards ceftazidime-avibactam is high,

especially in multidrug-resistant strains (68). These considerations led to the use of bacteriophage therapy.

Their ability to self-replicate and produce polysaccharide depolymerases makes them an attractive tool for combating *P. aeruginosa* biofilm-associated infections (36, 69). Several *in vitro* studies have shown a synergistic effect between antimicrobials and antipseudomonal bacteriophages; however, such phenomena are not always observed. This may be explained by the mechanism of action of antimicrobials and the difference in the required environmental factors for biofilm formation; the latter can also be a reason for the disparity in laboratory and clinical results (29, 70). Environmental factors such as temperature, oxygen level, pH, etc. also play an important role in the interaction between phages and antimicrobial agents, and have a significant impact on the biofilm formation ability of the bacterium (71, 72). Until now, phage therapy has been safe in treating infection; however, the data are limited and may raise concerns in the future (73, 74), if the bacteriophage preparation is prepared in accordance with the quality and safety criteria for phage preparations (35, 39). Therefore, phages, such as bacteriophage cocktail BFC 1.10, which are well-described and safe for patient care, should be applied for treating infections (34). An additive effect of BFC 1.10 and ceftazidime-avibactam for planktonic cell growth and biofilm prevention was observed. Furthermore, the antibiotic concentration required for biofilm prevention decreased to the MIC cut-off value according to the EUCAST standard, making the strain susceptible to ceftazidime-avibactam (Figure 2.5).

Six months after completing treatment, the patient's wounds remained dry and closed, and laboratory inflammatory markers remained stable within normal ranges. Despite the resolution of the proximal femur side infection, MDR *P. aeruginosa* and VRE infection persisted at the distal part of femur. Therefore, our treatment of bacteriophages and antimicrobials did not lead to resolution of

infection but led to eradication of osteomyelitis in acetabular bone, which allowed hip replacement. Finally, no side effects were observed during the treatment. Fortunately, distal segment infection after right hip endoprosthesis implantation was successfully treated with DAIR and suppressive therapy using colistin and fosfomycin. To avoid persistent infection in another bone segment more accurate investigation such as labelled leukocyte scintigraphy or PET/CT could be performed. Another solution might be bacteriophage systemic application; however, it is recommended to us phages topically if possible. Phage application in femoral canal could be helpful but, in this case, we did not perform because the canal consisted of sclerotic lesions, and it was not possible to insert an irrigation system in it. In case of endoprosthesis associated infections a hydrogel coating with impregnated bacteriophages could be used. Such approach has been described and can retain the implant; however, the data is very limited (75).

Experimental use of bacteriophages in the treatment of multidrug-resistant *Pseudomonas aeruginosa* LVAD driveline infection

LVAD implantation has become more common in recent years, mainly as a bridge to heart transplantation (76). In addition to right ventricular failure, bleeding, thromboembolism, and pump malfunction, infection is one of the common complications observed (77, 78). Only 58.9 % of patients with LVAD are estimated to not develop the first major infection in the first year after device implantation; at three years after device implantation, only 38.2 % of patients have not had a major infection (79). Driveline infections are among the most common infections associated with LVAD, with a prevalence of 18.8–100 % of all infections related to LVAD (80). Similar to other implanted device infections, LVAD-associated infection is difficult to treat and often surgical intervention is required (81). The standard treatment for driveline-associated infections involves

systemic antibiotics; however, commonly, such an approach leads to treatment failure, and surgical relocation of LVAD driveline is necessary. In a study in Warsaw, Poland, the primary success rate with antibiotics was only 27 %, driveline repositioning was needed for 73.1 % of patients, and the mortality in these patients was 11.5 % (81).

The primary pathogens causing such infections are Gram-positive cocci. However, Gram-negative bacilli are becoming a common and concerning problem for patients with LVAD because they are frequently having multidrug resistance (23, 82).. In the ASSIST-ICD study in which 19 centres were involved, *P. aeruginosa* infections were detected in 13.7 % of cases (83, 84).

Our results show that the topical and intravenous application of phages combined with antibiotics and surgical treatment can be most appropriate for the successful outcome of infections associated with LVAD. There are two more cases of MDR *P. aeruginosa* LVAD-associated infection treatment described using phages. In one case, the same treatment modality was used with clinical cure and bacterial eradication (85). In the other case, only phages and antibiotics were used, and the treatment failed, with relapse of infection and development of phage resistance during treatment (86). Our case also shows that intravenously applied phages in concentration 10^7 were safe and did not elicit side effects similar to the results presented by Aslam et al. (87). Therefore, for future phage applications, a dosage of 10^7 PFU/mL for antipseudomonal phages could be optimal.

The application of phages in an *in vitro* biofilm model yielded varying results. Both phages, when used in the host (maternal) strain CN573 could lyse planktonic cells and eradicate established biofilm. In contrast, for the patient isolate, PAP01, biofilm eradication was not achieved. One of the obstacles that could explain these results is the bacterial susceptibility to the phages used, which was better for CN573 than for PAP01; for instance, the EOP for PAP01

using PNM was only 0.0005. When pre-adapted to host bacteria, as was the case for PNM and PT07 in CN573, phages are more efficient. Therefore, the best phage therapy results are expected to be achieved using personalised phage preparations, containing phages that were selected, or even pre-adapted, to better target the patient's infecting strain(s) (88). However, such an approach is time- and cost-consuming, making phage therapy harder to apply in a clinical setting. However, it is important to note that last-resort antibiotic treatment is also highly expensive and not always available. Another critical factor is the structure of the biofilm; according to our results, P. aeruginosa strain CN573 produced greater biofilm than strain PAP01, assuming that it would be easier for phages to eradicate the biofilm of PAP01. However, this was not observed and could be explained by differences in biofilm density. *Hu et al.* showed that phage penetration depends on biofilm density (89). This is one of the limitations of our study because we did not determine the biofilm density, and other methods, such as confocal laser scanning microscopy, should be used to identify the biofilm density. This could clarify whether the weak biofilm eradication effect of the phages in PAP01 was associated with the density of the biofilm.

The development of phage-resistant bacterial strains can occur quickly both *in vitro* and *in vivo* (90); by reducing the density of the biofilm, the presence of phage-resistant strains can be detected (26). In our study, resistance to phages was observed in the biofilm eradication model, with an incidence rate ranging from 70 % to 100 % for PNM and PT07, as well as for their combinations, using three different concentrations. Therefore, this could explain their failure to eradicate the biofilm. Resistance was present in the biofilm prevention model, but it was less common; for PNM, it was 80 %–100 %; for PT07, 10 %–40 %, but when using a combination of these phages, it was 10 %–20 % (Figure 2.8). Strategies involving combined treatment of phages and antibiotics can lead to a better outcome because the development of resistance against one agent can

elicit increased susceptibility to another agent (91). A study by *Burmeister et al.* showed that there is even a trade-off between phage resistance and antibiotic resistance, which means that in phage-resistant strains, a possible susceptibility to antibiotics can evolve (92). However, the interaction of phages and antibiotics is complex and does not always exhibit a synergistic effect; on the contrary, even antagonistic effects could be observed. The effect of phage-antibiotic combinations depends on the administration order, the concentration of phage, and the antibiotic's and the phage's mechanism of action (29, 93). The results show that ceftazidime-avibactam alone is unable to kill planktonic cells, requiring an increase in antibiotic concentration, while a combination treatment of 12 h of phage PNM, PT07 or a combination of both followed by 12 h of antibiotic has a strong planktonic cell growth inhibition effect. In contrast, inhibition of bacterial cell growth in the biofilm required a 3-fold higher concentration of ceftazidime-avibactam reaching the threshold to define the bacterium as resistant to this antibiotic. Using a strategy where antibiotics are applied for a further 12 h after 12 h of phage exposure, biofilm eradication was observed in all experiments regardless of antibiotic concentration. These data suggest that a maximal antibiotic concentration is not necessary to achieve the desired biofilm eradication effect if phages are correctly combined with antibiotics, i.e., the duration and sequence of their application are precisely determined.

The current standard treatment of LVAD biofilm-associated infections involves debridement and antibiotic therapy. Surgical intervention is crucial to mechanically remove and eliminate biofilm from the driveline (80). Similarly, in our case, debridement and repositioning of the driveline were performed and, most likely, were the cornerstone for biofilm eradication; however, the formation of new biofilm and the development of bacteraemia or septicaemia from the residual bacterial cells were prevented with antibiotics and phages.

Another reason to supplement antibiotic treatment with phages is the rapid development of antibiotic resistance in P. aeruginosa. This happens due to the presence of intrinsic and acquired resistance mechanisms (94). Bacterial isolates of our patient also showed changes in the pattern of resistance to antibiotics throughout the time of infection (Table 2.5). Therefore, we cannot be sure that resistance to amikacin and ceftazidime-avibactam will not occur during treatment. This explains the need to use other effective agents as phages in MDR bacterial infections.

Critical assessment of the work

The study had several limitations, one of them being the low diversity of bacterial isolates, which could be increased by involving patients from different regions, countries. Lack of diversity may inaccurately assess the bacteriophage effect.

The pattern and conditions of detection of bacterial biofilm formation have a significant impact on the result obtained; it is difficult to predict and prove the existence of an identical result in an organism, therefore the results obtained only reflect the biofilm forming capacity of the bacterium.

The lytic effect of bacteriophages in humans is influenced by multiple factors such as pH, the production of phage neutralising antibodies, non-specific immunity factors, therefore the *in vitro* efficacy of phages is only partially indicative of their potential effect *in vivo*. The interaction of bacteriophages with bacteria in an infection is a dynamic process, during which both are subject to change, phage resistance can develop and natural phage adaptation to overcome resistance can occur. Therefore, the identified resistance outcomes and the adaptation to overcome resistance provide theoretical evidence for the existence of both processes and their potential impact on the outcome. The interaction of bacteriophages with antibiotic agents is diverse and not all possible combinations

and regimens of antibiotics and phages used were identified during the work, which may lead to a selective selection of the results obtained.

Only two patients with life-threatening multidrug-resistant infections were treated during the work, which does not allow broad conclusions on the efficacy and safety of phage therapy from a clinical point of view. Rather, the results add to existing data on phage therapy. A more accurate assessment would require the involvement of a larger number of patients and clinical trials.

Conclusions

1. The hypothesis of this thesis is partially confirmed, because bacteriophages and their combination with antibiotics have the ability to eradicate biofilms in some isolated bacteria. In bacterial biofilms where eradication was not observed, additional steps such as phage adaptation or specific bacteriophage and antibiotic regimens are required to achieve the desired result.
2. *S. aureus* colonisation is associated with higher mortality in PD patients.
    a. PD patients are colonised with phenotypically distinct *S. aureus* isolates.
3. Bacteriophages have the ability to eradicate bacterial biofilms and inhibit planktonic cell growth in both antibiotic-susceptible and resistant uropathogenic *E. coli*.
4. *P. aeruginosa* phage resistance develops relatively rapidly *in vitro* models and affects the outcome of biofilm eradication.
    a. Phage resistance in *S. aureus* and *E. coli* can be overcome by phage-bacterium adaptation.
5. Topical phage therapy and systemic therapy in combination with antibiotics are safe and do not cause serious side effects.
    a. BFC 1.10 phage cocktail has an additive interaction with ceftazidime-avibactam. The combination of these agents with surgical therapy may result in clinical cure of MDR *P. aeruginosa* osteomyelitis, but not in complete bacterial eradication.
    b. The PNM and PT07 phages in combination with ceftazidime-avibactam have greater antibacterial efficacy when the phages are used first, followed by the antibiotic. When combined with surgical therapy, these agents can cure MDR *P. aeruginosa* LVAD cable infection.

List of publications, reports and patents on the topic of the Thesis

Publications in international peer-reviewed journals

1. **Racenis, K.,** Kroica, J., Rezevska, D., Avotins, L., Skuditis, E., Popova, A., . . . Petersons, A. (2020). S. aureus Colonization, Biofilm Production, and Phage Susceptibility in Peritoneal Dialysis Patients. *Antibiotics, 9*(9), 582. doi:10.3390/antibiotics9090582

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Publications in local peer-reviewed journals

1. **Rācenis K.,** Kroiča J., Eglīte L., Mihailova I., Reinis A., Skadiņš I., . . . Petersons, A. (2017). Bakteriofāgu līdzskā efekta vērtējums biofilmu producējošās uropatogēnās E.coli kultūrās. *RSU Zinātniskie raksti,* 166–173.

Reports and theses at international congresses and conferences

1. **Racenis, K.** 2023. Phage therapy: a personalized approach in the treatment of multi-drug resistant infections. *PMNET Forum 2023.* Riga, Latvia. (oral presentation)

2. **Rācenis K.,** Kroiča J., Rezevska D., Mukāne L., Pētersons A. 2023. The clinical application of bacteriophages in complicated biofilm-associated infections. *RSU Research week 2023: Knowledge for Use in Practice*. Riga, Latvia. (oral presentation)

3. **Racenis K.,** Petersons A., Kroica J. 2022. Combination treatment of antibiotics and phages in biofilm-related infections. *44TH ICMM World congress 2021*. Brussels, Belgium. (oral presentation)

4. **Rācenis, K.**, Pētersons, A., Munkena, Z., Rezevska, D., Broks, R., Gailīte, L., & Kroiča, J. 2021. Risk factor detection for biofilm formation in *E. coli*. 275. *RSU Research week 2021: Knowledge for Use in Practice*. Riga, Latvia. (oral presentation)

5. **Racenis K.,** Kroica J., Petersons A. 2019. Detection of biofilm formation among uropathogenic ESBL-producing Escherichia coli. *29th European Congress of Clinical Microbiology and Infectious Disease.* Amsterdam, Netherlands. (poster presentation)

6. **Racenis K.** 2019. Bacteriophage use in bacterial biofilm treatment. Reintroduction of *Bakteriophages in today's medicine space. Phage Meeting Berlin 2019*. Berlin, Germany. (oral presentation)

7. **Racenis K.,** Kroica J, Mukane L., Petersons A., 2019. *E. coli* biofilm eradication using meropenem and INTESTI bacteriophage cocktail. *77th International Scientific Conference of the University of Latvia*. Riga, Latvia. (oral presentation)

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1. **Rācenis K.,** Kroiča J., Avotiņš L., Vītola L., Saulkane L., Puide I., Mihailova I., Pētersons A. 2018. S. aureus dekontaminācija nefroloģiskiem pacientiem, izmantojot dažādus bakteriofāgus. *Rīgas Stradiņa Universitātes Zinātniskā konference, 2018.* Rīga, Latvija. (oral presentation)

2. **Rācenis K.,** Eglīte L., Balode A.O., Pētersons A., Silda A., Kroiča J. 2017. Bakteriofāgu līdzīgā efekta izvērtējums no pacientiem izdalītās E.coli kultūrās. *RSU Zinātniskā konference, 2017*. Rīga, Latvija. (oral presentation)

3. **Rācenis K.** Bakteriofāgu līticskā efekta vērtējums biofilmu producējošās uropatogēnās E.coli kultūrās. 2017. RSU TIF rezidentu 20. zinātniski-praktiskā konference un konkurss "Mūsdienu aktualitātes medicīnā". Rīga, Latvija. (oral presentation)

4. **Rācenis K,** Eglīte L., Dišlers A., Klinklava V. O., Kroiča J. 2016. Bakteriofāgu līticsko spēju raksturojums *in vitro*. *Rīgas Stradiņa universitātes 15. Zinātniskā konference*. Rīga, Latvija. (poster presentation)

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