Master's Thesis

Potential of phage therapy against uropathogenic antibiotic resistant *Escherichia coli* biofilms

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Urinary tract infections (UTIs) are one of the most common bacterial infections in the world. The majority of UTIs are caused by uropathogenic *E. coli* (UPEC) which tend to live in surface-attached microbe communities, known as biofilms. Urinary tract infections can be cured with antibiotics, generally with good success. However, increased antibiotic resistance has led to a rise of recurrent UTIs and biofilms are in fact known to reduce the effect of antibiotics. One potential alternative to treat such infections in the future is phage therapy, i.e., using viruses that kill bacteria. The aim of this study was to find out how different bacteriophages (phages) can eliminate three different UPEC strains (EC7, EC10, EC38) growing in biofilms. Five different phages were produced for each strain. Biofilms of each strain were grown in the pegs of 96-well plate lids and then further treated with two different concentrations of phage lysates. In the experiment were studied not only the potential of individual phages, but also their combined effect in pairs and in a cocktail of five phages. The results were obtained by measuring the optical density at a wavelength of 600 nm every 30 minutes for 24 hours using Tecan Spark® microplate reader. The study shows that several treatments effectively reduce bacterial growth and thus, proves the potential of phage therapy as an effective novel approach. When the results are further consider together with a parallel antibiotic study, it is clear that phage therapy is a promising alternative for the treatment of bacterial infections, especially when combined with antibiotic treatment.

JYVÄSKYLÄN YLIOPISTO, Matemaattis-luonnontieteellinen tiedekunta
Bio- ja ympäristötieteiden laitos
Solu- ja molekyylibiologian maisteriohjelma

Telimaa Heta	Faagiterapia mahdollisena hoitomuotona uropatogeenisiä antibioottiresistenttejä *Escherichia coli* biofilmejä vastaan
Pro gradu tutkielma:	30 s.
Työn ohjaajat:	Matti Jalasvuori, PhD, Senior Lecturer Cindy Given, PhD
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Hakusanat: antibioottiresistenssi, infektio, bakteriofagit, virtsatietulehdus

Virtsatieinfektiot ovat yksi yleisimmistä bakteeri-infektioista kaikkialla maailmassa. Selvästi eniten virtsatieinfektioita aiheuttaa uropatogeeninen *E. coli* (UPEC). Kuten lähes kaikki bakteerit, myös UPEC elää mielellään pintaan tarttuneissa bakteeriyhteisöissä eli biofilmeissä. Virtsatieinfektioita hoidetaan antibiooteilla, yleisesti ottaen hyvällä menestyksellä. Antibioottiresistenssin lisääntyminen on kuitenkin lisännyt uusiutuvien virtsatieinfektioiden määrää ja biofilmien tiedetäänkin heikentävän antibioottiyhdisteiden tehoa. Yksi potentiaalinen keino hoitaa uusiutuvia virtsatietulehduksia on faagiterapia eli bakteereita tappavien viruksien terapeuttinen hyödyntäminen. Tämän työn tarkoituksena oli selvittää, kuinka eri bakteriofagit (faagit) pystyvät eliminoimaan biofilmeissä kasvaneita antibioottiresistenssejä UPEC kantoja (EC7, EC10, EC38). Koetta varten tuotettiin kutakin kantaa kohden viittä faagia. Bakteerikantojen annetiin muodostaa biofilmi 96-kuoppalevyjen kansipiikkeihin, jonka jälkeen ne altistettiin faageille. Kokeessa testattiin sekä yksittäisten faagien kykyä eliminoida biofilmejä, että kahden ja viiden faagin yhdistelmiä. Data saatiin mittaamalla optinen tiheys (600 nm) Tecan Spark® mikrolevyn lukijalla. Eri faagihoidoilla huomattiin olevan bakteereiden kasvua estävä vaikutus ja näin ollen tutkimus osoittaa, että faagiterapia on potentiaalinen vaihtoehto biofilmeissä elävien bakteereiden aiheuttamien infektioiden hoitoon. Kun tuloksia tarkastellaan yhdessä rinnakkaisen antibioottitutkimuksen kanssa, voidaan todeta, että erityisen lupaavia tuloksia saadaan kun käytetään sekä faageja että antibiootteja.

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TERMS AND ABBREVIATIONS

Terms

Antibiotic resistance	genetic change in the bacterium that gives it the ability to tolerate the effect of the antibiotic
Bacterial biofilm	bacterial community attached to the solid surface
Bacteriophage	group of viruses that infect bacteria
E. coli	bacterial species *Escherichia coli*
Phage	bacteriophage
Phage therapy	use of bacteriophages to treat bacterial infections
Uropathogenic	pathogen that is able to cause infection in urinary tract

Abbreviations

AMR	antimicrobial resistance
PAS	phage-antibiotic synergy
UTI	urinary tract infection
UPEC	uropathogenic *E. coli*
IBC	intracellular bacterial community
QIR	quiescent intracellular reservoir

1 INTRODUCTION

1.1 Urinary tract infections

Urinary tract infections (UTIs) are infections in the urinary system, including urethra, urinary bladder, ureters, and kidneys. The majority of UTIs are caused by bacteria, usually a member of Enterobacteriaceae family and predominantly *Escherichia coli* (*E. coli*) (Ikäheimo et al. 1996, Flores-Mireles et al. 2015) Being one of the most common bacterial infections worldwide (Kim et al. 2021), UTIs cause a vast burden on health care with an estimated 150 million annual infections (Mohiuddin & Nasirullah 2019).

UTIs can be classified in several ways, such as based on where in the urinary tract the infection occurs. In general, UTI develops when the pathogen ascends the urethra to the bladder, which is the most targeted site of UTIs (Terlizzi et al. 2017). The infection of bladder is known as cystitis. The number of bacteria cells in urine for the diagnosis is generally defined to be $\geq 10^5$ per ml (Kim et al. 2021). An untreated infection can lead to the pathogen entering the kidneys through the ureters, in which case the infection is classified as pyelonephritis. The symptoms related to cystitis are frequent and painful urination, suprapubic pain as well as bloody urine, whereas pyelonephritis might cause further symptoms such as fever, chills, backpain and nausea (Flores-Mireles et al. 2015, McLellan & Hunstad 2016).

UTIs are significantly more common in women than in men. A reason for this gender distribution is the shorter urethra in women, which makes it more favorable for bacteria to travel up the urethra. In addition, the distance between the anus and the urethra is shorter in women and thus, makes women more prone to infection, as the pathogen is most commonly from the patient's own gut. In menopausal women, the infection appears easily due to decreased estrogen levels. High estrogen levels help preserve healthy mucous membranes, which further benefits health promoting microbes (mainly lactobacilli) to maintain a low pH value. In addition to gender, other risk factors are sexual intercourse, infrequent urination, incomplete urination, current or history of catheterization, diabetes, pregnancy and less common factors such as HIV and renal transplant (Al-Hayek 2009).

There are multiple ways for the host to fight against UTIs, both natural and acquired factors. The urination flow already disposes most of the bacteria out of the human body. The transitional epithelium of the urinary bladder secretes glycosamines which create a mucin layer on the bladder walls, thus preventing attachment of bacterial cells (Kucheria et al. 2005). In addition, several compounds are released into the bladder lumen, such as antimicrobial peptides and lysozyme in order to make conditions unfavorable for invading bacteria (McLellan & Hunstad 2016). However, the human defense mechanisms are not

always able to prevent the progression of the bacterial infection and medical treatment is needed. The primary treatment for UTIs is antibiotics prescribed by a doctor, most commonly trimethoprim alone or in a combination with sulfamethoxazole as well as β-lactams, fluoroquinolones, nitrofurantoin and fosfomycin tromethamine (Jancel & Dudas 2002).

Sometimes UTIs tend to occur frequently. It could be due to a persistent strain of bacteria or a completely new infection. UTI is typically classified as recurrent when the infection occurs twice within six months or at least three times within one year (Kumar & Kumar 2018). Some patients suffer from the problem even more often. Frequent urinary tract infections are without a question a highly uncomfortable condition that can reduce the quality of life. Thus, and not surprisingly, in addition to physical symptoms, recurrent UTIs are associated with symptoms of anxiety and depression. (Medina & Castillo-Pino 2019)

1.2 Bacterial biofilms

Bacteria can live as a free planktonic cell or as a part of a bacterial biofilm. Bacterial biofilms are communities of bacteria attached to a solid surface in either biological or abiotic environment (Łusiak-Szelachowska et al. 2020). In case of UTIs, biofilms adhere to the surface of the urinary tract, mainly to the walls of the urinary bladder. Most of the biofilm structure is not actual bacterial cells, but a matrix, a slimy structure containing polysaccharides, proteins, lipids and extracellular nucleic acids produced by the bacteria (Łusiak-Szelachowska et al. 2020). However, the largest part of the matrix is water, and it constitutes up to 97 percent of the entire biofilm. Water takes care of the hydration of the biofilm and is important for bacteria to obtain the essential nutrients (Rather et al. 2021).

The transition from the planktonic bacterium becoming part of the biofilm is dependent on the ability of the bacterium to start producing the necessary adhesins and the above-mentioned extracellular matrix components. The formation of biofilms can be carried out via multiple routes and mechanisms. However, it seems that quorum-sensing plays significant role in such processes (Tolker-Nielsen 2014). Other likely necessary factors for biofilm formation are temperature, pH, gravitational and hydrodynamic forces, Brownian movement and for instance, different kinds of signaling molecules (Rather et al. 2021).

To simplify, the formation of biofilm can be divided into four main stages: attachment, microcolony formation, maturation and dispersion as illustrated below (Fig. 1). In favourable conditions, planktonic bacteria attach to the surface. There are two types of attachment, reversible and irreversible attachment. Reversible attached bacteria are only loosely attached whereas irreversible attached bacteria change the orientation becoming a flat structure and therefore already gaining protection against physical factors that are trying to inhibit biofilm formation. This is followed by formation of microcolonies as bacteria are multiplying and producing matrix components. These components then play a crucial role in maturation of biofilm. The mature biofilm has three different layers:

inner regulating layer, middle microbial basement layer and outer layer. The outer layer contains planktonic bacteria which are ready to start a new cycle as the biofilm structure finally go through dispersion (Rather et al 2021).

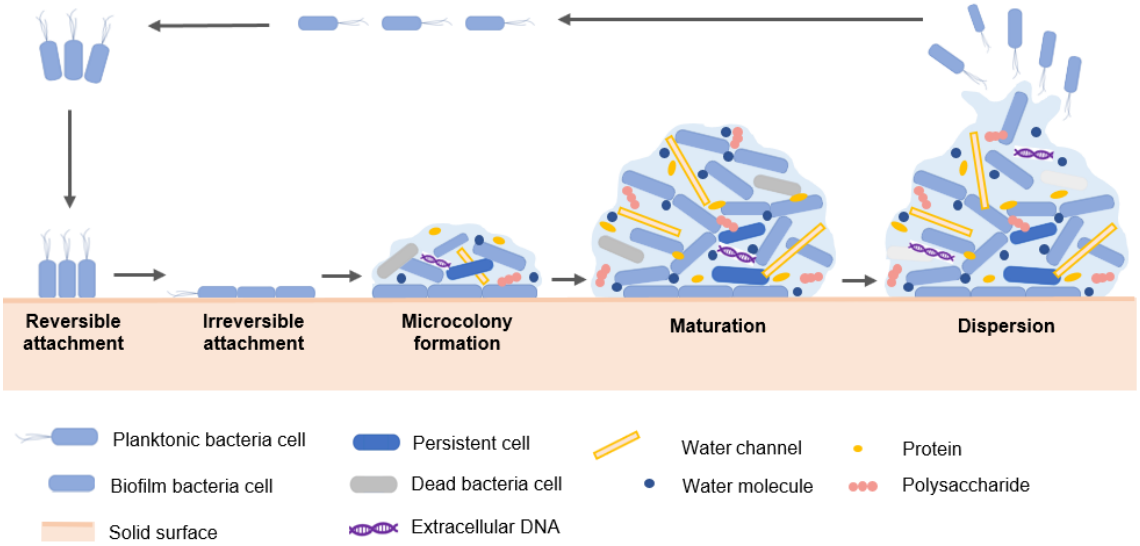

Fig. 1. Simplified illustration of bacterial biofilm formation. The four main stages are attachment, microcolony formation, maturation, and dispersion (Rather et al. 2021, reillustration).

1.3 Uropathogenic *E. coli*

Escherichia coli (*E. coli*) is a gram-negative and rod-shaped bacterium species, which has been studied quite extensively due to its ability to grow easily in laboratory conditions with low costs. Thus, it often serves as a model microbe for biological research such as biological engineering (Terlizzi et al. 2017). This harmless commensal of the gut (Kasper et al. 2004) causes most of all urinary tract infections throughout the world (Terlizzi et al. 2017). In fact, *E. coli* causes up to 90% of all UTIs in young women (Raeispour & Ranjbar 2018) *E. coli* strains causing UTIs are often collectively called uropathogenic *E. coli* (UPEC). It is a genetically heterogenous group which expresses several sorts of virulence factors (Sharma et al. 2016), such as adhesins, flagella, polysaccharides on the cell surface, toxins as well as iron-acquisition mechanisms (Terlizzi et al. 2017).

UPEC causes a urinary tract infection by entering the urinary system along urethra and ascending to the bladder lumen where it initially lives in a planktonic form in urine. This is followed by adhesion of bacterial cells to the bladder walls, which initiates an interaction with the epithelial defense system and formation of biofilms. Hereafter, bacteria start forming intracellular bacterial communities (IBCs) in underlying urothelium, where replication efficiency can go up to 10^5 new bacteria per cell. In IBCs, quiescent intracellular reservoirs (QIRs) are produced (Terlizzi et al. 2017). These are subsets of UPEC that stay hidden from the immune system. It has been suggested already a relatively long time ago that

persistent recurrent UTIs may often be due to QIRs instead of a new bacterial invasion (Mysorekar & Hultgren 2006). From the bladder, UPEC can eventually ascend all the way to the kidneys. This is exceptionally dangerous for the patient as the risk of septicemia (blood poisoning) is greatly increased (Terlizzi et al. 2017).

1.4 Antibiotic and antimicrobial resistance

Antibiotic resistance is without a question one of the most concerning threats to human health today, affecting all age groups in all countries of the world. Antibiotic resistance occurs due to a genetic change in the bacterium that gives it the ability to tolerate the effect of the antibiotic (WHO 2020). A broader term, antimicrobial resistance (AMR) is used a lot today, also covering the resistance of viruses, fungi and parasites to the drugs developed against them. However, the antibiotic resistance of bacteria is the most significant part of this larger phenomenon (WHO 2021). The first observation of antibiotic-resistant bacteria was already made in 1947, about twenty years after Alexander Fleming first discovered penicillin (Hunter 2020). However, antibiotic resistance has only emerged as a major problem in recent years, as rates of antibiotic resistance have risen rapidly throughout the world.

The development and spread of resistance genes are a natural process, but human behavior and actions have accelerated it significantly due the overuse and misuse of antibiotics in healthcare as well as in agriculture. In addition, insufficient waste management and environmental transmission provides favorable conditions for the spread of antibiotic resistance (Miethke et al. 2021). The problem occurs especially in hospitals and other health care units (Cassini et al. 2019), where people are commonly exposed to antibiotics, in addition to the fact that hospitals often have patients with impaired immune systems. The most worrying estimates claim that by 2050 up to 10 million people will die each year due to AMR (de Kraker et al. 2016). In Finland, the effectiveness of antibiotics is still at a good level, although around 90 people die each year from antibiotic-resistant bacterial infections (THL 2021). Therefore, the prevention of antibiotic resistance should be paid attention all over the world, especially as traveling spreads resistant strains.

Unfortunately, the discovery of new effective antibiotics has almost stopped, mainly due to the lack of funding. Large pharmaceutical companies have not focused on the development of new antibiotics for a long time, because the returns have been poor. Therefore, it is understandable that small and medium-sized companies struggle with profitability as well (Hunter 2020). While preclinical and clinical research still receives some attention, funding for early-stage research, often academic research, is particularly low. This is worrying, because finding effective antibiotics certainly requires the mapping of completely new candidates. The development of antibiotics would require major development steps in renewing the entire financing model and close cooperation between public, academic and industrial sectors (Miethke et al. 2021).

1.4.1 Antibiotic resistant UPEC biofilms

Antibiotic resistance plays an important role also in UTIs, as the number of UTIs caused by antibiotic resistant pathogens has increased by the time as well as the spectrum of resistance (Raeispour & Ranjbar 2018). This can be found to be a logical consequence, considering that *E. coli*, the most important cause of urinary tract infections, is known to have effectively developed resistance to various antibiotics, proofed by number of studies. In their study published in 2022, Nquyen et al. showed that ciprofloxacin, amikacin, piperallicin, meropenem, and imipenem were effective against *E. coli* whereas ampicillin, nalidixic acid and cotrimoxazole were not.

For instance, a study published in 2018 examined antibiotic resistance of 60 different UPEC strains isolated from patients who were hospitalized in Tehran due to UTIs (Raeispour & Ranjbar 2018). There were several antibiotic compounds that more than half of the strains were found to express resistance: cefepime (100%), cefalothin (74%), cefpodexime (67%), nalidixic acid (63%), cotrimoxazole (54%) and yet more antibiotic compounds with fewer percentages. However, no resistance was discovered against imipenem in this study. It is good to note that the antibiotic resistance status is generally worse in Iran than on average in the world (Antimicrobial Resistance Collaborators 2022).

There are multiple ways that UPEC can express antibiotic resistance, but one important way of any bacteria to protect themselves against antibiotics is to form bacterial biofilms (Tolker-Nielsen 2014). Shah et al. published an article 2020 about studying the pattern of biofilm formation and antibiotic resistance of UPEC. Among their 57 UPEC isolates, almost 88% were positive to form biofilm *in vitro.* They discovered a strong correlation between biofilm production and antibiotic resistance, especially in the case of multidrug resistant bacteria, as around 90% of biofilm producing UPEC was found to express resistance against more than three different classes of antibiotics. Yet effective antibiotics against biofilm producing UPEC isolates were found to be norfloxacin, amikacin, imipenem, and piperacillin/tazobactam (Shah et al. 2020). However, the results show that there is a rapidly increasing need to develop alternative treatments for bacterial infections.

1.5 Phage therapy

1.5.1 Bacteriophages

Bacteriophages, or phages as short, are viruses that infect bacteria cells. They make up the largest group of viruses known and are found everywhere, meaning water, soil and even air (Ackermann & Węgrzyn 2014). The very first documentation of phages was done already 1896 by Ernest Hankin, when he observed antibacterial activity against *vibrio cholera* in two rivers in India (Hewlett 1939). In 1910, Felix d'Herelle noticed clear round zones in bacterial lawns, and started calling them plaques, a term still used today for spots formed due to the lysis of bacteria by the phage. d'Herelle continued working with

phages, later giving these viruses the name bacteriophages, meaning "bacterium eaters" (Duckworth 1976).

The most important environment for phages seems to be lysogenic bacterium (Ackermann & Węgrzyn 2014). Phages have two possible life cycles: lytic and lysogenic cycle, but there are phages that only have lytic life cycle (Azam et al. 2021). The lytic cycle, also known as virulent cycle, implies that the phage is actively producing new infecting phages which eventually leads to the death of the bacterial cell (Fig 2). The lysogenic cycle, on the other hand, means that the phage inserts its genome as latent into the bacterial genome. This is then known as prophage. It replicates more or less but does not lead to the death of the host bacterium (Ackermann & Węgrzyn 2014).

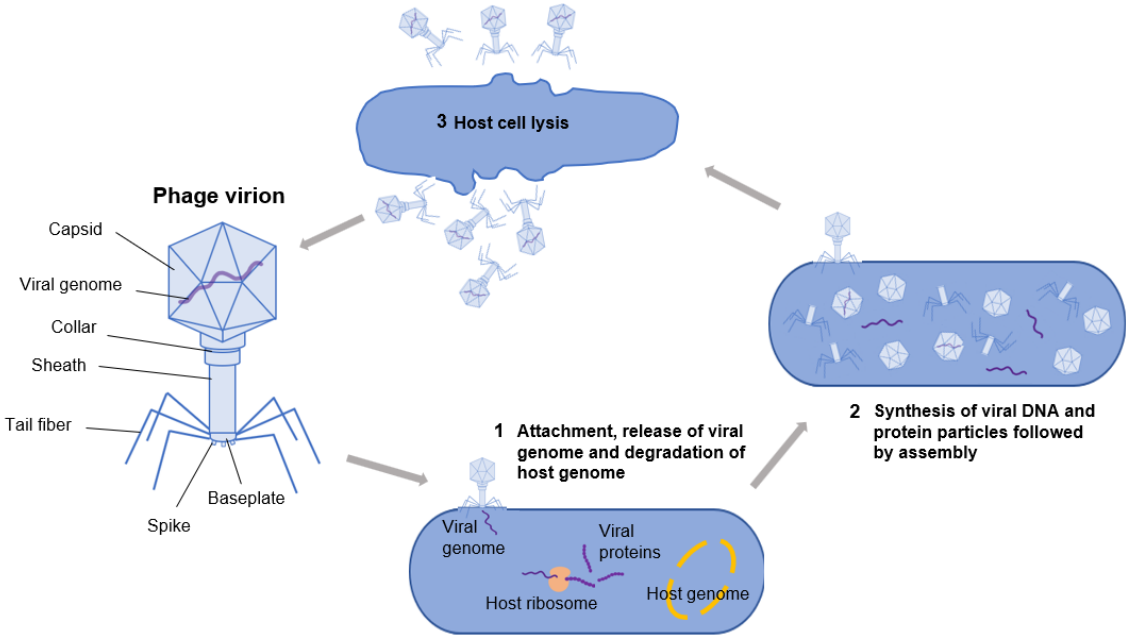

Figure 2. Lytic cycle of phages. (Luong et al. 2020, reillustration)

1.5.2 Clinical use of phages

The increased antibiotic resistance has forced the scientific community to consider alternative treatments for bacterial infections other than desperately aiming to develop new antibiotics. One of the most promising approaches is phage therapy, i.e. using phages as a drug against bacteria. In practice, this means introducing phages into the human body in order to infect and eventually destroy the pathogenic bacteria. This could be administered in several routes depending on the type of the infection, but for instance orally, intravenously or in case of wounds directly to the skin (Khalid et al. 2021).

In addition to using phages alone, phage therapy can be combined with antibiotics. The effect caused by phages and antibiotics together is called phage-antibiotic synergy (PAS), meaning that the combined efficacy to eliminate bacteria is better than the sum of using either of them separately. There is already plenty of evidence for PAS, but few observations have also been made of

antagonistic relationships between some phages and antibiotics (Łusiak-Szelachowska et al. 2022), emphasizing the importance of precisely studying the effect of both phages and antibiotics alone as well as together in order to guarantee the best treatment result.

Phages are believed to have many benefits for medical use. The main advantage is that the mechanism by which phages eliminate bacteria is completely different from the mechanism by which antibiotics destroy bacteria. Thus, phages are especially promising for treating infections caused by multi-resistant bacteria (Łusiak Szelachowska et al. 2022). Since they multiply when infecting, their number is expected to increase during treatment until there are no more bacteria cells (Loc-Carrillo & Abedon 2011). While the narrow host range might be challenging in order to find the right phage candidates, it also does not disturb the normal flora of the human body (Łusiak-Szelachowska et al. 2022). In addition, the potential for bacteria to develop resistance against phages is thought to be lower than it is against antibiotics (Loc-Carrillo & Abedon 2011).

Although phage research mostly stopped after the discovery of antibiotics, research continued in some countries, especially in the former Soviet Union and there many human phages therapy studies were performed, often with *E. Coli*. particularly (Abedon et al. 2011). Today, phage research is abundant, and phage therapy has been successfully used in cases where no other means have been found to treat antibiotic-resistant bacterial infections. As human trials have mostly been these kind of highly personalized single patient cases and usually combined with multiple antibiotic treatments, there is a lack of systematic and comprehensive human trial data (Luong et al. 2020). The Eliava Institute in Georgia was for a long time the only place where phage therapy was available for customers. Patients are either treated on site or phages are sent to countries where it is allowed (ELIAVA 2022). Regarding UPEC biofilms, Sanchez et al. published an article 2022 of their study, in which they aimed to develop phage cocktails against catheter-associated UTIs, where the formation of biofilms plays an even greater role. It proved that phage therapy for UPEC infections is a challenging task, but it clearly has a lot of potential as a future treatment option.

There are some main challenges regarding phage therapy, such as narrow host range and the possibility of bacteria to obtain resistance against phages. However, the use of phage cocktails and combining phages with antibiotics is believed to be an effective way to combat these obstacles as well as genetic manipulation of surface proteins of phages which bind to bacterial receptors. In addition, techniques such as CRISPR-Cas can be used to shut down antibiotic resistance genes, thus reducing the concern that phages would spread these genes from one bacterium to another. One concern is the low stability of phages in the blood stream, meaning that more doses may have to be given depending on the administration method. In addition to antibiotic resistant genes, different safety concerns are associated with potential toxin and virulence genes in phage genome. With whole-genome analysis and custom-made phages these issues can most likely be combated (Azam et al. 2021). The most optimal phages for therapeutics are strictly lysogenic, do not carry any bacterial genes and are stable

during storage and treatment. In addition, a good candidate would produce endonucleases which degrade the host chromosome (Fernández et al. 2019).

In summary, it is clear that the increase of antibiotic resistance threatens the entire world, and the development of novel treatment alternatives is essential. The aim of this study is to investigate the potential of phages as a treatment for infections caused by antibiotic resistant UPEC strains. Phage candidates are previously proofed to infect each strain, but only in a planktonic form. However, the hypothesis was that they also eliminate UPEC strains living in biofilms. This is particularly important to study, because biofilms and antibiotic resistance have been shown to have a strong positive correlation. UPEC biofilms were formed on the pegs of the special 96-well plate lids. The biofilms were treated with different phage treatments and later the bacterial growth was measured by OD600 using a plate reader.

2 MATERIALS AND METHODS

2.1 Materials

The bacterial strains EC7, EC10 and EC38 as well as the phages used in this study were received from Matti Jalasvuori research group (University of Jyväskylä). The bacteria have been originally isolated from urinary tract infection samples.

2.2 Methods

2.2.1 Safety management

The work included handling antibiotic resistance bacteria and thus, was carried out in BW Silver Line Biosafety cabinet (Kojair®) in BSL-2 facilities. The lab coat, shoes and gloves were changed when entering and leaving the area and all equipment disinfected with 85% ethanol. No personal belongings such as mobile phones were brough into the working area.

2.2.2 Phage lysate stock preparation

1 % Agar LB semi-confluent plates were prepared using overlay technique. Three plates were prepared for each phage and host combination. 100 µl of the o/n host culture and 50 µl or 100 µl of the original phage stock was added into the glass tube. The volume of the phage stock depends on the titer. 3 ml of soft agar (+55°C) was added into the tube and quickly poured onto plate. The top layer was left solidified. Plates were incubated o/n at +37°C.

Semi-confluent plates were then selected in order to make semi-agar. This was performed by gently scraping the top layer of the agar containing phages and host and transferring into 50 ml tube. 5 ml of sterile LB was added into the tube and incubated for 3 hours (200 rpm) at +37°C.

The tubes were centrifuged for 15 minutes, 6000 rpm at RT. The Agar and host cell debris were filtered out with 0.20 µm and 0.8/0.2 µm filter (Acrodisc® Supor® Membrane Sterile R, PALL®). The filtrate was collected into 50 ml tube. 100 µl of each filtrate was taken in order to make dilution series (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) to check the titer with overlay technique (o/n at 37°C). The remaining filtrate was further divided into 15 ml tubes and stored at +4°C.

The plaques were counted from the plates of different dilutions and the titer further calculated for each stock using the equation below.

$$\frac{PFU}{ml} = \frac{amount\ of\ plaques}{d \cdot V}, \quad (1)$$

where PFU means plaque forming units, d is the dilution and V is the volume of original phage stock addition (50 or 100 µl in this work).

2.2.3 Preparation of lysate dilutions for the experiments

Each phage lysate was diluted into two titers with a tenfold difference: 1.3E+10 PFU/ml and being 1.3E+9 PFU/ml. However, as the titers of fNenEC7p4, fNenEC6954p6 and fNenEC6p2 stocks of EC38 were not managed to get as high as aimed, their lysates were diluted into 1.3E+8 PFU/ml and 1.3E+7 PFU/ml. The lysate dilutions were stored at +4°C. Table 1 below presents the titers that are being used in the experiments.

Table 1. Phage lysate titers with a tenfold difference for each bacterial host.

	EC7		EC10		EC38	
Phage	10x (PFU/ml)	1x (PFU/ml)	10x (PFU/ml)	1x (PFU/ml)	10x (PFU/ml)	1x (PFU/ml)
fNenEC10p2	1.3E+10	1.3E+9	1.3E+10	1.3E+9		
fNenEC7p4	1.3E+10	1.3E+9	1.3E+10	1.3E+9	1.3E+8	1.3E+7
fNenEC19p1	1.3E+10	1.3E+9	1.3E+10	1.3E+9		
fNenEC6p1					1.3E+10	1.3E+9
fNen6954p6					1.3E+8	1.3E+7
fNenEC6p2					1.3E+8	1.3E+7
fNenEC10p1			1.3E+10	1.3E+9		
fTerEC10p1			1.3E+10	1.3E+9		
fTalEC7p1	1.3E+10	1.3E+9				
fNenEC7p6	1.3E+10	1.3E+9				
fTerEC38p2					1.3E+10	1.3E+9

2.2.4 Bacterial biofilm formation

The o/n liquid culture of host was added into 50 ml tube containing LB in ratio of 1µl/1ml, mixed and 150 µl further pipetted into the wells of 96-well plate (Nunclon™ Delta Surface, Sterile R, Thermo Scientific). The plate was closed with a peg lid (Nunc™ Immuno TSP Lids, Thermo-Fischer) and wrapped with parafilm. The bacteria were let to grow for four days at +37°C (no shaking). The workflow can be seen below (Fig. 3).

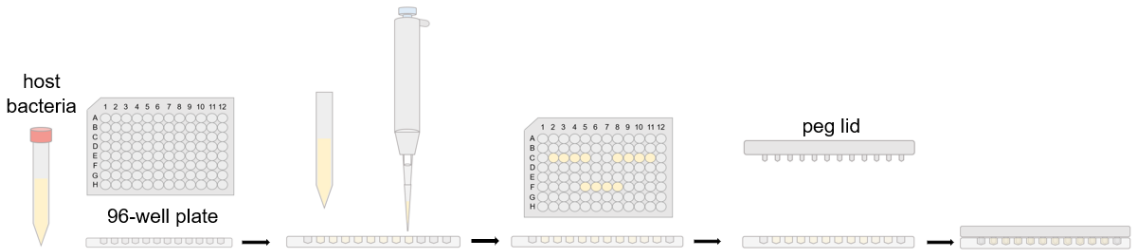

Figure 3. Bacterial biofilm formation workflow.

2.2.5 Phage treatment

Two washing plates were prepared by adding 180 µl of 1xPBS into wells of 96-well plates. The treatment plates were prepared differently according to the particular experiment. For this, see the details below. Each treatment plate contained 150 µl of LB as control in four replicates.

2.2.5.1 Individual phage treatment

150 µl of each phage lysate was pipetted into the wells of 96-well plate in four replicates as shown in Figure 4.

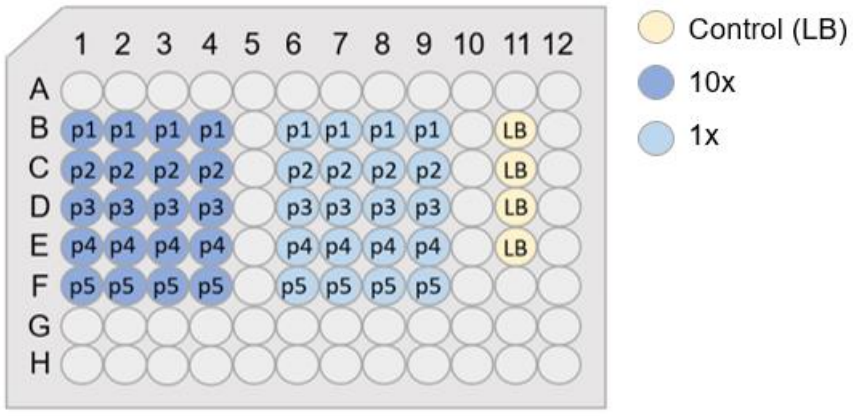

Figure 4. Treatment plate layout for the individual phage treatment plate.

2.2.5.2 Phage pair treatment

Only one of the two phage lysate concentrations were used in this experiment, according to the results of the previous experiment data of

individual phages. With EC7 and EC10, 1x dilutions were used and with EC38, 10x dilutions were used.

The pair solutions were prepared into Eppendorf tubes to contain lysate of both pairs in 1:1 ratio. The pair solutions were mixed and 150 µl pipetted into wells of 96-well plate in four replicates as shown in Figure 5.

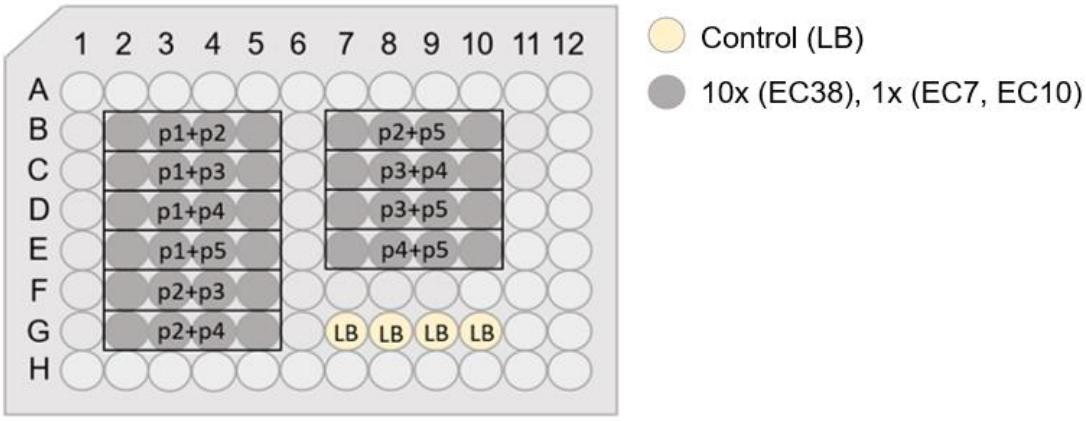

Figure 5. Treatment plate layout for the pair treatment plate.

2.2.5.3 Phage cocktail treatment

In this experiment, two cocktails containing five phages were prepared for each host strain. 10x and 1x cocktails were prepared by adding each phage lysate of particular concentration into the Eppendorf tube in 1:1 ratio. The cocktail solution was mixed and 150 µl of solution pipetted into the wells of 96-well plate in four replicates as shown in Figure 6.

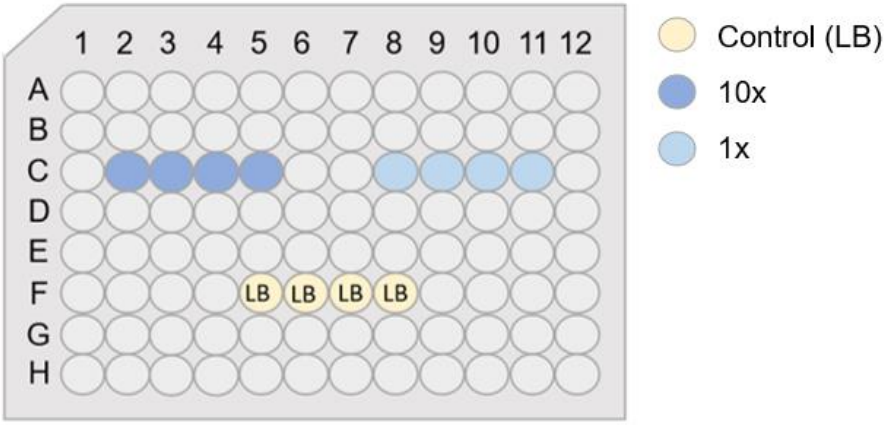

Figure 6. Treatment plate layout for the cocktail treatment plate.

The bacterial biofilm was washed by dipping the peg lid in both washing plates. The peg lid was then transferred to the treatment plate and wrapped with parafilm. The plate was left incubating for 24 hours at +37°C (no shaking).

2.2.6 OD measurement

Two washing plates were prepared by adding 180 µl of 1xPBS into the wells of 96-well plates. The OD checking plate was prepared by pipetting 180 µl of LB into the wells of 96-well plate. In addition, 180 µl of LB was pipetted into one well which will not be in contact with the biofilm. This blank sample serves as LB control.

The bacterial biofilm was washed by dipping the peg lid in both washing plates (see Fig. 7). The peg lid was then transferred to the treatment plate and incubated for 30 minutes at RT (no shaking).

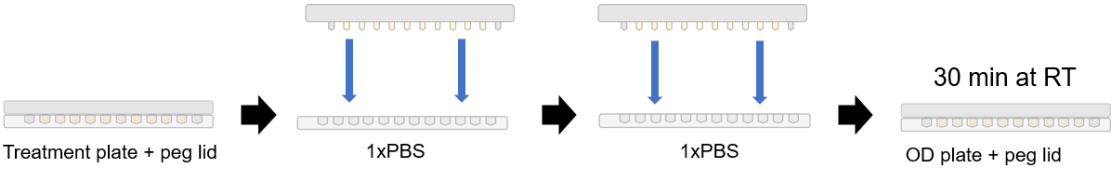

Figure 7. Washing the peg lids for OD measurement.

The peg lid was removed, and the OD checking plate closed with a normal lid. The plate was wrapped with parafilm and incubated for 2 hours at +37°C (no shaking). The optical density at 600nm was measured with microplate reader (Spark® multimode reader, Tecan) using SparkControl Magellan, Tecan, V. 3.1. software. The plate was left inside the plate reader for 24 hours and the measurement was set to occur every 30 minutes. The temperature was set +37°C. Workflow is seen in Figure 8. The data was then analysed in Excel.

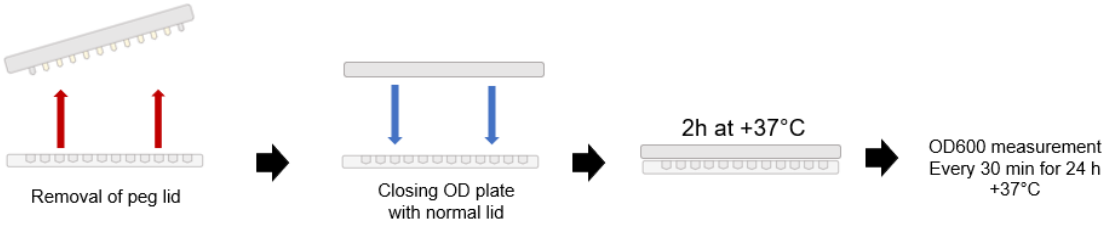

Figure 8. The preparation of OD measurement. The plate is wrapped in parafilm for the 2 hours incubation and measurement.

3 RESULTS

3.1 EC7 strain

The effect of individual phages and the combination of all five phages on EC7 growth was studied. Graphs were created from the measurement data for each individual treatment (Fig. 9A-E) and the cocktail treatment (Fig. 9F). The untreated sample does not contain any phages, thus acting as a control for viable bacteria cells. Phages fNenEC10p2, fNenEC7p4, fNenEC19p1 and fTalEC7p6 inhibited the bacterial growth, whereas fNenEC7p1 shows no effect. The cocktail treatment also inhibits the bacterial growth. 10x concentration (1.3E+10 PFU/ml) shows better efficiency than 1x (1.3E+9 PFU/ml), except with fNenEC7p4 that inhibits the growth similarly regardless of concentration and with cocktail treatment the lower concentration keeps bacterial growth low for longer time.

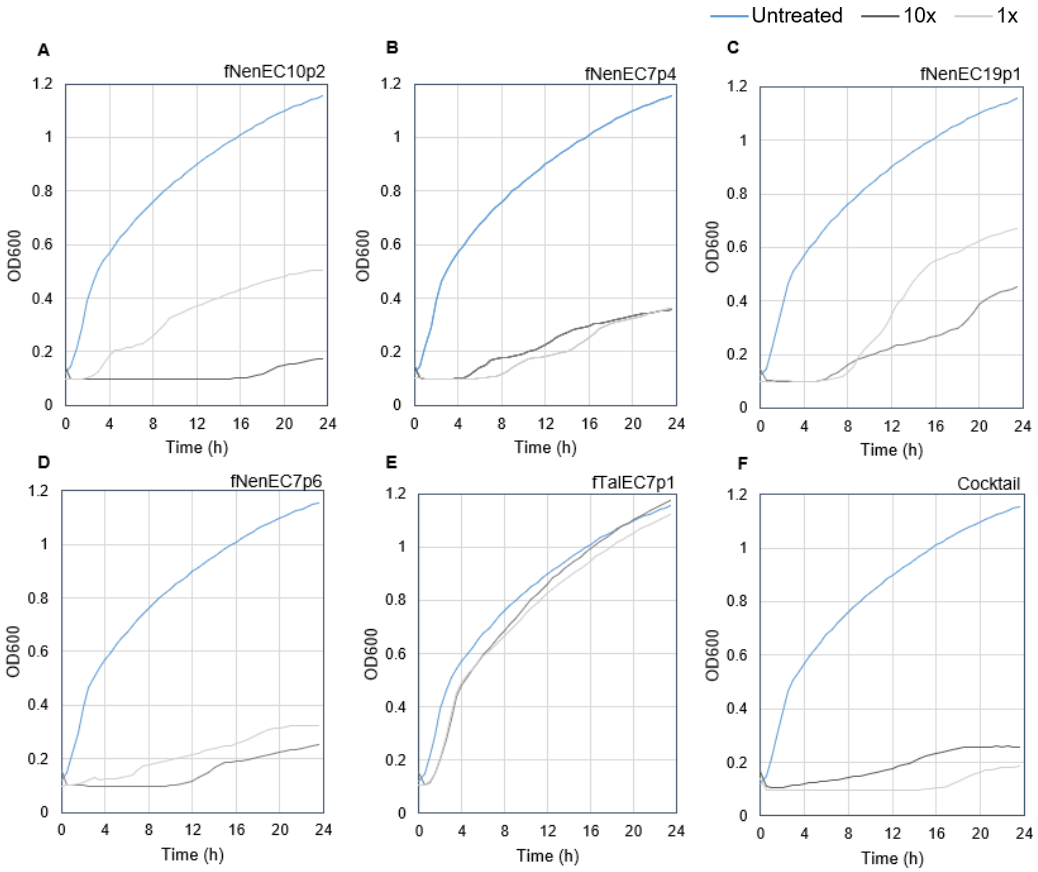

Figure 9. The effect of individual phages (A-E) and their combination (F) on the growth of EC7 cells in two different concentrations. The X-axis represents time, whereas the Y-axis describes the vitality of the bacterial cells. The untreated sample contains no phages. Phage concentrations are 1.3E+10 PFU/ml for 1x and 1.3E+9 PFU/ml for 1x.

The last measurements point (23.5 h) of above mentioned treatments were gathered into a single graph (Fig. 10), to which error bars (SD) were added to express the differences between the four replicates. It can be seen that fNenEC10p2 at a lower concentration does not guarantee an effective inhibition.

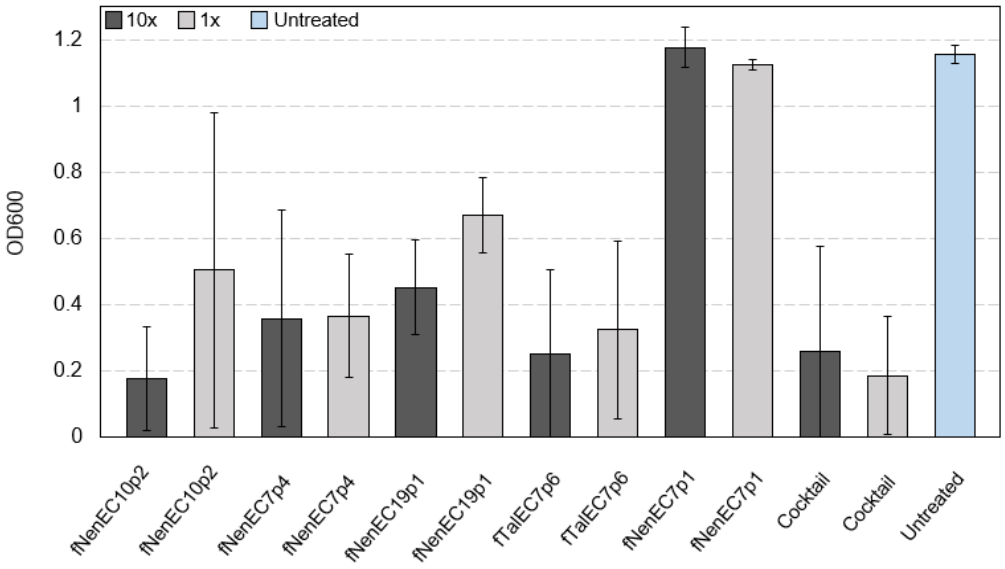

Figure 10. The last measurement points of phage treatments for EC7. Error bars present standard deviation between four replicates. The Y-axis describes the vitality of the bacterial cells. See the used concentrations on the previous page.

The effect of each phage was tested in pairs on EC7 growth. The 1x concentration (1.3E+9 PFU/ml) was used. Figure 11A shows all pair treatments measured every 30 minutes, whereas Figure 11B shows the end point (23.5 h) of the same measurements with error bars (SD). Untreated sample contains no phages. fNenEC10p2 combined with either fNenEC19p1 or fTalEC7p1 shows an excellent synergistic effect. The results show that many phages seem to inhibit the growth of bacterial cells better in pairs than individually or in combination.

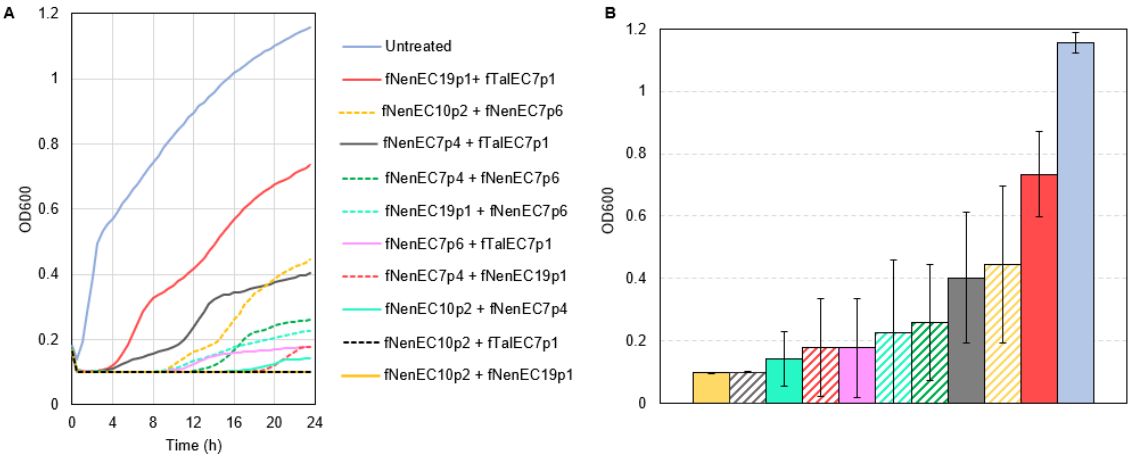

Figure 11. EC7 cells treated with phage pairs. The growth over time (**A**) and the vitality at the last measurement point (**B**) is shown. The Y-axis describes the vitality of the bacterial cells in both graphs.

3.2 EC10 strain

The effect of individual phages and the combination of all five phages on EC10 growth was studied. Graphs were created from the measurement data for each individual treatment (Fig. 12A-E) and the cocktail treatment (Fig. 12F). The untreated sample does not contain any phages, thus acting as a control for viable bacteria cells. Phages fNenEC10p2, fNenEC19p1, fNenEC10p1and fTerEC10p1 inhibited the bacterial growth, whereas fNenEC7p4 shows no effect. The cocktail treatment also inhibits the bacterial growth. The best result was obtained with 10x concentration (1.3E+10 PFU/ml) of fNenEC10p2. The concentration differences didn't show a big impact in inhibition effect, except with fNenEC10p1 which doesn't seem to decrease the bacterial growth as effectively with the lower concentration 1x (1.3E+9 PFU/ml).

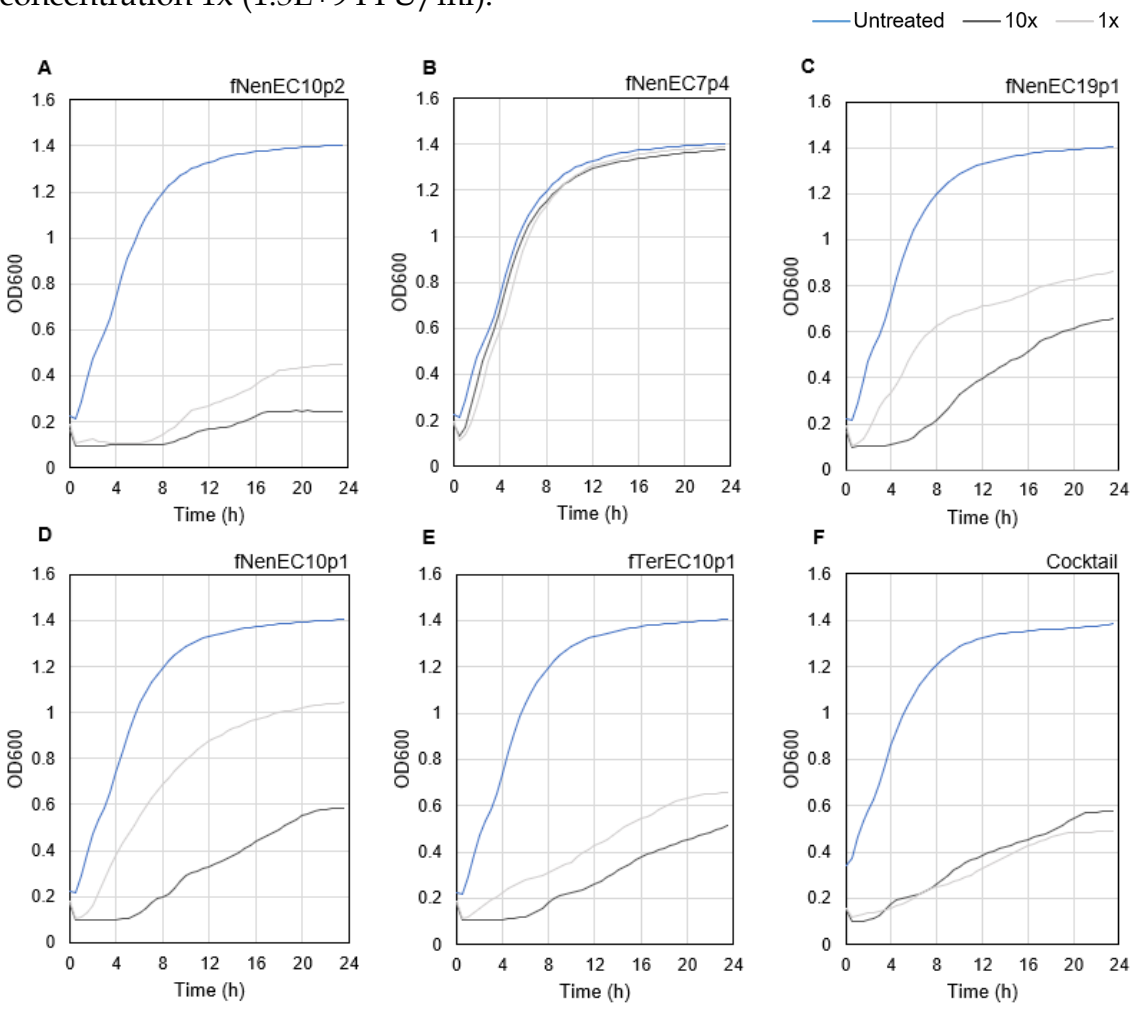

Figure 12. The effect of individual phages (**A-E**) and their combination (**F**) on the growth of EC7 cells in two different concentrations. The X-axis represents the time, whereas the Y-axis describes the vitality of the bacterial cells. Untreated sample contains no phages. Phage concentrations are 1.3E+10 PFU/ml for 1x and 1.3E+9 PFU/ml for 1x.

The last measurements point (23.5 h) of above mentioned treatments were gathered into a single graph (Fig. 13), to which error bars (SD) were added to express the relatively big differences between the four replicates. This confirms the previous observation of fNenEC10p2 being the most effective candidate.

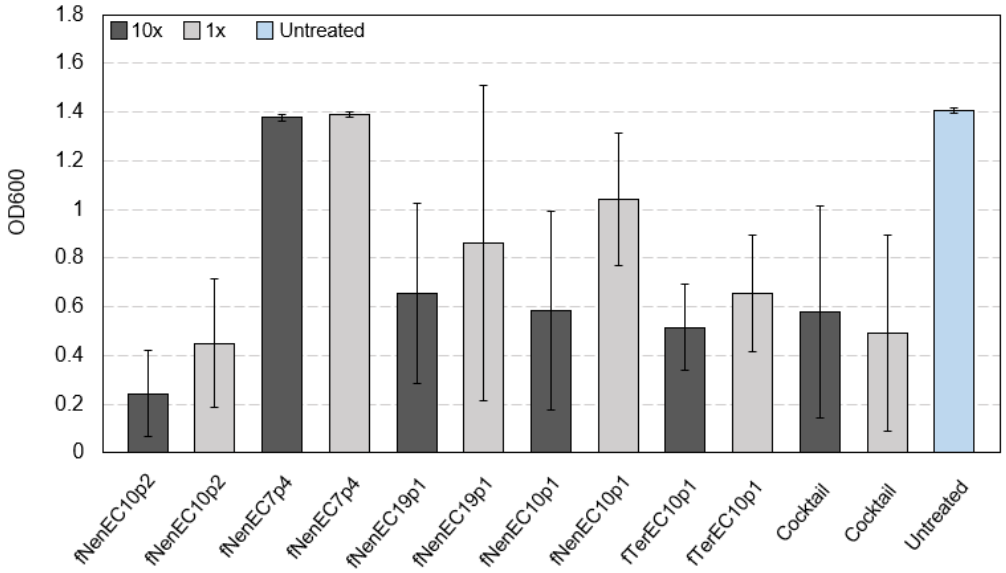

Figure 13. The last measurement points (23.5 h) of phage treatments for EC10. Error bars present standard deviation between four replicates. The Y-axis describes the vitality of the bacterial cells. See the used concentrations on the previous page.

The effect of each phage was tested in pairs on EC7 growth. The 1x concentration (1.3E+9 PFU/ml) was used. Figure 14A contains all paired treatments measured every 30 minutes, whereas Figure 14B shows the end point (23.5 h) of the same measurements with error bars (SD). Untreated sample contains no phages. fNenEC10p2 combined with other phage shows the best inhibition effect in pair treatments.

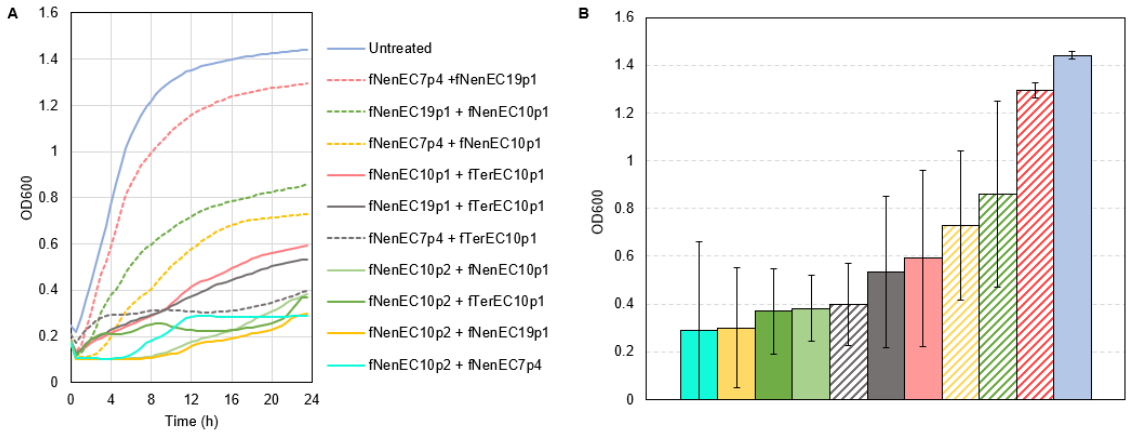

Figure 14. EC10 cells treated with phage pairs. The growth over time (**A**) and the vitality at the last measurement point (**B**) is shown. The Y-axis describes the vitality of the bacterial cells in both graphs.

3.3 EC38 strain

It should be noted that with EC38, the concentrations in all experiments vary depending on the phage. Phages marked with * have the following concentrations: 10x = 1.3E+8 PFU/ml, 1x = 1.3E+7 PFU/ml. Phages marked with ** have the following concentrations: 10x = 1.3E+9 PFU/ml, 1x = 1.3E+10 PFU/ml.

The effect of individual phages and the combination of all five phages on EC38 growth was studied. Graphs were created from the measurement data for each individual treatment (Fig. 15A-E) and the cocktail treatment (Fig. 15F). The untreated sample does not contain any phages, thus acting as a control for viable bacteria cells. None of the treatments show a great inhibition effect against EC38. However, slight inhibition is seen with all phages. In addition, bacterial growth seems to stop with many of the treatments at a certain point, after which it no longer increases.

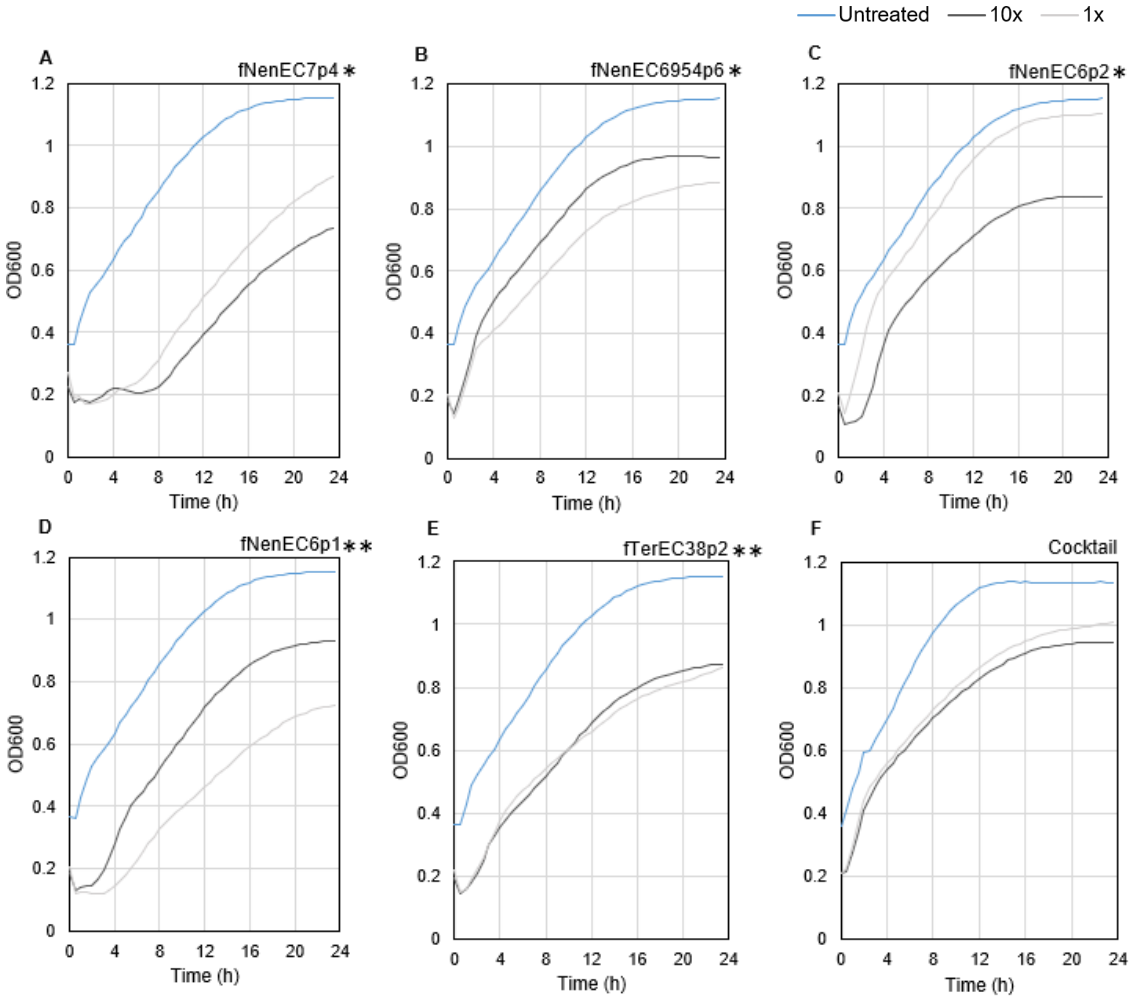

Figure 15. The effect of individual phages (**A-E**) and their combination (**F**) on the growth of EC7 cells in two different concentrations. The X-axis represents the time, whereas the Y-axis describes the vitality of the bacterial cells. Untreated sample contains no phages.

The last measurements point (23.5 h) of above mentioned treatments were gathered into a single graph (Fig. 16), to which error bars (SD) were added to express the differences between the four replicates. The graph shows the same as the previous observations: none of the treatments effectively inhibits the growth of bacteria, but a small effect can be seen.

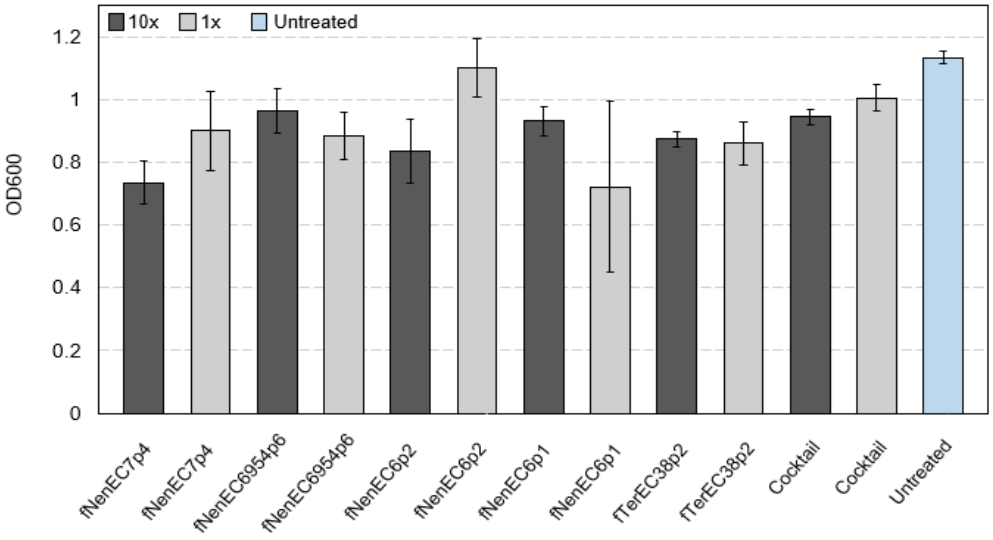

Figure 16. The last measurement points (23.5 h) of phage treatments for EC38. Error bars present standard deviation between four replicates. The Y-axis describes the vitality of the bacterial cells. See the used concentrations on previous the page.

The effect of each phage was tested in pairs on EC38 growth. The 10x concentration (1.3E+8 PFU/ml or 1.3E+10 PFU/ml) was used. See the 10x values on the previous page. Figure 17A contains all paired treatments measured every 30 minutes, whereas Figure 17B shows the end point (23.5 h) of the same measurements with error bars (SD). Untreated sample contains no phages. Paired treatments do not inhibit bacterial growth as desired. The pair treatments giving the best average value contain a lot of variation between replicates.

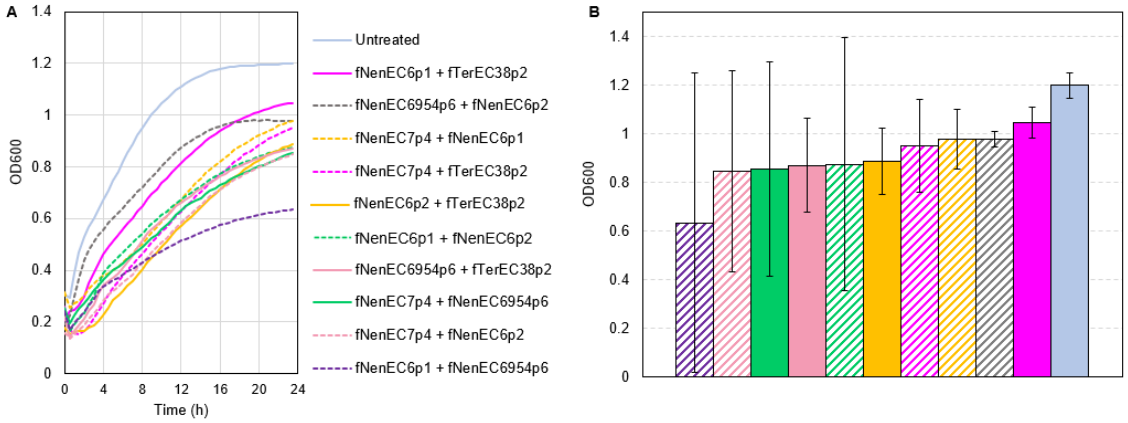

Figure 17. EC38 cells treated with phage pairs. The growth over time (**A**) and the vitality at the last measurement point (**B**) is shown. The Y-axis describes the vitality of the bacterial cells in both graphs.

4 DISCUSSION

Due to the growing trend of antibiotic resistance, phage therapy has gained a lot of interest as the treatment alternative for resistant infections However, a huge amount of preliminary research is needed in order to widely use such treatment in clinical means. The main purpose of this study was to obtain information on which phages could possibly work therapeutically, including the understanding of how they interact with each other (in pairs and cocktails). The data gives an early stage understanding, yet leaving a lot for interpretation. There are multiple possible explanations for different interactions between phages and bacteria. This should be kept in mind when interpreting the results and thus, it is important to not make too direct conclusions based on the graphs presented in results section.

In general, most of the phages show an inhibiting effect on bacterial growth. This was expected as the phages were selected among previously tested phages by the research group. These phages were proofed to infect the particular strain in planktonic form. Thus, this study shows that they have potential to eliminate these bacteria also in biofilms. On the other hand, some phage treatments did not show any eliminative effect. There is no inhibition of EC7 with fNenEC7p1 (Fig. 9E, Fig. 10) and similarly no inhibition of EC10 with fNenEC7p4 (Fig. 12B, Fig. 13). Perhaps these phages are not capable to destroy biofilm, or some external factors have been influencing the phage lysate quality, making phages incapable to infect. For all other treatments, the inhibition of bacterial growth occurred more or less. It is known that the older biofilms are harder to fight against with phage therapy (Łusiak-Szelachowska et al. 2022) and thus, would be worth considering to test the potential of the phage therapy also with older than 4 days old EC7, EC10 and EC38 biofilms (at the time of treatment) as was done in this study and further compare the inhibition efficiencies.

The interpretation of the data requires observation from several perspectives. Thus, two different graphs were made to describe each treatment. The first type of graph (Fig. 9, 11A, 12, 14A, 15 and 17A) shows the development of the bacterial density in samples over time, giving the idea of the pattern that the possible phage infection follows. In induvial phage experiments performed with EC7 strain, the curve mostly followed a trend of slower bacterial growth (Fig. 9A-E). This means that the bacteria are able to grow but not as effectively as in normal conditions. Similar pattern can be seen in the cocktail experiment (Fig. 9F) and many pair treatments of EC7 (Fig. 11A). In addition, the most desirable trend was seen in pair experiment when fNenEC10p2 was combined with either fNenEC19p1 or fTalEC7p1. There is no evidence of bacterial growth as the curve remains steadily down throughout the measurement period. This is an ideal situation when considering possible phage therapy for the patient. Interestingly, a different trend is seen with most of the experiments done with EC38 strain (Fig. 15 and 17A). The bacterial growth tends to still increase fast after treatment,

followed by slowing down and for some it eventually stops completely, as seen with fNenEC6p2 for instance (Fig. 15C).

The behaviour of the bacterial growth curve is something worth considering. It is, also in the case of early stage research, a matter of speculation that which kind of trend is more desired: initial delay in the bacterial growth that still keeps increasing slowly over time or no significant decrease of the bacterial growth in the beginning but later a slowdown followed by possible shut off. It is possible that temporarily suppressing the bacterial growth enables the patient to cure with the help of the body's own immune defences. However, it may be that this is not enough, and the bacterial growth starts to rise after the phage treatment leaving the infection remain persistently in the body. Thus, it could be thought that the phage treatment causing bacterial growth to stop later is better, even if the curve does not go as low. In this case, the recovery of a patient might take more time but in the end the treatment leads to a better outcome, ideally clearing the bacterial infection and obtaining a full recovery. Of course, it would be an ideal situation if the phage acts quickly and prevents growth throughout the treatment. However, this can possibly be the case in a real situation when the human immune defense and the phages hopefully work together in order to get rid of an UPEC infection. It has been shown with multidrug resistant *Pseudomonas aeruginosa* in a mouse model, that the outcome for bacteria elimination in phage therapy is dependent especially on phage-neutrophil synergy, which led to a cure of an acute infection (Roach et al. 2017).

The other type of the graphs shows the bacterial density of each treatment at the last measurement point (Fig. 10, 11B, 13, 14B, 16 and 17B). These graphs were created in order to describe the differences between the four replicates of each treatment. This was done by adding the error bars with standard deviation in every column. Error bars tend to remain small when there is no phage infection seen, highlighting the fact that there is no inhibiting effect in any of the samples. However, when observing the deviation withing the most promising candidates, there error bars tend to be relatively large. Thus, the average values used in the graphs do not describe accurately the individual replicates, meaning that there is a big variation in the inhibition responses. It seems that the progress of phage infection is somewhat random. However, large error bars do not necessarily mean that these phages are bad candidates. For most treatments, the inhibition is still notable compared to the control.

It can be considered as a general assumption that a higher phage concentration leads to a better inhibition because a larger number of phages are able to infect the bacterial cells in the biofilm. However, this is not always the case. Although most of the treatments in this study were more effective at the higher concentration, some worked better at the lower dose. This can be seen with fNenEC6p1 that inhibits the growth of EC38 cells better with 1x concentration than 10x concentration (Fig. 15D). The growth of bacterial cells is delayed with the lower concentration and does not later on increase as fast as with the higher concentration. However, this was not the case with all the replicates with the lower concentration, which is seen as larger error bars at the

last measurement point (Fig. 16). The question remains, however, why do some replicates have a better effect with the lower concentration, instead of practicing the expected dose-dependent manner? Surely one can speculate that the 10x lysate has been affected in some way during preparations and therefore does not show a good response. More likely the reason lies in the interaction between the bacterium and the phage, such as if the exposure of the bacterium to many phages causes some kind of defense response which at lower concentrations is not profitable trade-off enough to be expressed.

In addition, many experiments in this research show that a ten-fold difference in concentration of phage titers does not necessarily lead to any notable differences. When treating EC10 cells with the cocktail (Fig. 12A) or EC38 cells with fTerEC38p2 (Fig. 15E), there is only a very little difference in the responses obtained between 1x and 10x concentrations. Determining the optimal doses in phage therapy is not easy anyway. Phage is a self-replicating drug, so the amount should increase when it reaches the target cells and starts infecting. On the other hand, it is known that many phages never reach the target cells. This also depends a lot on how the phages are administered. In general, injection is an effective method, whereas oral administration is prone to lose a notable amount of phages to other parts of the body. Thus, further research is needed to find out what kind of doses should be used. Using a lower concentration would certainly be more ideal, as it means lower costs in phage production, at least if the purpose is to produce larger stocks which could possibly be further modified.

A phage that rapidly suppresses bacterial growth but afterwards allows it could be a potential candidate when considering repeat dosing. In this study, fNenEC10p2 is a good example of such a phage for EC7 (Fig. 9A) and EC10 infections (Fig 12A). It is able to keep the bacterial growth low for many hours compared to the control, especially at the higher concentration. Although bacterial growth eventually begins to grow, repeated doses can potentially keep it down, leading to the complete eradication of the bacteria. In a study performed in 2018, Manohar et al. examined the effect of dose repeats of a phage that infects a strain of *E. coli* and found out that four doses in 24 hours can lead to 100% recovery of *G. mellonella* larvae suffering from *E. coli* infection (Manohar et al. 2018). Thus, dose repeating is one promising option when considering phage therapy as a clinical treatment.

One of the aims of this study was to gain information on the interactions between two phages as well. This is important especially when optimizing the effect of phage cocktails. There is a possibility that some of the phages do not infect and eliminate the bacteria effectively when they are combined with another phage. These interactions are most likely the reason why the cocktails of all five phages for each strain did not automatically lead to the best responses. In the experiments done with EC38 strain, some negative interactions can be seen when combining fNenEC6p1 and fTerEC38p2 in 10x concentrations (Fig. 17), as there is more bacterial growth compared to using each of them alone (Fig. 15D-E). There may be some sort of competition between phages that makes the infecting process difficult for both of them, in case they have to share resources.

However, sometimes the presence of another phage individuals seems to be essential in order to eliminate the bacteria. Interesting data related to this phenomenon can be obtained in experiments done with EC7 strain (Fig. 12). When fNenEC10p2 is combined with either fNenEC19p1 or fTalECp1 (1x concentrations), the bacterial growth is pushed down across all the time points, resulting in a desired outcome as mentioned earlier in discussion. However, when you combine fNenEC19p1 and fTalECp1 together, the response is notably worse, being the least effective pair combination among the five phages. The individual experiments (Fig. 9) show that these phages alone do not have a good response, especially fTalECp1 which does not seem to have any effect to eliminate the bacteria. Thus, it is rather interesting that these phages are still useful when combined with fNenEC10p2, as it shows a better response than using fNenEC10p2 alone (Fig. 9A). Perhaps fNenEC10p2 is the one being able to destroy the biofilm effectively, whereas fNenEC19p1 and fTalEC7p1 are not capable of this but once getting into contact with bacterial cells, can effectively infect and further result in the total elimination of the bacteria.

One of the most promising methods of using phage therapy is to combine it with antibiotic therapy. Thus, along with this thesis, a parallel study was carried out in which the possible synergistic effect of antibiotics and phages was tested on the same bacterial biofilms EC7, EC10 and EC38 (Valkonen 2023). The phage lysates used were identical to 1x concentrations in this study, thus making the comparison rather convenient. The antibiotics used in the parallel study were trimethoprim (25mg/ml), sulfamethoxazole (20 mg/ml) and nitrofurantoin (6.25 mg/ml). EC7, EC10 and EC38 biofilms were tested to be resistant to all these antibiotics. However, when combining phage therapy with the antibiotics, notable reduction of bacterial growth was observed. In the case of EC7, its growth is effectively reduced or stopped when any phage of the five phages except fTalEC7p1 is combined with any of the three antibiotics. In the case of EC10 however, the responses were not as desirable, as fNenEC7p4 and fNenEC19p1 did not inhibit the bacterial growth when combined with any of the antibiotics. This is consistent with fNenEC7p4, as it did not show any inhibition alone. Interestingly, fNenEC19p1 did show inhibition alone but when antibiotic is added, there is no visible change in the bacterial growth compared to the control. This result gives a valuable reminder of possible antagonistic effects of antibiotic-phage therapy. It's been previously discussed that the antibiotic compound may disturb some bacterial compound that is crucial for phage infection and thus, leading to a poor outcome (Łusiak-Szelachowska et al. 2022) Since phage treatment did not show a low bacterial density of EC38 strain, it was interesting to know how the addition of antibiotics affects the inhibition effect. Fortunately, fNenEC7p4 showed a good inhibition effect when combined with the antibiotic, especially nitrofurantoin. The use of nitrofurantoin seemed to work with other phages of EC38 experiment as well.

The explanation for such PAS obtained with the parallel study is most likely due to efficiency of the treatment to break down the biofilm. As mentioned, the bacterial resistance is often due to biofilm structure being a barrier protecting the

bacterial cells in the inner layers. Compared to phages, antibiotics are rather large compounds and thus, not able to just penetrate throughout the biofilm. Alone or with the help of antibiotics, phages might be able to break down the biofilm structure enough to make the environment accessible for antibiotics and furthermore, infect the bacteria cells themselves. PAS has been proved earlier in several studies. Recently, Eskenazi et al. 2022 reported a case of a man who underwent surgery and got infected by *Klebsiella pneumoniae* that was resistant to all known antibiotics. After long-term antibiotic treatments (>2 years), phage therapy was added along with antibiotic therapy, leading to major improvements in patient's wounds and overall condition. The paper also showed the high effect of phage therapy against the biofilms of particular strain *in vitro*, thus reinforcing the notion that phage therapy can be used to fight against the challenges posed especially by biofilms. In addition, Kumaran et al. 2018 showed with *Staphylococcus aureus* biofilms that the benefit of phage-antibiotic therapy is increased when phages are given before antibiotic treatment. This set up could be an interesting option to test in the future with EC7, EC10 and EC38 as well, as now the antibiotic-phage treatment was carried out simultaneously.

In summary, the potential of phage therapy in order to fight against multidrug resistant uropathogenic EC7, EC10 and EC38 biofilms was shown in this study. Different patterns and outcomes were managed to observe with different experiments. The study confirmed that the interaction withing different phages can lead to positive or negative outcomes on reducing bacterial growth. From a clinical perspective it is important to highlight the benefit of phage therapy combined with antibiotic treatment. Overall, this study gives hope that persistent recurrent urinary UTIs can be cured effectively in the future, reducing the suffer of numerous patients and bringing us as one step closer to defeat the AMR that plays a role as one of the most worrying threats for mankind. However, more research and systematic clinical trials are needed to make this possible

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Jyväskylä May, 2023
Heta Telimaa

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