Master's Thesis

Analysis of the therapeutical properties of a phage against *Staphylococcus aureus* pig isolates, and phage isolation against *Pseudomonas aeruginosa* from hospital waste waters

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PREFACE

The work depicted in this thesis was carried out in collaboration with the University of Jyväskylä and the University of Helsinki between May and August 2016. The project started in the group of Matti Jalasvuori in the University of Jyväskylä and the main part of the work took place in the group of Mikael Skurnik in Haartman Institute in Helsinki.

I would like to thank the group leaders and my supervisors Mikael Skurnik, Matti Jalasvuori and Saija Kiljunen for this opportunity, their help and guidance during the whole endeavor that was this project. Supporting specialists Darren Smith and Zao Fang for helping with sequencing and TEM imaging, respectively. Pentti Kuusela for providing culturing plates, Annamari Heikinheimo and the team for the isolated pig *S. aureus* strains. Maria Pajunen for all the valuable advices. Sari Mattila and Pilvi Ruotsalainen for getting me started and for the massive ground work that was put to the finding of the phages. My lab mates Henni Tuomala, Suleman Qasim, Annelie Carron, Sheetal Patpatia and Tomasz Benedyk for the support and for making the nice atmosphere during my whole stay in Helsinki.

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Tiivistelmä:

Antibioottiresistenttiys on kasvava ongelma, jonka selättämiseksi tarvitaan uusia keinoja. Prekliinisten tutkimustulosten perusteella bakteereja infektoivien virusten eli faagien on havaittu olevan hyvä vaihtoehto antibiooteille. Sairaalanäytteestä löydettyä metisilliinille resistenttiä *Staphylococcus aureus* (MRSA) -kantaa vastaan eristetty bakteriofagi karakterisoitiin sekvensoimalla ja läpäisyelektronimikroskoopilla, jotta sen mahdollista terapeuttista käyttöä voitaisiin selvittää. Faagilla havaittiin olevan keskikokoinen 43,098 bp:n pituinen genomi ja faagin morfologian perusteella se lukeutuu *Siphoviridae*-heimoon kuuluviin hännällisiin faageihin. Faagi puhdistettiin anioninvaihtokromatografian avulla, jotta puhdistusmenetelmän soveltuvuutta faagipartikkelien puhdistamiseen terapiakäyttöön voitaisiin arvioida. Lisäksi faagin infektiokykyä testattiin sikakantoihin. Sekvensointitulosten perusteella faagi lukeutuu elinkierroltaan lauhkeisiin (engl. temperate) faageihin, joten se ei ole sopiva käytettäväksi faagiterapiassa. Faagin havaittiin infektoivan heikosti sioista eristettyjä MRSA-kantoja, huolimatta sen laajakirjoisuudesta ihmiskantoja vastaan. Syy heikkoon infektiivisyyteen jäi epäselväksi. Faagin avulla saatiin kuitenkin tärkeää tietoa lauhkeiden faagien käyttäytymisestä ja anioninvaihtokromatografian tehokkuudesta faagien puhdistuksessa. Lopuksi kokeiltiin uuden faagin eristämistä sairaalajätevedestä sellaisia *Pseudomonas aeruginosa* kantoja vastaan, joille ei vielä ollut löytynyt yhtään faagia. Jätevedestä onnistuttiin löytämään faagi, joka infektoi jopa 63 % testatuista 43:sta bakteerikannasta. Infektiokyky testattiin myös seitsemältä ennalta eristetyiltä *P. aeruginosa* faagilta. Uuden faagin nimeksi annettiin fHoPae01 ja se talletettiin myöhempiä tutkimuksia varten.

Avainsanat: Faagi, bakteeri, terapia, MRSA, HPLC, antibioottiresistenttiys, *Siphoviridae*

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Abstract of Master's Thesis

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Abstract:

The increasing antibiotic resistance is proving to be a major issue and new methods for combating multidrug-resistant bacteria are desperately needed. Based on recent promising preclinical results, the therapeutic use of viruses that infect bacteria (phages) seems to be a good alternative for antibiotics. The therapeutic potential of a phage, which was obtained from an isolation attempt against human methillicin resistant *Staphylococcus aureus* (MRSA) strain isolated from a hospital sample, was tested against 54 strains of MRSA isolated from pigs. The phage was characterized using sequencing and transmission electron microscopy imaging to have intermediate sized 43,098 bp long genome and to belong to the tailed *Siphoviridae* family, respectively. The phage was purified using anion exchange chromatography for imaging purposes and to evaluate how the purification method affects small and delicate virus particles. Based on the sequencing data and sensitivity tests the phage could be classified as a lysogenic (temperate) phage that cannot be used as a therapy phage due to the risk of transferring resistance and virulence factors to the target bacterial strain. The phage showed decreased infection efficiency against the majority of the tested *S. aureus* strains isolated from pigs. The reason for the reduced infectivity is still under investigation. The laboratory tests done on the phage gave valuable information of the behavior and characteristics of temperate phages and of the viability of liquid chromatography based purifying of small virus particles for therapeutic use. Finally, the isolation of novel phage against *Pseudomonas aeruginosa* strains, that were missing corresponding phages, was successfully performed from hospital waste water samples. The host range of the newly obtained phage fHoPae01 was tested against 43 *P. aeruginosa* strains together with seven preisolated phages. The results indicate that the novel phage was able to lyse almost 63 % of the target strains and it was stored to be studied further.

Keywords: Phage, bacteria, therapy, MRSA, HPLC, antibiotic resistance, *Siphoviridae*

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ABBREVIATIONS

AEC	Anion exchange chromatography
BLAST	Basic local alignment research tool
Chipster	Versatile data analysis platform with interactive visualizations and workflows
HPLC	High performance liquid chromatography
ESKAPEE	*Enterococcus. faecum, Staphylococcus. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter species, Escherichia. coli*
GMO	Genetically modified organism
IGV	Integrative genomics viewer
MDR	Multidrug-resistant
MRSA	Methillicin-resistant *Staphylococcus aureus*
MWCO	Molecular weight cut-off
NEBcutter V2.0	Virtual digestion tool provided by NewEngland BioLabs®Inc.
LPS	Lipopolysaccharide
LOS	Lipooligosaccharide
PFU	Plaque forming unit
RAST	Rapid annotation using subsystem technology
SA14P1	*Staphylococcus aureus* strain 14 phage 1
TEM	Transmission electron microscopy
WHO	World Health Organization

1. INTRODUCTION

1.1 The current status of antibiotic resistance

Antibiotic resistance is the hot topic of the 21st century as a result of the ever-increasing number of hospitalization due to multidrug-resistant (MDR) bacterial infections. A state of emergency has been acknowledged all around the world and during 2012 World Health Organization (WHO) warned of the possibility of entering an era where the antibiotics lose their power over controlling the bacterial infections (WHO, 2012). The Review on Antimicrobial Resistance published in 2016 and funded by Welcome trust and UK government states that in 2014 antimicrobial drug resistant strains caused at least 700 000 deaths annually worldwide. This number is expected to reach at least 10 million in 2050, unless action is taken (O'Neill, 2016). The urgent need for effective antimicrobial drugs has started several studies towards finding other remedies for combating the growing threat of pathogenic bacteria. From the alternative approaches, the use of clinical products containing bacteriophages shows great promise. Based on the recent preclinical testing, phage therapy seems highly selective and relatively safe augmentation or an alternative for antibiotics (Merabishvili et al., 2009; Weber-Dabrowska et al., 2016; Sarker et al., 2016).

1.2 Short historical overview of phage therapy

Phage therapy is the use of bacterial viruses, bacteriophages (or just phages) in the treatment of bacterial infections (Abedon et al., 2012). The power of this form of therapy comes from the natural pathogen-host interaction between phages and bacteria. The interaction also confers to the relatively narrow host range or infectivity of the phages and to the vast abundance, which is estimated to be 10^{30} to 10^{32} phages in the world (Whitman et al., 1998; Abedon et al., 2012). Before the availability and effectiveness of modern antibiotics, the phage research was blooming, but plagued by poor understanding about phage biology which lead to inconclusive results. Due to the lack of significant clinical trials and the discovery of penicillin in 1947, the phage research practically ended in the Western European countries and in the U.S. (for reviews, see Wittebole et al., 2011 and Parracho et al., 2012).

Today the scientists are desperately trying to find effective methods against the MDR strains of bacteria that have been emerging because of the use of antibiotics for several

decades (Reese et al., 2011). As the antibiotics are starting to lose their efficacy and the antibiotic pipeline runs dry, the interest in phage therapy has been rejuvenated. One reason for the increased interest in phages is that the resistance of bacterium to multiple antibiotics does not necessarily confer protection against phages. This means that the use of phage therapy can be focused to fight against highly pathogenic and dangerous strains to give hope for individuals with untreatable or mortal infections. So far, phage therapy has gained a firm foothold only in a few East European countries such as Poland and in the former Soviet Union countries like Georgia, where the regulatory climate is favorable and phage research is more readily funded (Wittebole et al., 2011).

1.3 Phages

1.3.1 Phage morphology and characteristics

The bacterial viruses, like many other viruses, measure from tens to hundreds of nanometers in size. Phages, however, have unique morphology which usually composes of two main protein structures: DNA filled hexagonal head-like capsid and a syringe-like tail fiber (Ackermann, 1975; Ackermann and Kropinski, 2007). Tailed phages, which are divided in to three groups – *Myoviridae*, *Shiphoviridae* and *Podoviridae* – each with varying tail morphology, forming almost the entirety (96 %) of over 6000 currently known phage species. The rest are polyhedral, pleomorphic or filamentous phages. Only a few of these phages have lipid constituents, such as lipid membranes, which makes them susceptible to solvents (Hendrix et al., 1999; Ackermann and Kropinski, 2007; Poranen et al., 2015).

Phages are lifeless outside of cells, but recognize their host bacteria based on the unique surface proteins and receptors by binding to them using specific proteins usually located in the tail fiber (Duckworth et al., 1987). The small size of phages gives them many useful attributes such as the possibility to permeate through the blood-brain barrier and fine chromatography filters. Because phages are generally robust and thrive in places where there are any bacteria present, the waste water plants, sewers, soil and outdoor water sources serve as the perfect environmental reservoir for finding phages (Lobocka et al., 2014; Mattila et al., 2015).

1.3.2 Phage life cycle

Phages multiply in bacteria by hijacking the molecular machinery of the host to form new phages. There are mainly two distinct paths that the phage reproduction life cycle can take: lytic (virulent) and lysogenic (temperate). In the lytic life cycle, phage multiplies in its host and the newly assembled phages immediately escape from the cell using enzymes to lyse the bacterial cell wall. In the case of lysogenic life cycle, the phage enters the cell and incorporates its genome to the bacterial DNA (Canchava et al., 2003). During the lysogenic cycle, phages can remain dormant in the genome indefinitely until the reproduction is triggered by suitable environmental conditions, outside signal or small-molecule communication between phages. (Duckworth et al., 1987; Oppenheim et al., 2005; Erez et al., 2017). Since phages can have reproduction life cycle that is lytic, lysogenic or a mixture of both, due to the lytic or lysogenic (temperate) nature of phages, it is important to study the therapy phages carefully to ensure efficacy and prevent adverse effects or even boosting the bacterial resistance (Merabishvili et al., 2009). Problems of lysogenic phages arise when a phage introduces new genes and coding elements to the bacterial genome in the form of virulence factors through lysogenic conversion (Bae et al., 2006). Due to prevalent issues in safety, only phages with lytic life cycles can be used in antibacterial treatment.

1.3.3 Phage host specificity

Phages have evolved to recognize the unique receptors, proteins and lipid components of the outer membrane of their host bacteria. This attributes to the fact that phages can be found in diverse pheno- and genotypes with drastically different host ranges, and basically every strain of different bacteria has their own corresponding phages (Clockie et al., 2011). The highly selective host specificity explains why the phages cannot infect or multiply in eukaryotic cells. The host specificity of phage also means that the phages only multiply in the area where the host bacteria are present, thus making them efficient combating the bacterial population locally. Thanks to the advantages of the highly specific pathogen-host interaction, phages compare favorably with relatively unspecific antibiotics since phages are less harmful for the normal bacterial flora and thus cause less collateral damage during treatment. This is imperative as by maintaining the healthy flora during treatment, the risk of opportunistic bacterial infection is considerably lower (Loc-Carrillo and Abedon, 2011).

1.4 Multidrug-resistant bacteria

Bacteria defend themselves from the environment by using many different regulatory pathways, molecular machinery, shielding and by rapidly evolving. In MDR strains these defense mechanisms are the top of the line and constantly upgraded. The key to the rapid increase of MDR bacterial strains lies in the bacterial capability to rapidly reproduce and adapt with the ability to incorporate useful genes into their plasmid DNA repertoire almost effortlessly (Alberts et al., 2008; Reese et al., 2011). These genes are referred to as virulence factors, which include genes residing in plasmids, transposons, pathogenicity islands and lysogenic bacteriophages (Bae et al., 2006; Diep et al., 2008; Baba et al., 2008). As a consequence of careless use of powerful antibiotics for decades, we have provided the perfect environment i.e. breeding grounds for the bacterial species to evolve and to gain resistance against even the most potent antimicrobial drugs. Nowadays, the antibiotic resistance is no longer uncommon outside hospital settings. Because of this the risk of spreading the antibiotic resistance to new strains due to horizontal gene transfer and transfer of pathogenicity and virulence factors is imminent and will cause severe problems in the near future (Reese et al., 2011; Alibayov et al., 2014).

1.5 *Staphylococcus aureus*

1.5.1 Short history of *S. aureus*

It is estimated that over 20 % of the human population is colonized by *S. aureus*. Although MRSA strains can cause mild or even deadly infections in the right circumstances when there is a break in the protective skin barriers or in the case of lowered immune defense, they can be fatal to otherwise healthy and young individuals (Miller et al., 2005; Somerville and Proctor, 2009).

Methillicin-resistant *S. aureus* (MRSA) (historically named after gaining methillicin resistance) are one of the most problematic antibiotic resistant strains of *Staphylococci*. The first clinical MRSA isolate was found already in 1961, only one year after methillicin was released (Parker and Jevons, 1964). Today, these strains generally include *S. aureus* strains that are resistant to all β-lactam agents and even the last resort vancomycin (Gordon and Lowy, 2008). MRSA bacteria are the common cause of skin and respiratory tract infections especially after hospital treatment (Ayliffe, 1997; Chambers, 2001). Prolonged

S. aureus infections are also prominent in the farming industry, where MRSA strains plague livestock and present a threat of cross infections in workers. For example, in pigs the strain expresses major risk to the farmers due to the genetic similarity of pigs and humans and the proximity between the animals, feces and workers (Armand-Lefevre et al., 2005; Schook et al., 2015).

1.5.2 *S. aureus* morphology

S. aureus is an opportunistic 0.7–1.2 μm diameter gram-positive coccal bacteria commonly found on the human skin and respiratory tracks as a part of the normal bacterial flora and as a foodborne pathogen, and it is prominent in all mammals in general. *S. aureus* is catalase positive, facultative anaerobic and forms distinct microscopic grape-like clusters (Archer, 1998; Somerville and Proctor, 2009).

1.5.3 *S. aureus* cell wall

Like other gram-positive bacteria, *S. aureus* has a thick cell wall that offers protection against multiple antimicrobial drugs. The cell wall is made of peptidoglycan, proteins and teichoic acids which form a robust three-dimensional structural network. The rigidity and the toughness of the wall come from the glycan strands that are composed of six disaccharide subunits which have a high degree of cross-bridging (Somerville and Proctor, 2009). The teichoic acids provide integrity and confer to the pathogenicity and colonization of *S. aureus* (Swoboda et al., 2010).

1.5.4 *S. aureus* genome

The typical *S. aureus* genome consists of roughly 2600 genes and several thousands of strains have been sequenced (Geer et al., 2009). Based on the genomes of nine different *S. aureus* strains that have been fully sequenced (Feng et al., 2007), the sequence analysis revealed the strains have approximately 78 % core genome similarity and the 22 % difference in genes comes from accessory genomes such as virulence factors (Lindsay and Holden, 2004). *S. aureus* strains can withstand harsh conditions exceptionally well and currently there are strains that are resistant to at least 8 different antibiotics (Feng et al., 2007).

1.6 *Pseudomonas aeruginosa*

1.6.1 Morphology and characteristics

Another significant opportunistic pathogen that can survive in a variety of hostile ecological niches whilst enduring disinfectants and antibiotics is *Pseudomonas aeruginosa* (Hardalo and Edberg, 1997). This highly adaptive, ubiquitous pathogen is accountable up to over 20 % of the infections in hospital settings (Bouza et al., 1999). *P. aeruginosa* is a rod-shaped gram-negative bacterium measuring at 0.5–0.8 µm by 1.5–3.0 µm and it forms persistent polysaccharide-encased surface-attached biofilms**.** This nosocomial pathogen is one of the main causes of infections in immunocompromised hosts, patients with catheterized urinary tracks, burn wounds and cystic fibrosis. (Bodey et al., 1983)

1.6.2 *P. aeruginosa* genome

P. aeruginosa has a large and complex genome composed of 5.7–7 Mbp genome supplemented with variable accessory segments, making the first fully sequenced *P. aeruginosa* genome of PAO1 (6.3 Mbp), one of the largest bacterial genomes ever sequenced (Stover et al., 2000). Over 90 % of the genome consists of highly conserved core genome which is shared between different *P. aeruginosa* strains. The remaining accessory segments have major clinical importance as they can significantly differ between strains. These unique accessory genomes of *P. aeruginosa* encompass virulence factors and confer to the strains resistance against the multiple classes of antibiotics (Mesaros et al., 2006; Kung et al., 2010).

1.7 Virulence factors and toxigenesis

The reason why pathogenic bacteria present a threat to humans and other organisms in general is mostly due to the virulence factors and vast abundance of substances such as pigments, toxins, enzymes and cell wall components, latter of which are produced and secreted by the bacterial cells. Some strains also form persistent biofilms that help the bacterial colonization and harm the host. Virulence factors increase the infectiveness of the invading pathogens in the form of special molecules that help in the invasion, evasion of the host cell defenses and in obtaining nutrients from the host cell. Toxins trigger the inflammatory processes when recognized by the immune system of the host. In severe

incidents or when exposed to large toxin quantities, this can cause harm to the organism in the form of cell death, tissue destruction and toxic shock or sepsis (Alberts et al., 2008; Reese et al., 2011).

Gram-positive and gram-negative strains of bacteria produce toxins, which can be divided into exo- and endotoxins, which are substances formulated and excreted by the bacteria or are present in the structures of bacterial cells, respectively. Exotoxins are proteins that work mostly enzymatically and can inhibit host cell RNA translation, target the intestine and/or cause cell damage from a distance. Both gram-positive and gram-negative bacteria can produce protein exotoxins that are excreted outside the bacterial cell, but only gram-negative bacteria produce cell wall-derived endotoxins in the form of lipopolysaccharide (LPS) or lipooligosaccharide (LOP) (Reese et al., 2011).

The bacterial toxins are biomolecules which are usually extremely small, very robust, difficult to detect and thus hard to eliminate. Although the toxicity of the biomolecule can be removed, it can still function as an antigenic substance that can cause inflammation. Toxins produced by or present in the cell are unique to different bacterial strains, which makes the individual eradication tedious (for reviews, see Gill, 1982; Middlebrook and Dorland, 1984). To ensure that the current safety requirements are met, all bacteriophage-drug substances are subjected to strict quality parameters and guidelines which include the test methods and acceptance criteria (Parracho et al., 2012). For example, in the case of bacterial endotoxins that are present everywhere where gram-negative bacteria are found, legislation has been made to limit the toxin amount measured as EU (endotoxins units) in pharmaceuticals to 0.5 EU/ml or 5 EU in an hour per kg of body weight. This is considered a maximum level of endotoxin in intravenous applications (Parracho et al., 2012).

1.8 Purification of phage-substances

For pharmaceutical purposes, the concentration of bacterial toxins must be lowered onto a safe level in all clinical products, such as intravenous or -muscular drugs and externally applied lotions (for review see Parracho et al., 2012). Phage solution preparation processes usually encompass the use of pathogenic host bacteria for phage propagation, which causes several problems in the form of bacterial remnants such as debris and high concentrations of toxins in the raw supernatant. Due to the small size and diversity of the toxic biomolecules, the basic size-exclusion filtration is commonly used, but it is in some cases

insufficient in removing all the toxic compounds. Conventionally time-consuming procedures, such as high speed centrifugation (CsCl gradient) and aftermarket purification kits, are used in phage purification (Drucker et al., 1992; Boulanger 2009). These methods have proven to be effective, but high price, phage deterioration, low yields and the low purification capacity were found to be limiting factors in the mass production of purified phages (Adrianssens et al., 2012).

The purification of phage solutions with a high-performance liquid chromatography (HPLC) systems with anion exchange chromatography (AEC) columns, have been successful in the rapid removal most of the impurities and boasting phage yields up to 100 % (Drucker et al., 1992; Adrianssens et al., 2012; Bourdin et al., 2014). In this purification approach, the lysate components are selectively bound and unbound based on the charge differences between biomolecules and phages in the solution (Adrianssens et al., 2012). This type of chromatographic tool can be effectively utilized when the overall net charge of phage is calculated from the sequence data derived from the genome of the phage. The estimation is usually more accurate when it is based on the most abundant structure of the phage particle i.e. the large head capsid structure. Although HPLC may be sufficient for large scale clinical production, the current impediment lies in the high pharmaceutical legislation and regulation demands for the equipment used in the detox process, which in turn makes the starting of professional clinical phage purification more demanding (Parracho et al., 2012).

1.9 Relevance of the study

So far, the advances in phage therapy have been greatly hindered by the lack of high quality research, strict pharmaceutical legislation and the loss of interest in finding other alternatives due to the relatively good efficiency of antibiotics. This has left the phage therapy in its infancy in most countries although back-up methods combating the ever-increasing antibiotic resistance of bacteria are desperately needed (Wittebole et al., 2011). Using phage therapy in the treatment of animal-borne infections is a promising starting point due to the less strict legislation of animal pharmaceuticals and the promising results already gained on animal models (Carmody et al., 2010; Monk et al., 2010). Despite that there are broad host range phages against human derived *S. aureus*, there have been

difficulties in finding suitable phages that infect and lyse even a fraction of the currently known *S. aureus* strains isolated from pigs (Tuomala, 2016).

2. AIMS OF THE STUDY

The phage collection of the University of Jyväskylä contained three preisolated broad host range *S. aureus* phages which were found during a phage research project (Mattila et al., 2015). Here, we describe the *S. aureus* phage SA14P1, which was chosen for further characterization including sensitivity and purification tests, sequencing and transmission electron microscopy (TEM) imaging, to determine the usability of phage as a potential therapeutic phage against 54 MRSA pig isolate strains. Finally, we set out to find novel phages against clinically important human infecting *Pseudomonas aeruginosa* strains from hospital waste water samples and to test the host range of seven preisolated phages using 43 MDR *P. aeruginosa* strains as potential targets.

3. MATERIALS AND METHODS

3.1 Phages and bacterial strains

S. aureus and *P. aeruginosa* phages and their corresponding host bacteria used in this research were obtained from the freezer stocks of the University of Jyväskylä. The phages were isolated from various waste water and soil samples during a two-year phage extraction project against the strains of bacteria derived from various hospital samples (Mattila et al., 2015). After a brief preliminary characterization, the phages were stored in -70 °C in 30 % glycerol for later use. The bacterial strains of 54 pig isolate *S. aureus* and 43 human *P. aeruginosa* were provided by Heikinheimo et al. and HUSLAB, respectively. Phages, host strains and bacterial strains used in the experiments are described in detail in Appendix 2, 3 and 4, respectively.

3.2 Bacterial growth media and conditions

Bacteria were grown in 5 ml liquid Luria-Bertani (LB) cultures at pH 7.5 or LB-agar plates. The plates were provided by Pentti Kuusela and made by HUSLAB (Elatusaineyksikkö). For making LB-soft, agar was added into the culture medium to a final concentration of 0.4 %. Media were autoclaved before use and stored at RT or 4 °C.

Liquid culture incubations were done on a shaker at 230 rpm at 37 °C and plates at 37 °C in an incubator. Solutions are described in detail in Appendix 1.

3.2.1 Phage propagation

High titer phage stocks (crude phage lysates) were made using semiconfluent plate method. Semiconfluent plates were obtained using phage dilution series and dilution of overnight cultured host bacteria in LB. The amount of host bacteria was calculated using the formula 90/A600 after measuring the absorbance of bacterial liquid culture at 600 nm using a spectrophotometer (BioPhotometer, Eppendorf). Bacterial solution containing the phages was plated with 3 ml of melted soft agar on a premade LB-agar plate and the plates were cultivated overnight at 37 °C. 3 ml of SM-buffer was applied on semiconfluent plates and incubated for 30 min at RT before the bacterial lawn was scraped to a 50 ml Falcon tube using an L-shaped rod. Live bacteria were removed from the lysate by incubating the solution in 6.7 % chloroform on a shaker for 1 h in RT, centrifuging at 4000–5000 rpm for 10 min and 0.22 µm filtering of the supernatant (Millex GV durapore, PVDF membrane, Merck Millipore Ltd.). The titer of the stock was measured by making a dilution series from the lysate and plating 100 µl the dilutions with the corresponding host bacteria. The amount of bacteria was calculated as described above. The stocks were stored at 4 °C.

3.2.2 Drop test

The host specificity of the phage was evaluated using a drop test method, where known amount of bacteria was plated using LB-agar overlay method and after solidifying, the 10 µl droplets of phage lysate (with known titer) were added on specific spots. The plates were incubated overnight at 37 °C. Clear plaques would form in the droplet, if bacterium was suitable host for the phage.

3.2.3 Phage lysate concentration

SA14P1 phage lysate was centrifuged at 40 000 rpm for 2 h to a concentration of roughly 1 x 10^8 PFU/ml. Ultracentrifuge (Beckman L7-80, Ordior) with SW55 Ti swing-out rotor. 5 ml centrifuge tubes (Ultra-Clear™ Centrifuge Tubes, 172 x 2 in., 13x51 mm, Beckman Instruments Inc.) were used as centrifuge vessels. After centrifugation phages were pooled to 1 ml and stored at 4 °C.

3.3 SA14P1 phage DNA

3.3.1 DNA isolation

SA14P1 phage DNA was extracted from 400 µl of crude 0.22 µm filtered SA14P1 phage lysate, with a titer of 10^8 PFU/ml in SM-buffer, using a slightly modified version of the manual phenol extraction procedure depicted in the general laboratory manual Molecular Cloning by Joseph Sambrook (See Appendix 5). The purity and concentration of isolated DNA was measured using NanoDrop-1000 spectrophotometer (Thermo Scientific).

3.3.2 DNA digestion

The phage DNA digestion was performed using 249.1 µg of SA14P1 phage DNA and four different restriction enzymes: EcoR1-HF (New England Biolabs) and Sca1, Sma1, Xba1 (Thermo Scientific). Digestion was performed at 37 °C for 2 h, as in the protocol provided by the manufacturer. Digested DNA samples were mixed with a 6 X loading dye buffer and run in 0.8 % agarose gel in a 1 x TAE running buffer for 2.5 hours at RT, voltage 64 V. 1 kb plus DNA ladder (50 ng/µl, Invitrogen) was used as a standard. The gel running device was HE99X Submarine electrophoresis unit (Amersham Pharmacia Biotech Inc.). The gel was stained using MidoriGreen staining solution (7.5 µl/100ml in H_2O, Nippon Genetics) for 30 min and washed with dH_2O for 30 min in 200 rpm shaker covered from light at RT. The bands were visualized using Molecular Imager® Gel Doc™ XR+ (Bio-Rad) and Image Lab™ imaging software (Bio-Rad).

3.3.3 Sequencing analysis

The 10 µl sample of the isolated phage DNA (concentration of 348.8 ng/µl), was sent for Darren Smith in Northumbria University (Newcastle, UK) for next generation sequencing (NGS) in a MiSeq PE300 sequencing device (Illumina Inc.).

For SA14P1 phage sequence analysis, many different open source computer softwares were utilized. First, advanced information was gathered and the sequence was assembled. Data analysis was carried out remotely by processing via supercomputer Taito (https://www.csc.fi/fi). The genome went through Biokit-module which contained the A5 pipeline (Tritt et al., 2012) and other genomic software which require high computing performance. The sequenced genome was processed using RAST (Aziz et al., 2008;

Overbeek et al., 2013; Brettin et al., 2015). The software is fully-automated service for annotating complete or nearly complete bacterial and archaeal genomes, and it provides the tools for rapidly distinguishing the most important parts in the genome of the phage. The contig files were chosen for a basic local alignment research tool (BLAST) search based on the size and number of reads to find similar virus genomes from the NCBI database (Geer et al., 2009). Because the genome was received from the sequencing in three separate pieces, the genome was rebuild using Chipster -software (Kallio et al., 2011). As the sequence was 99 % identical with the NCBI entries 23MRA and USA300 TCH1516, the final assembly of the SA14P1 genome was carried out using the *Siphoviridae* phage 23MRA (GenBank: KJ452292.1) as a template.

3.3.4 Determination of cohesive ends

For determining the presence of cohesive DNA ends, SA14P1 phage DNA was digested with XbaI or NsiI restriction enzymes (Thermo Scientific) according to the protocol of the manufacturer. Both XbaI and NsiI treated DNA was split into two separate eppendorf tubes. One set of tubes were placed at 4 °C and the other set of tubes were heated for 3 min to 60 °C and put on ice before loading the samples into the gel. The gel was prepared, run and imaged as described above.

3.4 *S. aureus* sensitivity tests against SA14P1

SA14P1 host strain specificity determination was conducted using a 96-well microplate (SpectraPlate-96 TC, PerkinElmer) and 1:100 dilutions of bacteria from overnight cultures in LB-, TSB- or TSA-medium. The amount of bacteria was calculated with 90/A600 for each tested bacterial strain. The tested strain was added in to six wells each as 200 µl of the bacterial dilution and to three of them 10 µl of crude phage lysate with a titer of 10^8 PFU/ml was added. The progression of the infection was monitored using a multimode microplate reader (FLUOstar OPTIMA, BMG Labtech) that measured absorbance of the phage-bacteria mixture at 600 nm. In the case of bacterial lysis, the optical density started to rapidly decrease as the bacterial cells start to break down. Since phages do not absorb at 600 nm wavelength, the increase of phage particles does not affect the optical density of the sample at the selected range. The measurement was conducted at the time points of 0, 1, 2, 3, 4, 5 and 24 hours post infection. The plate was kept on a 200 rpm shaker at 37 °C

between the measurements. The SA14P1 host strain SA14 (known to be sensitive) served as a positive control strain. Other positive control was lytic and previously tested phage fRu-Sau02 and its host strain 5676. The addition of SA14P1 into culturing medium (LB) was used as a negative control. The results were presented in the form of mean graphs, where the change in absorbance is easy to detect and evaluate accurately. Graphs were made using Microsoft Excel software.

3.5 SA14P1 phage purification

3.5.1 Salt tolerance test

To determine if the phage particles can withstand the high salt concentrations during the HPLC purification, the phage lysate was subjected to a varying concentration of NaCl in 20 mM Tris-Cl pH 7.5 buffer. The phage lysate was incubated for 1 h at RT in 0, 0.1, 0.25, 0.5, 0.75 or 1.0 M of NaCl. After incubation, 10 µl of each treated phage lysate was titrated using the drop test method as described above.

3.5.2 Anion exchange chromatography

Purification was done from 4 ml of SA14P1 crude phage lysate with a titer of 1.39 x 10^8 PFU/ml. The lysate was purified with a two-step process: First, in 100K molecular weight cut off (MWCO) column (Amicon ultra, Merck Millipore Ltd.) for pre-purification and then purified with HPLC equipment (Äkta purifier, GE Healthcare). The lysate was concentrated and washed two times in a Amicon ultra centrifugation tube with 3 ml of buffer A (20 mM Tris-Cl pH 7.5) using 4000 rpm at 4 °C until 1 ml of lysate was remaining. The 1 ml of ultrafiltrated phage lysate was loaded in HPLC instrument into a 1000 µl injection loop. The phage particles were separated from the lysate using AEC with CIM® Tube monolithic QA-Column with 6 µm pore size (BIA separation GmbH) to bind the negatively charged phage particles to the positively charged column. The phages were washed using the low concentrations of NaCl in 20 mM Tris-Cl pH 7.5 to remove impurities. The elution of the phage particles was achieved by increasing the NaCl concentration linearly from 0 to 550 mM and finally to 1 M and collecting 1.5 ml fractions. All fractions collected from distinct chromatogram peaks were titrated using the drop test method to confirm the presence or the absence of phage particles.

3.6 TEM imaging of SA14P1 phage

Before the imaging, the high titer HPLC-purified SA14P1 stock was further concentrated using a table top centrifuge (Centrifuge 5415R, Eppendorf) for 90 min at 16.1 REF at 4 °C to reach a high phage count of roughly 1 x 10^9 phages/ml. The phage pellet was resuspended in 50 µl of 0.1 M ammonium acetate. The carbon coating of the TEM copper grid was conducted just before the addition of the sample, using a glow discharge method to produce a thin hydrophilic surface for the sample to sit on (K100X, Emitech). TEM-grid containing 10 µl of phage stock was stained on a droplet of 1 % uranyl acetate for 60 seconds. TEM imaging was done using Jeol JEM-1400 (Jeol Inc.). The acceleration voltage of 80 kV and current of 48,5 µA were used throughout the imaging session. Images were by a 11-megapixel side-mounted CCD camera (11 MegaPixel Morada, Olympus). Phage morphology analysis, particle size measurements and image processing was done using the FIJI image processing package software version 1.50g (Schindelin et al., 2012).

3.7 *Pseudomonas aeruginosa* phages

3.7.1 *P. aeruginosa* phage isolation

The isolation of a novel phage from hospital waste water samples was done against MDR *Pseudomonas aeruginosa* strains. Detailed water sample information is described in Appendix 7. The waste water samples were pooled to a single 15 ml falcon tube containing the MDR strains 5513, 5514, 5525 and 5551 (See Appendix 4). These strains were chosen for the isolation attempt as they did not have corresponding phages. The mixture was incubated at 37 °C overnight and inspected for visible change in transparency and/or consistency. Crude lysate was prepared using the methods described above. The titer of the lysate and the presence of phages were analyzed using the drop test technique and target bacteria strain 5525. The newly isolated phage was named fHoPae01 and stored at – 70 °C in either 8 % DMSO or 20 % glycerol. Phage lysate was stored at 4 °C.

3.7.2 Host range assessment for *P. aeruginosa* phages

The infectivity of the newly isolated phage was characterized to determine the *P. aeruginosa* strain host specificity in conjunction with seven phage isolates from the University of Jyväskylä (Mattila et al., 2015). These phages included PA1P1, PA5P1,

PA8P1, PA10P2, PA11P1 PA12P1 and PA14P1 which are described in more detail in Appendix 4. The phages were propagated using the semiconfluent plate method described above. Preliminary host range assessment for *P. aeruginosa* phages was performed using the drop test method.

4. RESULTS

4.1 Sensitivity of *S. aureus* against SA14P1

Sensitivity assay using SA14P1 phage was conducted against 54 *S. aureus* pig isolates. From these isolates, 48 were successfully tested. The remaining strains could not be tested due to poor growth despite multiple optimization efforts. Based on the results, SA14P1 phage was unable to lyse most of the pig MRSA strains and in the case of possible infection, only the growth speed of the bacteria was slightly hindered. According to the results, 16 out of 48 MRSA strains showed the signs of sensitivity to SA14P1 phage. The sensitivity test method worked as intended for both control strains and the decrease of sample absorption gave a clear indication of the ability of the phage to lyse the bacterial cells. Examples of visualized sensitivity test results are shown in Figure 1.

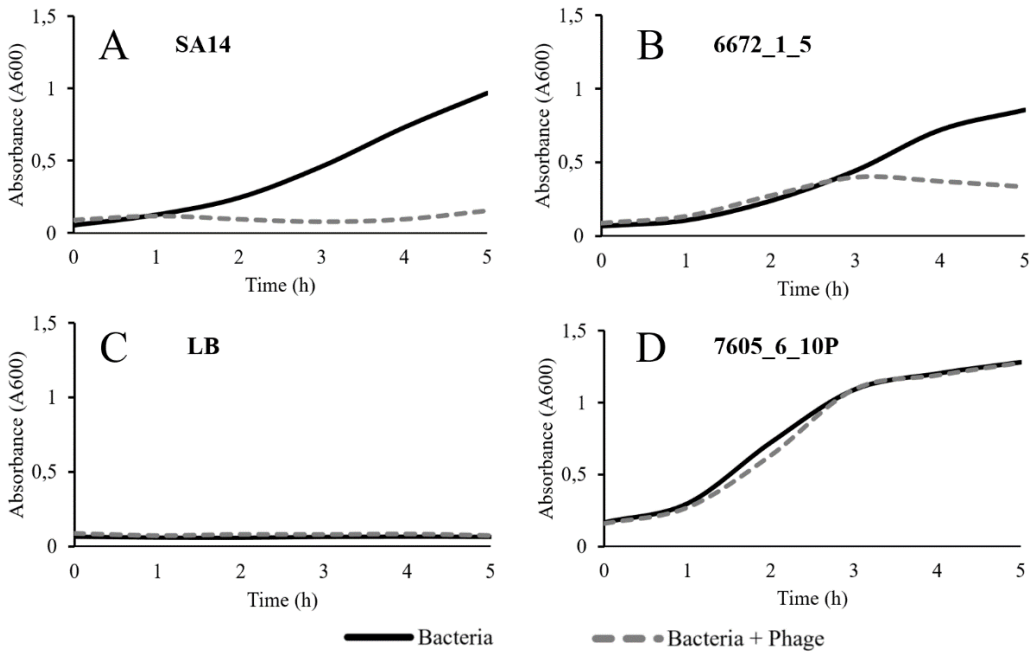

Figure 1. **SA14P1 sensitivity test.** The sensitivities of the pig isolate *S. aureus* strains were studied measuring the change in bacteria growth speed when phage lysate was added. The increase in bacterial cell count increases the optical density and the addition of phage lysate decreases or slows down the growth. The results can be classified to four scenarios: (A) lysis effect, (B) slowed growth, (C) negative control and (D) unaffected growth of resistant strain. Here are shown the actual results, that were obtained from the

sensitivity test. The bacterial strains used in the experiments are shown in the graphs. LB is Luria Bertani-medium.

Previously DNA sequence analysis of the protein A gene variable repeat region (spa typing) has been used to rapid and accurate method to discriminate *Staphylococcus aureus* outbreak isolates. From the 54 tested strains, 12 have been reported transmittable to human hosts (Heikinheimo et al., 2016). Here the CC398 strains are *spa* types t034 and t108 of which t034 is responsible for 72 % of the recent cases of the dominant CC398-associated infections in Finland (https://www.thl.fi/en/web/thlfien) (Fluit, 2012). The strains with *spa* type t108 are also transmittable from human to human, which makes them especially interesting in this research. There were in total 7 *spa* t034 and 5 *spa* t108 strains in this research, from which four and three showed susceptibility to SA14P1, respectively. The results indicate that the strains with *spa* type t034 or t108, that can infect humans, are more susceptible to SA14P1 phage, which can be seen in Figure 2 as the relatively high portion of human infecting strains slowed by the addition of the phage. The strains that did not grow in the sensitivity test conditions consist entirely of strains that infect only pigs.

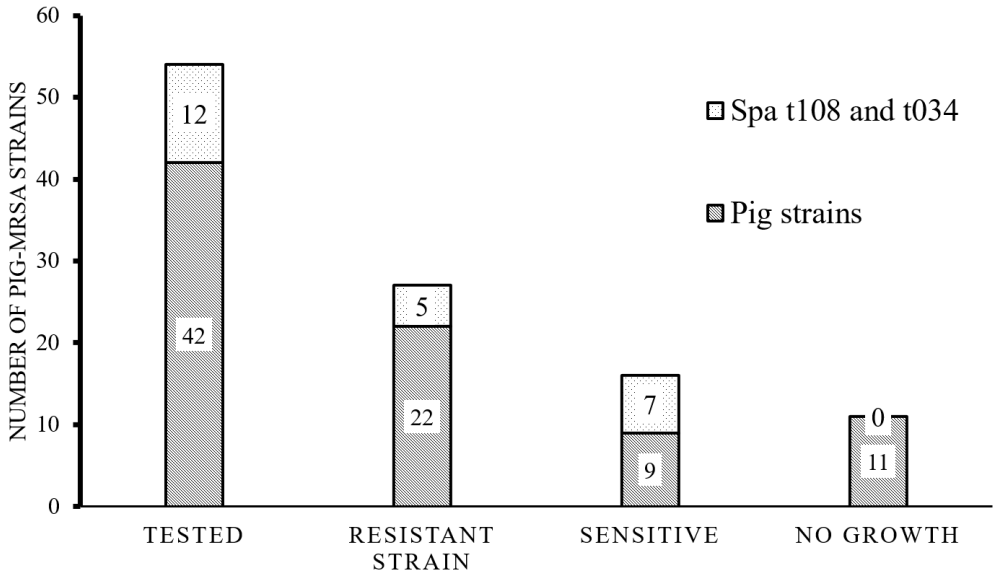

Figure 2. **Pig-MRSA sensitivity to SA14P1.** The *S. aureus* sensitivity to SA14P1 phage was tested using a plate reader to monitor the growth of *S. aureus* strains in liquid cultures with and without SA14P1 phage. The test was done using three duplicate growths, from which three were added a 10 µl volume of SA14P1 crude phage lysate at a titer of 10^8 PFU/ml. The majority of the strains tested did not display any signs of lysis or even hindered growth. Some strains, however, presented up to a twofold decrease in growth speed when phage lysate was added. Due to poor pig MRSA growth, 11 strains could not be tested. From the 54 strains tested, 12 were from strains with *spa* types t108 and t034 which reportedly are transmittable to humans and five of these (t108) are transmittable from human to human (Heikinheimo et al., 2016). These data indicate that the *spa* types t034 and t108 are more sensitive to SA14P1 phage than other pig isolates.

4.2 The genome and DNA features of SA14P1

4.2.1 Genome size estimation

After SA14P1 DNA was extracted from crude phage lysate using manual phenol extraction, DNA concentration was measured to be relatively high, 348.8 ng/µl. The results verify that DNA isolation was successful.

The phage DNA was digested using various suitable restriction enzymes to roughly estimate the size of the genome of the phage. The genome was successfully cut with EcoRI-HF, ScaI and XbaI. The only restriction enzyme that did not cut the genome was SmaI (Figure 3). The digestion using EcoRI-HF in well 3 gave an estimated genome size of 45 kbp which corresponds to the size of *Siphoviridae* or *Podoviridae* genome (Figure 3). There were some impurities in the samples that are visible as cloudiness at the bottom of the gel.

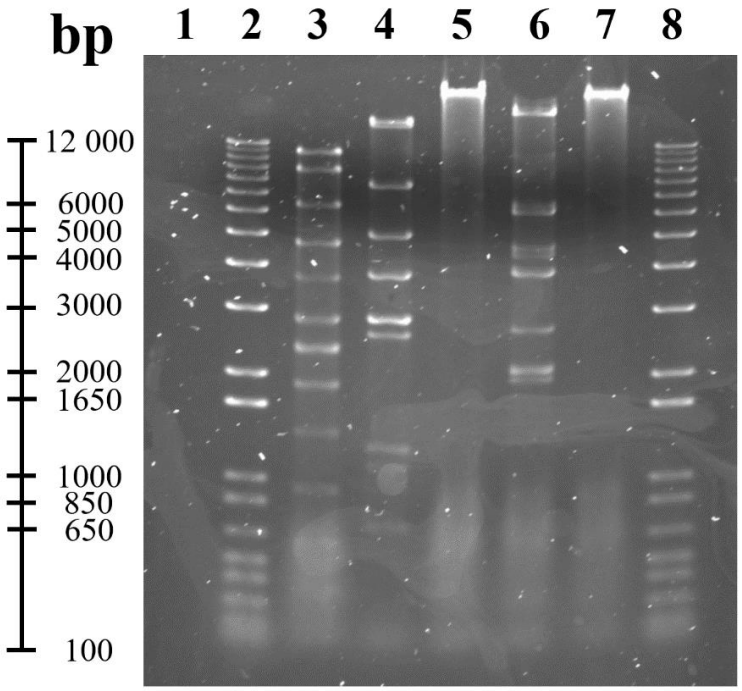

Figure 3. **SA14P1 genome size.** The preliminary genome size estimation was done using various restriction enzymes to digest the SA14P1 DNA. The 10 µl sample volume containing 5 µl of a 6X running buffer with different digestions were loaded into the wells 3 – 6 and 7 µl and 3.5 µl of DNA ladder were loaded into the wells 2 and 8, respectively. Restriction enzyme digestions with EcoRI-HF, ScaI, SmaI and XbaI were loaded in to the wells 3, 4, 5, 6, respectively. Undigested DNA was loaded into the well 7. The 0.8 % agarose gel was run for 2.5 h using 64 V in 1 x TAE running buffer at RT. The gel image shows that SmaI did not cut the DNA. From the EcoRI-HF digestion, the size of the genome was estimated to be roughly 45 kb in size.

4.2.2 Sequence analysis

Preliminary sequence analysis revealed that the phage sequence was in eight contigs, from which three (0, 1, 3) contained the actual phage genome. The rest of the contigs contained small amounts of contaminating bacterial DNA. The genome size measured 43 098 bp long and the GC-content was 32.9 %. The BLAST search of the largest sequenced SA14P1 phage contig revealed that the phage was 99 % identical to a previously found USA300 TCH1516 and 23MRA siphoviruses (GenBank: CP000730.1 and KJ452292.1, respectively). These phages were found to be *S. aureus* prophages whose expression is inhibited when cultured in media with blood (Tenover et al., 2009; Santiago-Rodriguez et al., 2015). The differences between the SA14P1 and 23MRA were found to be minor point mutations that were mainly one amino acid insertions and duplications throughout the genome. There were homologs to other *Staphylococcal* phages in many of the identified open reading frames and high overall similarity with Phage 77 (GenBank: 2743812.) (Liu et al., 2004). The main features of SA14P1 genome are presented in Table 1.

Table 1. **Main information of SA14P1 genome.** Genome features were elucidated using IGV, RAST and ExPASy ProtParam bioinformatics tools.

General features	
Genome size	43 098 bp
GC-content	32.9 %
Number of coding sequences	70
Major capsid protein size	1146 bp
Major capsid protein isoelectric point	5.0005
Phage tail length tape-measure protein	4533 bp*

*Tape-measure protein in two pieces.

Using the bioinformatics tools of integrative genomics viewer (IGV) and RAST, the sequence was analyzed to find proteins that are encoded by the open reading frames. Many coding sequences for proteins typical of a temperate phage were found. These included sequences such as DNA integration, antibiotic resistance and superantigen-like protein coding genes. These encoded proteins are listed in the Table 2. It is possible that the phage genome harbors other virulence factors, such as staphylokinase which was found during the analysis.

Table 2. **Phage genome annotation results.** The SA14P1 sequence was analyzed using RAST 2.0 to study the contents of the genome and the proteins that are encoded. The aim was to identify prophage associated structures, virulence factors and functional proteins that would give clear evidence of the phages temperate nature.

Product	Length (bp)	Function
DNA integration related proteins		
DNA-binding protein, phage associated	180	DNA binding
Phage integrase	1026	Mediates site-specific recombination between two different sequences
Antibiotic resistance		
Metallo-beta-lactamase superfamily domain protein in prophage	486	β-lactamase resistance
Superantigen-like proteins		
Hypothetical SAR0365 homolog in superantigen-encoding pathogenicity islands SaPI	183, 249*	Defense against immune system
Exoenzymes		
Staphylokinase	492	Disrupts fibrin meshwork

*Superantigen coding protein might be in two pieces.

The genome study revealed that there was a relatively long repetitive sequence for the phage tail tape-measure protein, which would indicate long tailed phage (Mahony et al., 2016).

4.2.3 Cohesive ends

Based on genome sequencing results and the preliminary digestion with different restriction enzymes for size estimation, it was presumable that the phage genome has cohesive ends that affect the genome digestion results displayed in the gel in Figure 3. This difference in the band location can be visualized in the XbaI restriction enzyme digestion results in well 6 (Figure 3). To elucidate how the digested end fragments of the genome bind to each other, the digestion was repeated with a heat treatment procedure shown in Figure 4 A, and using XbaI and NsiI restriction enzymes. The aim was to see if there is a difference in band locations between virtual digestion, heat-treated (H) and untreated digestions. The results are presented in Figure 4 B.

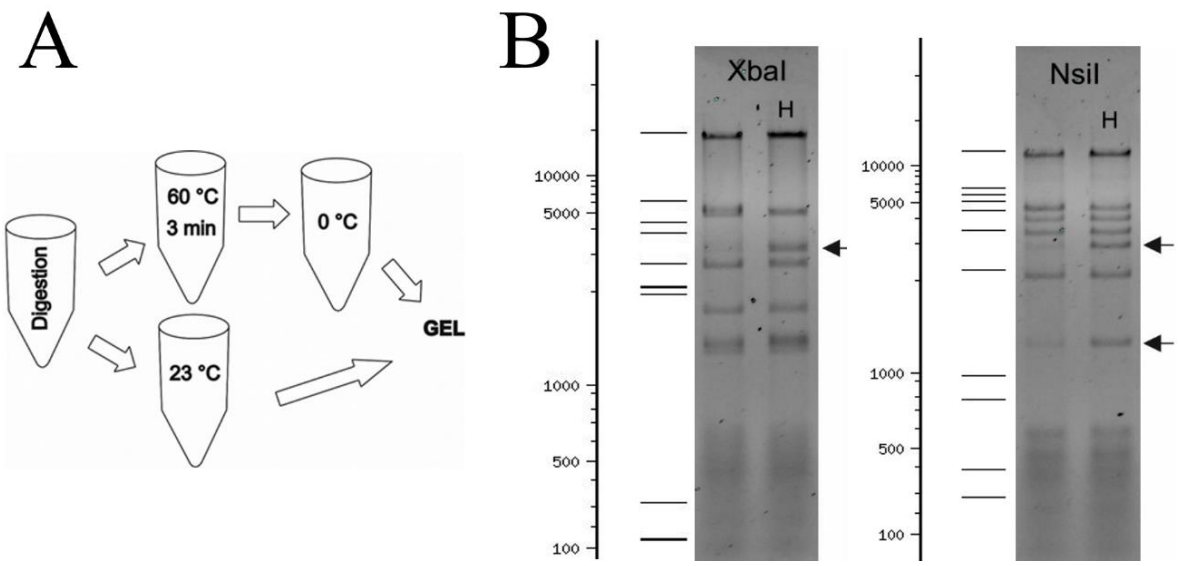

Figure 4. **Cohesive DNA ends digestion results.** A) Heat treatment protocol. B) The DNA digestions of SA14P1 were analyzed and compared using virtual digestions from NEBcutter V2.0 software (New England BioLabs Inc.) to evaluate the real-life digestion results (on the right) with the expected virtual digestion results (on the left). Heat-treated digestions are marked with H. When using XbaI restriction enzyme, the left end fragment is cut to a 4292 bp long fragment. This fragment is missing in the untreated sample. When the digested DNA is heat-treated the fragment becomes visible. The right end fragment is considerably smaller (141 bp) when using XbaI, and thus it becomes almost impossible to see in the gel. According to the virtual digestion of NsiI, the left end of the DNA sequence forms a 4423 bp long segment which is missing in the untreated sample. Also, the right end fragment which is cut to a length of 2183 bp is missing in the untreated sample. After heat-treatment, the missing DNA fragments become visible as seen in the virtual digestion. Differences are highlighted with arrows.

After testing the hypothesis of cohesive DNA ends using heat-treatment protocol, the results indicate that there was a significant difference between the band locations of the heat-treated and the untreated DNA digestions. The heat-treatment unbinds some DNA fragments, that are formed after the DNA is digested, from each other. When the virtual digestions were compared with the actual digestion results, there was a significant difference in the way the restriction enzyme digests the SA14P1 phage DNA (Figure 4 B). When the real-life untreated digestions on the gel were analyzed and compared with the virtual digestion results, the XbaI restriction enzyme gave a different result in the 4292 bp region. The gel image presented that there was a band missing in the untreated sample at 4300 bp range (Figure 4 B). Instead, there seemed to be a double band at around 5500 bp. According to the virtual digestion of NsiI, the left end of the DNA sequence forms a 4423 bp long segment which is missing in the untreated sample. Also, the right end fragment which is cut to a length of 2183 bp is missing in the untreated sample. All of these fragments appeared after the DNA was heat-treated.

By estimating the sizes of the observed bands that appeared in the heat-treated sample, the results indicated that the bands matched the end pieces of the phage DNA which were cut by the NsiI and XbaI restriction enzymes, as seen on Figure 3. This is strong evidence that the phage DNA has cohesive (sticky) ends. The DNA ends bind together after digestion and when heat-treated are released again (Figure 4 B).

4.2.4 Purification viability based on genomics

In order to purify the phage using AEC, the overall charge of the phage particle was first determined. This was done by recognizing the major capsid protein sequence from the sequence data using RAST-software annotations and generating valuable information about the functional proteins and structures found in the corresponding DNA. The overall charge of the phage particle was estimated by calculating the net charge of the major capsid protein of the phage. The isoelectric point of the major capsid protein at pH of 7.5 was determined using ExPASy ProtParam tool- software (Artimo et al., 2012). In the case of the SA14P1, the isoelectric point of major capsid protein was 5.0005, which corresponds distinctly negative charge at pH 7.5, thus making the concept of using anion exchange chromatography a feasible option in phage purification.

4.3 Anion exchange chromatography purification results

To ensure that the high salt concentrations in HPLC do not affect the infectivity or structural integrity of the phage, the phage was subjected to the high concentrations of NaCl and the infectivity was checked. SA14P1 did not show a decrease in phage titer after the salt durability test, which ensured that the phages could survive in the high NaCl concentration subjected during HPLC purification.

The HPLC results indicated that the bulk of the phage eluted at rather high NaCl concentration of 350 mM, but there were still some phage particles in other fractions as well (Figure 5). The chromatogram peaks indicated that high amounts of impurities were washed from the lysate already at the start of the purification. This could be visualized as a tall and wide peak at 0–3 ml of buffer (fractions A1–A4) (Figure 5). After the NaCl concentration started to increase, lower amounts of debris washed away which was shown as multiple smaller peaks in 5–16 ml volumes (fractions A8, A9 and A13). The strongest and narrowest individual peak at 16–17 ml (fractions B15 and B14) at 350 mM NaCl

turned out to contain the majority of the phage particles at around 10^7–10^8 PFU/ml (Figure 5). At the highest NaCl concentration there were some impurities and only small amounts of phage particles still present. Based on the total number of phages recovered in a single peak, the yield was calculated to be roughly 80–90% which is unusually high for an experimental set-up. Considering that at the starting point the phage titer was 1.39 x 10^8 PFU/ml, the results indicated that majority of the phages were recovered.

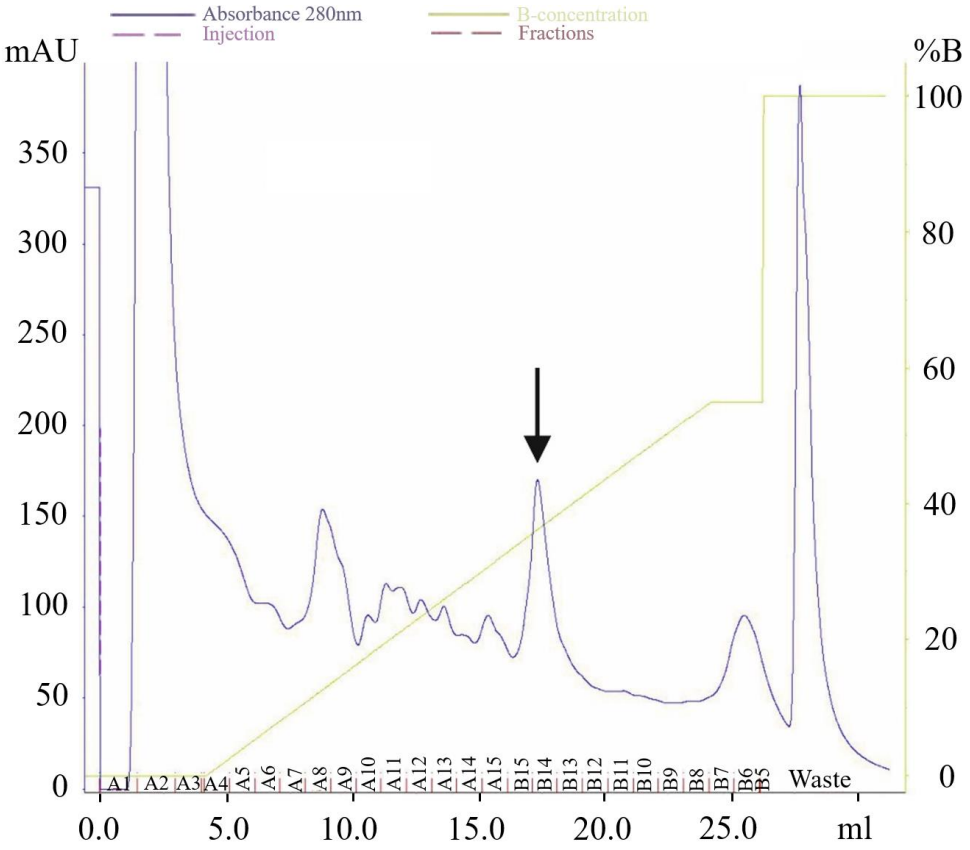

Figure 5**. Anion-exchange chromatogram of SA14P1 lysate**. The phage lysate was purified using anion exchange chromatography. The 1 ml ultra-filtrated phage lysate was bound to the column and washed using 5 ml of 20 mM Tris-Cl pH 7,5 buffer solution. After washing step, the phage was eluted from the column by linearly increasing buffer solution concentration from 20 mM Tris-Cl pH 7,5 to 1 M NaCl. Elution fractions were collected and tested with the drop test method. The peak corresponding the phages is marked with an arrow in the chromatograph. Blue line depicts A280 of the sample and NaCl concentration is marked with green line.

The fractions were tested using the drop test method. Based on the test results, the first fractions did not contain any phages, thus the high peak seen in the chromatogram in Figure 5 (fractions A2 and A4) contained only impurities or deactivated phage particles. As the NaCl concentration was increased, the amount of eluted phages started to rise, but the small peaks (fractions A8, A9 and A13) contained only small amounts of phages.

When the NaCl concentration reached 350 mM a distinct sharp peak appeared in the chromatogram (fractions B15 and B14) which contained the majority of the phages. After the salt concentration was increased to 1 M there was a slight increase of phages that still eluted from the column (Figure 6). At low NaCl concentrations, no significant amounts of phages were eluted, but when looking at the tall peaks occurring at A2 and waste in Figure 5, it is shown that large amounts of debris and some phages were eluted from the column.

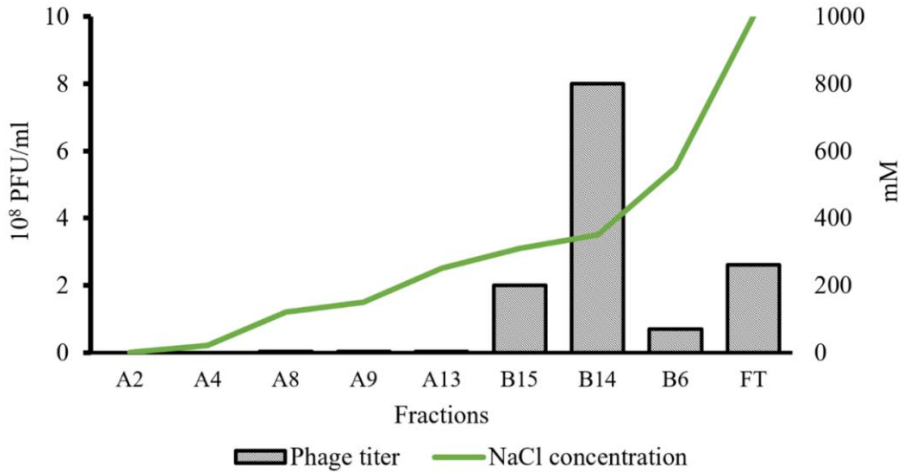

Figure 6. **HPLC purification results.** The amount of phages in the HPLC-purified fractions was determined using drop test method. The results showed that the largest amount of phages was eluted roughly at 350 mM NaCl concentration. When the NaCl concentration was increased to 1 M there was still some phages eluted, but compared with the phage containing fraction, the amount was more than three times lower. After calculating the phage yield in the single peak at B14, the total yield was estimated to be almost 90 %, which is high for an experimental set-up.

4.4 TEM-imaging

4.4.1 Phage morphology

The high magnification of the TEM made it possible to determine the morphology of the phages and to evaluate their condition after the purification steps. Based on the findings the phage can be categorized into order *Caudovirales* (tailed phages) and more specifically to the family *Siphoviridae* due to the distinct long non-contractile tail and round and/or hexagonal head capsid (Figure 7 A and Figure 8). Phage head capsids sized roughly 60 nm in diameter and the tail fiber measured to be over 200 nm long and 8 nm wide. The length of the longest whole phage particle measured was 420 nm. The measurements were done on 10 intact phages and the results are shown in Table 3. The results indicated that the average tail length of the phages is over 200 nm which would indicate that the SA14P1

phage belongs to the serogroup F. Overview of the purified lysate is shown in Figure 8 and individual phage is shown detail in Figure 7.

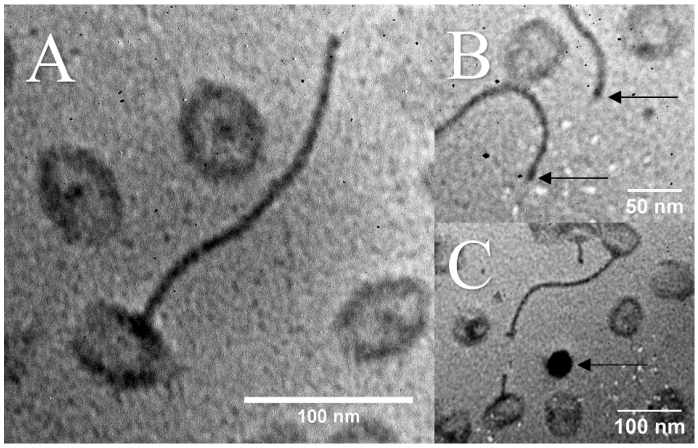

Figure 7. **Individual SA14P1 phage particles.** SA14P1 phage morphology was studied using high magnification TEM images. Head capsid sized roughly a 60 nm diameter and the tail measured roughly 200 nm long. Among the intact phages there were a lot of phages that had lost their tail completely or part of it. A) The overall features of SA14P1 showing the round possibly hexagonal head capsid and the long flexible tail. B) The attachment head of the phage at the end of the tail. The attachment heads of two phages are marked with arrows. C) The phages that had broken head capsid were colored black by the uranyl acetate negative staining solution, which can be seen marked with an arrow.

4.4.2 Purification efficacy and phage dimensions

Based on the imaging results, the uranyl acetate staining worked, but the majority of the phages were positively stained. The lysate did not contain distinct impurities such as salt crystals or bacterial debris, which indicates that the purification using a AEC method with HPLC instrument was successful.

Table 3. **Phage dimensions.** Phage dimension were measured from TEM images using FIJI open source image processing package. The measurement was done on 10 intact, phage particles and averaged.

Measured	nm
Overall length	344.4 ± 59.6
Head capsid width*	58.3 ± 6.1
Tail width	8.4 ± 0.8
Tail length	207.4 ± 60.2

*Averaged result of measurements that were done horizontally and vertically to each phage.

The TEM images revealed that the negative staining of individual phage particles with 1% uranyl acetate was partially successful and the phages were easily distinguished from the background. The phages were however mostly positively stained. Due to the staining

method, some phage head capsids were dark colored which would indicate that there was a break in the capsid structure. Based on the overview images of the lysate in Figure 8, it was observed that many of the phage particles were damage in some part of the purification process. This was seen as a complete or partial loss of a tail fiber or dark colored head capsid. Many of the damaged phages were completely missing the tail, which might indicate that the tail-capsid junction is a prominent weak point in the phages structure. However, the infectivity of the phage did not change after HPLC purification.

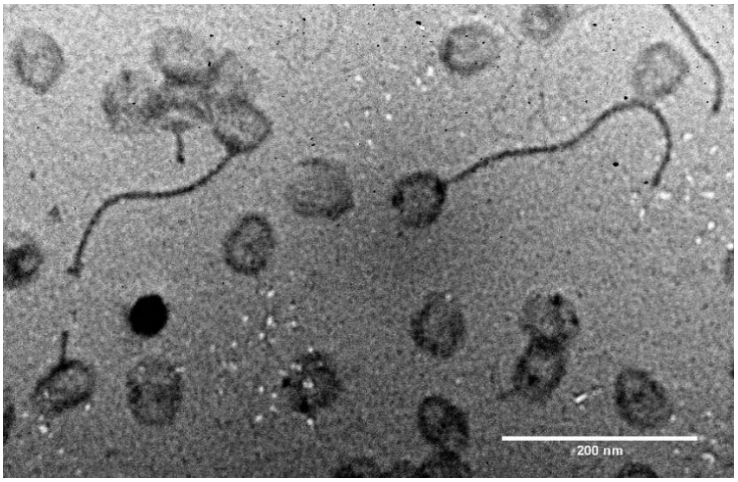

Figure 8. **TEM image of purified SA14P1 phage lysate.** TEM image of the uranyl acetate stained HPLC-purified phage lysate showed that the lysate contained tailed and tailless phage particles. The tailed particles corresponded to the *Siphoviridae* species and the round particles seemed to be the same species, but the tail part had been detached from the head capsid. The intact phages were measured to have 250 nm–300 nm overall length from the head to the tip of the tail. Uranyl acetate staining was partially successful as many phages were positively stained and only a few phages had been over stained. Some of the overstained particles had lost their tail, which might indicate a break in the head capsid.

4.5 Phage isolation against *P. aeruginosa* and strain specificity tests

The concept of isolating novel phages from hospital waste water samples was tested to find potent phages against *P. aeruginosa* strains. Phage isolation attempt was successful: a phage against MDR *P. aeruginosa* strain 5525 was found in the hospital waste waters and it was named fHoPae01. The phage formed medium sized clear plaques, and a crude lysate stock with a titer of 2.2 x 10^{10} PFU/ml was reached using semiconfluent plate -method (Figure 9 A). Further sensitivity testing showed that the phage could lyse at least 27 of the *P. aeruginosa* strains from the 43 tested. Results of the preliminary phage host specificity test (Figure 9 B). All phages that were tested performed well and the PA8P1 proved to

have the broadest infectivity range of 37 strains. In total, the phages could infect up to 80 % of the strains that were tested (See Appendix 6).

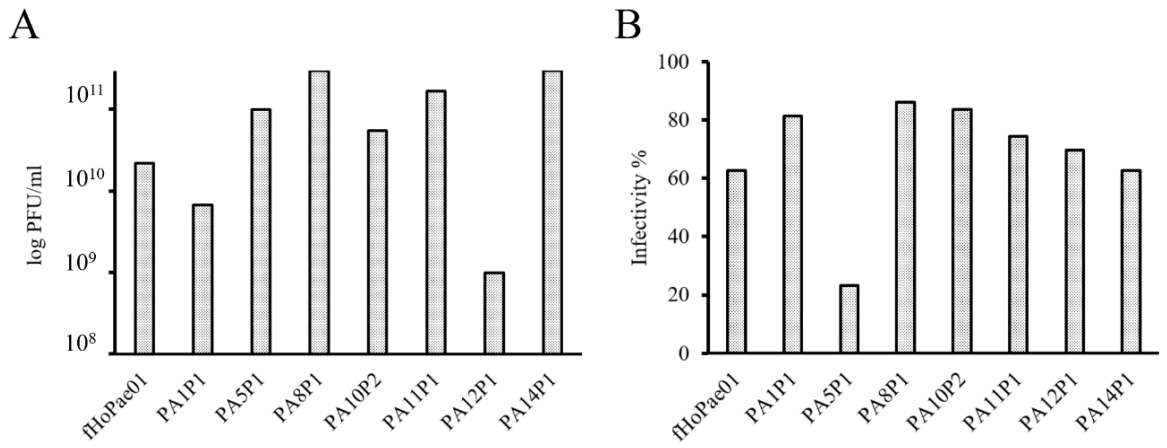

Figure 9. **Strain specificity of *P. aeruginosa* phages.** *P. aeruginosa* phages were propagated and tested for strain specificity using the semiconfluent plate method and the drop test method, respectively. Phages were scored based on the ability to lyse the target bacteria which consisted of 43 isolates of *P. aeruginosa*. A) The titration results indicate that the *P. aeruginosa* phages reach relatively high titers of over 10^9 PFU/ml without additional concentration steps. B) With these crude lysates the phages were able to lyse up to 80 % of the *P. aeruginosa* strains tested. The PA5P1 proved to have the narrowest host range with the lysis of 23 % (10 strains) of the strains tested and the PA8P1 proved to have the broadest host range with 86 % (37 stains). Newly isolated phage fHoPae01 lysed 63 % of the target strains.

5. DISCUSSION

5.1 Therapy potential of SA14P1

Based on the data gathered from the SA14P1, it is certain that the phage cannot perform as a therapy phage as it has the characteristics of a temperate phage. Although SA14P1 has rather broad host range and it forms clear plaques in bacterial cultures, there is a high probability of horizontal gene transfer when the phage goes into a lysogenic state and integrates into the genome of the host bacteria. This conclusion is backed by the presence of the phage associated integrase and DNA-binding proteins in the SA14P1 genome (Table 3), which further increase the risk of augmenting the pathogenicity or the resistance of the host bacteria through lysogenic conversion (Bae et al., 2006; Diep et al., 2008; Baba et al., 2008). SA14P1 genome was also found to encode staphylokinase which is a vital immune modulator for *S. aureus* strain bacteria against host innate immunity (Bokarewa et al., 2006).

5.2 SA14P1 genome

When comparing the SA14P1 genome with the 23MRA genome found in BLAST-search, they were found 99 % identical. 23MRA was described as a phage that was found as integrated prophage in the genome of a MRSA strain from a human blood sample (Santiago-Rodriguez et al., 2015). The genome of *S. aureus* isolates usually encompasses lysogenic bacteriophages from which most belong to the *Siphoviridae* phage family (Highlander et al., 2007; Santiago-Rodriguez et al., 2015). There is a high probability that the SA14P1 is an induced prophage from *S. aureus* isolate. Integrated phages have a significant role in conferring to the pathogenicity of the bacteria by encoding toxins, immune modulator staphylokinase or superantigens that cause toxic shock syndrome and host tissue destruction. It is possible that SA14P1 phage influences the pathogenicity of *S. aureus* strains through lysogenic conversion. Because bacterial genomes encompass prophages and other virulence factors, it is critical to use sequencing and database searches when determining the therapeutic suitability of isolated phages.

5.3 Purification of phages

5.3.1 AEC purification efficacy

The detailed evaluation of the phage revealed some interesting results of the effectiveness of AEC purification. Based on the phage particles net-charge, the elution of the phage could be roughly estimated based on previous trials. The phage was seen to elute as hypothesized at a relatively high salt concentration of 350 mM NaCl, which was expected due to the distinct negative charge of the major capsid protein of the phage particle. When the fraction expected to contain the phage was used in titer determination the results showed up to 80–90 % yield, which is high for an experimental set-up (Adrianssens et al., 2012; Bourdin et al., 2014). By using a step gradient when increasing the salt concentration instead of a linear increase used in our experiment, this result can be improved even further. This would give more time for washing the impurities and the elution of the phage. The linear increase in the NaCl concentration, however, gave valuable information on the phages elution from the column, and these results can be used as preliminary guidelines when purifying similar phages in the future. With a fully optimized HPLC purification method and suitable phages, the yields have been reported as high as 100 % (Adrianssens

et al., 2012; Bourdin et al., 2014). The TEM imaging confirmed that the studied HPLC-purified phage lysate was highly concentrated and free of impurities.

HPLC purification can also contribute to phage lysate concentration, as phages are selectively eluted at a certain NaCl concentration (Adrianssens et al., 2012; Bourdin et al., 2014). The preliminary purification tests described in this experiment were carried out with the liquid chromatography method only to see does the major head capsid charge gives a clear indication of phage elution parameters and to verify if delicate virus particles such as SA14P1 could be purified using this method. The method should be optimized for making more detailed evaluation of phage concentration and purification efficiency. The phage purification results should also be analyzed using full plates for titration instead of the drop test to further increase the accuracy of the results.

5.3.2 Significance of pre-filtration steps

Using the centrifuge Amicon ultra 100 000 MWCO column as a prepurification method turned out to be an important part of the purification process because of the high amount of bacterial debris in the crude lysate. By using the column, the phage-containing lysate was concentrated, washed from the bulk of the impurities and the remains of the culture medium were changed to a more suitable running buffer for the HPLC method. This helps to keep the fine filters inside the HPLC clean and prevents bacterial remnants from clogging the AEC column. However, the additional centrifugation steps have some definite drawbacks when working with rather delicate virus particles such as SA14P1 of *Siphoviridae* species. TEM-images revealed that the sample contained a lot of broken virus particles that had lost their tail. This was likely due to the last concentration step of the phage lysate before TEM imaging, because the infectivity of HPLC-purified phage lysate remained unchanged (data not shown). This deterioration of phage particles could have been avoided by using higher titer phage stock.

5.4 SA14P1 chromosome and cohesive ends

DNA digestion gave a preliminary estimation phage genome size of 45 kb. Based on this information alone, the chances of the phage being temperate are high, based on the high number of temperate *Siphoviridae* phages, which have genome sizes of 40-45 kb (Santiago-Rodriguez et al., 2015). During the genome analysis of SA14P1 phage, it was

found that the phage DNA had cohesive ends. All currently known tailed phage virions contain a single, from 18 to 500 kb long linear dsDNA chromosome. Based on the phages DNA replication strategy and terminase actions during DNA packaging, the DNA strand can have one of six type of termini. These include circularly permuted direct terminal repeats, exact direct terminal repeats (short or long), terminal host DNA sequences, covalently bound terminal proteins and cohesive ends. All termini types except terminal proteins are generated by phage-encoded enzyme terminase in nucleolytic cleavage (Feiss and Widner, 1982: Casjens and Gilcrease, 2009). SA14P1 DNA digestion was studied using heat treatment and a agarose gel electrophoresis method (Figure 3). The results revealed that the phage DNA has cohesive ends, which bind together after digestion.

5.5 *S. aureus* pig isolate strain sensitivity

MRSA bacterial strains isolated from pig samples showed to have overall better resistance against phages derived from human samples when compared with the rather broad host range and lysis characteristics of the SA14P1 against human derived MRSA strains. This outcome is puzzling, as the close genomic similarity between human and pig could convey to the similarity in infecting pathogens (Armand-Lefevre et al., 2005). In addition, the farm animals are considered one of the main cause of zoonoses in the form of direct infections and food-borne pathogens (Armand-Lefevre et al., 2005). The results gained in this research would suggest that the pig MRSA strains differ significantly from the human strains. The pig MRSA strains have been shown to have an overall low sensitivity to the SA14P1 and to other phages that were isolated against human strains (Tuomala, 2016). This might be an indication of different surface receptors on pig MRSA cells compared with the human bacterial strains inhibiting the recognition and attachment of phages. This would explain why the growth of the bacteria was either unaffected or just hindered. The varying results are also linked to the temperate nature of the phage which might explain why the phage did not immediately cause complete lysis. The results of sensitivity testing after 24-hour incubation showed some promising outcomes in a longer time scale (data not shown).

5.6 Newly found phage fHoPae01

The attempt at phage isolation from hospital waste waters against *Pseudomonas aeruginosa* strains was successful. The newly found phage fHoPae01 displayed great characteristics as it could infect many diverse and highly resistant strains, from which some were missing corresponding phages. Based on the results from the preliminary tests, the phage seems lytic and should be characterized and sequenced together with all the phages tested to find out what kind of a phage it is. The fact that the phage was found in water samples collected near hospitals gives a good indication of the potential places where to find phages.

5.7 MRSA growth conditions in sensitivity tests

When studying the sensitivity of MRSA pig isolates against SA14P1, the culturing of the bacteria on LB, THB and TSA plates proved to be a major challenge (Figure 2). These bacterial strains were derived from nostril or carcass swab samples of pigs and initially cultivated on blood agar (Heikinheimo et al. 2016). During the project, the strains were taken from the -70 °C freezer storage and cultivated on mainly on LB plates and in LB media liquid cultures at 37 °C overnight without CO_2 regulation. After cultivation, the number of bacterial colonies was low in many cases, which hindered the project due to an insufficient number of bacteria for the sensitivity tests. The main reasons for fairly slow bacterial growth might have been the use of unsuitable growing medium, incubation temperature, harsh freezer conditions and/or the lack of CO_2 regulation. MRSA and other heteroresistant strains are known to be difficult to cultivate due to poor growth and different growth requirements compared with other bacterial strains. Typical MRSA grows at 33– 35 °C and some require CO_2 regulating (CLSI, 2013). For better growth results, the culturing parameters need to be further optimized in the form of growth media, the temperature and atmospheric regulation. This would, however, require special equipment which would suit MRSA strains, but not all other bacteria. Special culture media, such as BACTEC™ Plus aerobic broth (Becton, Dickinson and Company), brain-heart infusion medium and plates have been used for culturing MRSA, and these could be useful when faced with demanding MRSA strains in the future (Santiago-Rodriguez et al., 2015).

5.8 Phage implementations

5.8.1 Modified phages

The safe use of the SA14P1 phage for pharmaceutical purposes would require genetic and/or other type of modification. This would include the removal of all virulence factors potentially beneficial for the host bacteria. However, since phages are abundant in the nature, it would be more feasible to search for better phages for phage cocktail treatment purposes. The fact that genetically modified organisms (GMO) in pharmaceutical products have stricter legislation further hinders the use of a modified phages over phages in their natural state. Despite the antagonistic stance to GMO, modified phages can and have been utilized in various scenarios in bacterial treatment and typing, vaccination, gene delivery and even in treating drug addiction (Meola et al., 1995; Dickerson et al., 2005). Many of these applications rely on the virus capsid that can be modified and used as a carrier, or on recognition by the immune system and on the identification of host bacterial species *in vitro* and *in vivo* applications respectively (Watson and Eveland, 1965; Molenaar et al., 2002). Temperate phages, such as SA14P1, could be used as gene transporters that could for example deliver susceptibility genes for specific antibiotics.

Some antibacterial applications utilize only specific parts of the phage particle. These include the use of “empty” phages that do not have genome, separate phage tails and phage derived proteins, enzymes and lysins that break down the bacterial cells (Boulanger and Letellier, 1988; Loessner et al., 1994; Šmarda and Benada, 2005; Fenton et al., 2010; Drulis-Kawa et al., 2015). These approaches are considered less active as the phages cannot replicate in the bacterial cells. Individual phage structures and substances can be preferable in numerous different situations where the only suitable phages are lysogenic or the most effective antibacterial property of the phage is related to the structures or enzymes they produce. Most of these phage implementations have however the same caveat, which is the problem of ensuring the absence of active phage particles which are able to reproduce.

5.8.2 Other implementations

In addition to the use as antibacterials, phages can be used as tools in bacterial typing and as universal surface disinfectants. Bacterial typing application is based on the relatively

narrow host range of the phages, which in turn can be used to interact with bacterial strains usually aiming to the clear lysis of bacterial cells. This gives to arise many different applications where the phages are used as identifiers to rapidly and precisely gain knowledge of the unknown bacterium. The bacterial strain can be recognized with minimal sample preparation from various samples (see review by Clark and March, 2006).

5.9 Phage challenges and possibilities

5.9.1 Eradication of bacterial toxins

There are multiple problems that are still left to be solved if phages are to be used for pharmaceutical purposes. One of the most problematic aspects in phages is the propagation step, where pathogenic host bacterial species are used. These bacteria can include for example the resistant variants of *Enterococcus faecum, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter species* and *Escheria coli* or (ESKAPEE) strains, that are now considered the most problematic clinically (for review, see Navidinia, 2016). Although the bacterial cells can be in most cases killed and filtered from the phage lysate by using chloroform and suitable filters, the eradication of bacterial endotoxins, exotoxins and cell debris is a lot more tedious work. From these, the endotoxins (LPS and LOS) that are present in the cell walls of gram-negative bacteria, are released after bacterial cells are killed, which makes the prospect of the rapid destruction of a large population of bacteria rather dangerous for the patient as the toxins cause severe inflammation and have other properties of potent toxins. LPS compounds are also highly heat-resistant and almost impervious to chemicals which makes the purification extremely difficult (Masignani et al., 2006).

5.9.2 Legislation

Advances in phage therapy are greatly hindered due to the business-oriented pharmacy industry, mainly because of problems in the intellectual property- and patent rights concerning phage products (Clark and March, 2006). This is a significant deterrent to the major drug corporations, as there is a high degree of uncertainty in making profit with over a 100-year-old discovery. Phages are readily found in the nature and thus every company could produce their own variant of the said product and eventually bring competition to the field of phage medicine market. At the moment, the patent right for the rights to a certain

phage does not exist, which makes the prospect of owning phage products a lot more uncertain (Clark and March, 2006). Another major factor holding back the clinical use of phages is the lack of universal guidelines and framework relating to the administration and classification of phage products (Verbeken et al., 2009). Hopefully the legislation in the Western world becomes more suitable for phage medicine production and commercialization, so that it could provide the required interest for the research and production of new and more efficient phage-derived clinical products in the future.

5.9.3 Limited host range and bacterial immunity

The novel nature of phages contributes to a wide spectrum of applications for the treatment of bacterial infections in previously unseen ways. This is mainly due to the highly selective host range, which opens the possibility to treat infections without harming the normal bacterial flora. Unfortunately, high specificity is also a significant drawback because of the meticulous process of identifying the invading pathogen and choosing the right phage for the job. This, on the other hand, increases the chances of success, but also dramatically increases the costs of the phage medicine. Therefore, phage therapy is currently considered a great application in personalized treatment, but it still lacks in being the magic bullet that solves all the problems. The recent advent of multiple phages-containing cocktails has improved the effectiveness of the treatments by increasing the chance of finding phage-bacterium match when many different strains of bacteria are targeted simultaneously and the individual bacteria are subjected to a barrage of different phages at the same time (Oliveira et al., 2010). The use of phage cocktails might also be useful in the form of phage synergy, which means that a phage that seems insufficient at combating the infection on its own can work when it is used in combination with different phages. The increase in efficacy can be greater than when using the phages separately. This also minimizes the chances of bacteria gaining immunity against phages used in the treatment (Schmerer et al., 2014).

5.9.4 Phage safety in clinical products

The most controversial aspect about phages is their safety. Although phage medicines are sold freely in East-European countries, until today, there has been less than a handful of well-made preclinical experiments that have shown only minimal side effects when phages

were applied superficially or orally in humans (Bruttin and Brüssow, 2005; Sarker et al., 2016). Phages have been for long since used successfully in animal models and their use has been recognized to be beneficial (Carmody et al., 2010; Monk et al., 2010). When the safety of phage containing clinical products is discussed, one should also consider the fact that humans digest unknown quantities of phages in different food products produced by fermentation (bacteria). The majority of the speculations towards the safety of phage originates from the poorly done phage experiments from the time before antibiotics and thus the final verdict of phage safety should be given only after clinical trials have been completed and the results have been thoroughly analyzed.

6. FUTURE PERSPECTIVES

6.1 The next step

The research done in this thesis provided some preliminary data for the future experiments. The next logical research topic would be to find suitable phages against MRSA pig isolate strains, to study and compare them with the human-derived counterparts. To find phages against pig MRSA, the search should start from the source (farms) of the bacterial infections and the bacteria. The liquid chromatography purification method performed well in phage purification for TEM imaging. The future tests should include detailed analysis of the toxin levels of AEC-purified phage lysates and study the prospects of AEC in the purification of other phage types. *P. aeruginosa* phages studied briefly in this research showed great characteristics, and should be further studied in the form of genome sequencing and general characterization to evaluate their potential in phage therapy. The newly isolated phage fHoPae01 should also be studied and added to the genome database if the phage turns out to be an entirely new finding.

6.2 Future developments

There is still a lot of research to be done in the field of phage therapy. One of the most important research areas are the possible side effects of active phage-containing solutions. The fact that phage antigens are recognized by the immune system can confer to undesired inflammation and in the worst cases allergic reactions and lowered immunity. This might also lead to decrease in the infectivity and function of phages (Fogelman et al., 2000; Górski et al., 2012). On top of this, the bacterial cell death that is induced by the lytic

phages might also lead to harmful inflammation. This makes the administration of phages a major problem. Researchers are currently trying to find ways for the package and delivery of phages in order to shield them from the immune defense system and the inhospitable conditions inside the body. These methods include degradable capsules and maybe in the future even extracellular vehicles which would greatly improve the durability of phages, selectivity and maximize the efficacy of the therapy. As phages are found to be seemingly harmless when administrated orally, the next new phage treatment concept could be the isolation of broad-host-range phages against *Streptococcus mutans*, which is one of the harmful bacterial strains inside the mouth. This would be a good way to prevent the formation of biofilms and eventually caries (Dalmasso et al., 2015). The interest in isolating phages against plant pathogens has also been revived, providing treatments for plant diseases in an organic way (Obradovic et al., 2004).

7. CONCLUSIONS

The in-depth characterization of SA14P1 revealed that the phage cannot be used as a potential therapy phage. This was due to the lysogenic (temperate) life cycle of the phage, which makes the probability of increasing the pathogenicity of the target bacteria high. The sensitivity assay showed that the bacteria that infect pigs seem more resistant to phages that are found to have human infecting *S. aureus* hosts. This indicates that there are differences between the *S. aureus* bacterial strains infecting humans or only pigs. The efficacy of the phages against human infecting *P. aeruginosa* showed great promise by infecting in total almost 90 % of the 43 bacterial strains tested. The attributes of the newly isolated phage fHoPae01 make it interesting subject for further studies.

Phage research has been revitalized and the recent well-described studies show the potential of phage therapy against several different bacterial infections in various therapy settings. Moreover, the increasing phage medicine selection in East-European countries already offers a wide range of remedies. The unique attributes of phages make them highly diverse tools with almost limitless possibilities. In the future, the spectrum of phage-derived medicine is undoubtedly going to widen, and as the biology of the phages starts to unravel, the potency of phage therapy will increase. Hopefully, the fast-developing molecular biology techniques and genetic analysis of phages provides the help needed in the fight against MDR infections, as the post-antibiotic era is rapidly approaching.

8. REFERENCES

Abedon S.T., S.J. Kuhl, B.G. Blasdel and E.M. Kutter. 2011. Phage treatment of human infections. *Bacteriophage*. 1, (2): 66-85. doi: 10.4161/bact.1.2.15845.

Ackermann H.W. 1975. Classification of the bacteriophages of Gram-positive *cocci*: *Micrococcus*, *Staphylococcus*, and *Streptococcus*. Pathol. Biol. 23, (3): 247–253. PMID: 124850.

Adriaenssens E.M., S.M. Lehman, K. Vandersteegen, D. Vandenheuvel, D.L. Philippe, A. Cornelissen, M.R.J. Clokie, A.J. García, M. De Proft, M. Maes and R. Lavigne. 2012. CIM® monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles. *Virol.* 434, (2): 265-270. doi: 10.1016/j.virol.2012.09.018.

Alberts B., A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walters. 2008. Molecular biology of the cell 5th edition. Garlan Science, Taylor & Francis Group, LCC. 711 Third Avenue, 8th floor, New York, NY 10017, USA.1485-1524. ISBN: 978-0-8153-4106-2.

Alibayov B., L. Baba-Moussa, H. Sina, K. Zdeňková and K. Demnerová. 2014. *Staphylococcus aureus* mobile genetic elements. *Mol Biol Rep.* 41: 5005-5018. doi: 10.1007/s11033-014-3367-3.

Archer G. L. 1998. *Staphylococcus aureus*: A Well-Armed Pathogen. *Clin Infect Dis.* 26, (5): 1179-1181. doi: 10.1086/520289.

Armand-Lefevre L., R. Ruimy and A. Andremont. 2005. Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. *Emerg. Infect Dis.* 11, (5): 711-714. doi: 10.3201/eid1105.040866.

Artimo P., M. Jonnalagedda, K. Arnold, D. Baratin, G. Csardi, E. de Castro, S. Duvaud, V. Flegel, A. Fortier, E. Gasteiger, A. Grosdidier, C. Hernandez, V. Ioannidis, D. Kuznetsov, R. Liechti, S. Moretti, K. Mostaguir, N. Redaschi, G. Rossier, I. Xenarios, and H. Stockinger. 2012. ExPASy: SIB bioinformatics resource portal, *Nucleic Acids Res*, 40: (W1): 597-603.

Ayliffe G.A.J. 1997. The Progressive Intercontinental Spread of Methicillin-Resistant *Staphylococcus aureus*. *Clin. Infect. Dis.* 24: (S79) 74-79. doi: 10.1093/clinids/24.Supplement 1.S74.

Aziz R.K., D. Bartels, A.A. Best, M. DeJongh, T. Disz, R.A. Edwards, K. Formsma, S. Gerdes, E.M. Glass, M. Kubal, F. Meyer, G.J. Olsen, R. Olson, A.L. Osterman, R.A. Overbeek, L.K. McNeil, D. Paarmann, T. Paczian, B. Parrello, G.D. Pusch, C. Reich, R. Stevens, O. Vassieva, V. Vonstein, A. Wilke and O. Zagnitko. 2008. The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics.* 9, (75): 1-15. doi: 10.1186/1471-2164-9-75.

Baba T., T. Bae, O. Schneewind, F. Takeuchi and K. Hiramatsu. 2008. Genome Sequence of *Staphylococcus aureus* Strain Newman and Comparative Analysis of *Staphylococcal* Genomes: Polymorphism and Evolution of Two Major Pathogenicity Islands. *J Bacteriol.* 190, (1): 300-310. doi: 10.1128/JB.01000-07.

Bae T., T. Baba, K. Hiramatsu and O. Schneewind. 2006. Prophages of *Staphylococcus aureus* Newman and their contribution to virulence. *Mol. Microbiol.* 62, (4): 1035-1047. doi: 10.1111/j.1365-2958.2006.05441.x.

Bodey G.P. 1983. Infections caused by *Pseudomonas aeruginosa*. *Rev.Infect.Dis.* 5, (2): 279-313. PMID: 6405475.

Bouza E., F. Grcia-Garrote, E. Cercenado, M. Marin and M.S. Diaz. 1999. *Pseudomonas aeruginosa*: a Survey of Resistance in 136 Hospitals in Spain. *Antimicrob Agen Chemother*. 43, (4): 981-982. PMCID: PMC89243.

Boulanger P. 2009. Purification of bacteriophages and SDS-PAGE analysis of phage structural proteins from ghost particles. *Methods Mol Biol*. 502: 227-238. doi: 10.1007/978-1-60327-565-1_13.

Boulanger P. and L. Letellier. 1988. Characterization of Ion Channels Involved in the Penetration of Phage T4 DNA into Escherichia coli Cells. *J Bio Chem*. 263, (20): 9767-9775. PMID: 2454920.

Bourdin G., B. Schmitt, L. M. Guy, J-E. Germond, S. Zuber, L. Michot, G. Reuteler and H. Brüssow. 2014. Amplification and Purification of T4-Like Escherichia coli Phages for Phage Therapy: from Laboratory to Pilot Scale. *Appl. Environ. Microbiol.* 80, (4):1469-1476. doi: 10.1128/AEM.03357-13.

Brettin T., J.J. Davis, T. Disz, R.A. Edwards, S. Gerdes, G.J. Olsen, R. Olson, R. Overbeek, B. Parrello, G.D. Pusch, M. Shukla, J.A. Thomason, R. Stevens, V. Vonstein, A.R. Wattam and F. Xia. 2015. RASTtk: A modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci Rep*. 10, (5): 8365. doi: 10.1038/srep08365.

Bruttin A. and H. Brüssow. 2005. Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy. *Antimic Agen. Chemo.* 49, (7):2874-2878. doi: 10.1128/AAC.49.7.2874-2878.2005.

Canchaya C., C. Proux, G. Fournous, A. Bruttin and H. Brüssow. 2003. Prophage genomics. *Microbiol. Mol Biol Rev.* 67, (2): 238-473. doi: 10.1128/MMBR.67.3.473.2003.

Carmody L. A., J. J. Gill, E.J. Summer, U.S. Sajjan, C.F. Gonzalez, R.F. Young and J. J. Liuma. 2010. Efficacy of Bacteriophage Therapy in a Model of *Burkholderia cenocepacia* Pulmonary Infection. *The J Infect Dis.* 201, (2): 264-271. doi: 10.1086/649227.

Casjens S.R. and E.B. Gilcrease. 2009. Determining DNA packaging strategy by analysis of the termini of the chromosomes in tailed-bacteriophage virions. *Methods Mol Biol.* 502: 91-111. doi: 10.1007/978-1-60327-565-1_7.

Dickerson T.J., G.F. Kaufmann and K.D. Janda. 2005. Bacteriophage-mediated protein delivery into the central nervous system and its application in immunopharmacotherapy. *Expert Opin Biol Ther*. 5, (6): 773–781. doi: 10.1517/14712598.5.6.773.

Chambers H.F. 2001. The changing epidemiology of *Staphylococcus aureus*? *Emerg Infect Dis.* 7: 178-182. doi: 10.3201/eid0702.700178.

Clark J.R. and J.B. March. 2006. Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. *Trends Biotechnol.* 24, 85): 212-218. doi: 10.1016/j.tibtech.2006.03.003.

Clinical and Laboratory Standards Institute (CLSI). 2013. Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Third Informational Supplement. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19085 USA. CLSI document M100-S23. ISBN: 1-56238-865-7.

Dalmasso M., E. de Haas, H. Neve, R. Strain, F.J. Cousin, S.R. Stockdale, R.P. Ross and C. Hill. 2015. Isolation of a Novel Phage with Activity against *Streptococcus mutans* Biofilms. *PloS One.* 23, (9): e0138651. doi: 10.1371/journal.pone.0138651.

Diep B. A., G.G. Stone, L. Basuino, C. J. Graber, A. Miller, S-A. des Etages, A. Jones, A. M. Palazzolo-Ballance, F. Perdreau-Remington, G. F. Sensabaugh, F. R. DeLeo and H. F. Chambers. 2008. The Arginine Catabolic Mobile Element and *Staphylococcal* Chromosomal Cassette mec Linkage: Convergence of Virulence and Resistance in the USA300 Clone of Methicillin-Resistant *Staphylococcus aureus*. *J Infect Dis.* 197, (11): 1523-1530. doi: 10.1086/587907.

Drucker D.B., H.A Aluyi, J.A. Morris, D.R. Telford, B.A. Oppenheim and B.A. Crawley. 1992. Possibility of separating toxins from bacteria associated with sudden infant death syndrome using anion exchange chromatography. *J Clin Pathol*. 45, (9): 802-805. PMID: 1401212.

Drulis-Kawa Z., G. Majkowska-Skrobek and B. Maciejewska. 2015. Bacteriophages and Phage-Derived Proteins - Application Approaches. *Cur Med Chem.* 22, (14): 1757-1773. doi: 10.2174/0929867322666150209152851.

Erez Z., I. Steinberger-Levy, M. Shamir, S. Doron, A.S. Stokar-Avihail, Y. peleg, S. Melamed, A. Leavitt, A. Savidor, S. Albeck, G. Amitai and R. Sorek. 2017. Communication between viruses guides lysis-lysogeny decisions. *Nat*. 541. 488-493. doi:10.1038/nature21049.

Feiss M. and W. Widner. 1982. Bacteriophage λ DNA packaging: Scanning for the terminal cohesive end site during packaging. *Proc Natl Acad Sci USA.* 79, (11): 3498-3502. PMCID: PMC346448.

Feng Y., C. Chen, L. Su, S. Hu, J. Yu and C. Chiu. 2008. Evolution and pathogenesis of *Staphylococcus aureus*: lessons learned from genotyping and comparative genomics. *FEMS Microbiol Rev.* 32, (1): 23-37. doi: 10.1111/j.1574-6976.2007.00086.x.

Fenton M., O. McAuliffe, J. O'Mahony and A. Coffey. 2010. Recombinant bacteriophage lysins as antibacterials. *Bioeng Bugs.* 1, (1): 9-16. doi: 10.4161/bbug.1.1.9818.

Fluit A.C. 2012. Livestock-associated *Staphylococcus aureus*. *Clin Microbiol Infect.* 18, (8): 735-744. doi: 10.1111/j.1469-0691.2012.03846.x.

Fogelman I., V. Davey, H.D. Ochs, M. Elashoff, M.B. Feinberg, J.A. Mican, J.P. Siegel, M. Sneller and H.C. Lane. 2000. Evaluation of CD4 super (+) T cell function in vivo in HIV-infected patients as measured by bacteriophage phiX174 immunization. *J Infect Dis.* 182, (2): 435-441. doi: 10.1086/315739.

Geer L.Y., A. Marchler-Bauer, R.C. Geer, L. Han, J. He, S. He, C. Liu, W. Shi, S.H. Bryant. The NCBI BioSystems database. 2010. *Nucleic Acids Res.* 38: Database issue: D492-D496. PMID: 19854944 Database Website: https://www.ncbi.nlm.nih.gov/biosystems. Accessed 1.10.2017.

Gill D.M. 1982. Bacterial Toxins: a Table of Lethal Amounts. *Microbiol Rev.* 46, (1): 86-94. PMID: 6806598.

Gordon R.J. and F.D. Lowy. 2008. Pathogenesis of Methicillin-Resistant *Staphylococcus aureus* Infection. *Clin Infect Dis.* 46 (1): S350-359. doi: 10.1086/533591.

Górski A., R. Międzybrodzki, J. Borysowski, K. Dąbrowska, P. Wierzbicki, M. Ohams, G.Korczak-Kowalska, N. Olszowska-Zaremba, M.Łusiak-Szelachowska, M. Kłak, E. Jończyk, E. Kaniuga, A.Gołaś, S. Purchla, B. Weber-Dąbrowska, S. Letkiewicz, W.Fortuna,, K. Szufnarowski,, Z. Pawełczyk, P.Rogóż and D. Kłosowska. 2012. Phage as a Modulator of Immune Responses Practical Implications for Phage Therapy. *Adv. V. Res.* 83: 41-71 doi: 10.1016/B978-0-12-394438-2.00002-5.

Hardalo C. and S.C. Edberg. 1997. *Pseudomonas aeruginosa*: Assessment of Risk from Drinking Water. *Crit Rev in Microbiol*. 23, (1): 47-75. doi: 10.3109/10408419709115130.

Heikinheimo A., S. Johler, L. Karvonen, J. Julmi, M. Fredriksson-Ahomaa and R. Stephan. 2016. New dominant spa type t2741 in livestock-associated MRSA (CC398-MRSA-V) in Finnish fattening pigs at slaughter. *Antimic Resis Infect Control*. 5, (6): doi: 10.1186/s13756-016-0105-8

Hendrix R.W., M.C.M. Smith, R.N. Burns, M.E. Ford and G.F. Hatfull. 1999. Evolutionary Relationships among Diverse Bacteriophages and Prophages: All the World's a Phage. P*roc Natl Acad Sci U.S.A.* 96, (5): 2192-2197. doi: 10.1073/pnas.96.5.2192.

Parker M.T. and M.P. Jevons. 1964. A Survey of Methillicin Resistance in *Staphylococci. Postgrad Med J.* 40: 170-178. PMID: 13957735.

Kallio M.A., J.T. Tuimala, T. Hupponen, P. Klemelä, M. Gentile, I. Scheinin, M. Koski, J. Käki and E.I. Korpelainen. 2011. Chipster: user-friendly analysis software for microarray and other high-throughput data. *BMC Genomics*. 14, (12): 507. doi: 10.1186/1471-2164-12-507.

Lindsay J.A. and M.T.G. Holden. 2004. *Staphylococcus aureus*: superbug, super genome? *Trends Microbiol*. 12 (8): 378–385. doi: 10.1016/j.tim.2004.06.004.

Liu J., M. Dehbi, G. Moeck, F. Arhin, P. Bauda, D. Bergeron, M. Callejo, V. Ferretti, N. Ha, T. Kwan, J. McCarty, R. Srikumar, D. Williams, J.J. Wu, P. Gros, J. Pelletier and M. DuBow. 2004. Antimicrobial drug discovery through bacteriophage genomics. *Nat. Boitechnol.* 22, (2): 185-191. doi: 10.1038/nbt932.

Loc-Carrillo C. and S.T. Abedon. 2011. Pros and cons of phage therapy. *Bacteriophage.* 1, (2): 111-114. doi: 10.4161/bact.1.2.14590.

Loessner M.J., A. Schneider and S. Scherer. 1995. A new procedure for efficient recovery of DNA, RNA, and proteins from Listeria cells by rapid lysis with a recombinant bacteriophage endolysin. *Appl. Environ. Microbiol.* 61, (3): 1150-1152. PMCID: PMC167370.

Mahony J., M. Alqarni, S. Stockdale, S. Spinelli, M. Feyereisen, C. Cambillau and D.V. Sinderen. 2016. Functional and structural dissection of the tape measure protein of lactococcal phage TP901-1. *Sci Rep*. 6, (8): 36667. doi: 10.1038/srep36667.

Masignani V., M. Pizza and R. Rappuoli. 2006. Bacterial toxins. Editors: Dworkin M., S. Falkow, E. Rosenberg, K-F. Schleifer and E. Stackebrandt. The Prokaryotes A Handbook on the Biology of Bacteria. Third edition. *Springer*. New York USA. 6 (2): 893-899. ISBN: 10: 0-387-25499-X.

Mattila S., P. Ruotsalainen and M. Jalasvuori. 2015. On-Demand Isolation of Bacteriophages Against Drug-Resistant Bacteria for Personalized Phage Therapy. *Front Microbiol.* 6: 1271. doi: 10.3389/fmicb.2015.01271.

Meola A., P. Delmastro, P. Monaci, A. Luzzago, A. Nicosia, F. Felici, R. Cortese and G. Galfre. 1995. Derivation of vaccines from mimotopes. Immunologic properties of human hepatitis B virus surface antigen mimotopes displayed on filamentous phage. *J Immunol.* 1995, 154, (7): 3162-3172. PMID: 7534789.

Merabishvili M., J. Pirnay, G. Verbeken, N. Chanishvili, M. Tediashvili, N. Lashkhi, T. Glonti, V. Krylov, J. Mast, L. Van Parys, R. Lavigne, G. Volckaert, W. Mattheus, G. Verween, P. De Corte, T. Rose, S. Jennes, M. Zizi, D. De Vos and M. Vaneechoutte. 2009. Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. *PloS One.* 4, (3) :e4944. doi: 10.1371/journal.pone.0004944.

Mesaros N., P. Nordmann, P. Plesiat, M. Roussel-Delvallez, J. Van Eldere, Y. Glupczynski, Y. Van Laethem, F. Jacobs, P. Lebecque, A. Malfroot, P.M. Tulkens and F. Van Bambeke. 2007. *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. *Clin Microbiol & Infec.* 13, (6): 560-578. doi: 10.1111/j.1469-0691.2007.01681.x.

Middlebrook J.L and R.B. Dorland. 1984. Bacterial Toxins: Cellular Mechanisms of Action. *Microbiol Rev.* 48, (3): 199-221. PMID: 6436655.

Molenaar T.J., I. Michon, S.A.M. de Haas, T.J.C. van Berkel, J. Kuiper, and E.A.L. Biessen. 2002. Uptake and Processing of Modified Bacteriophage M13 in Mice: Implications for Phage Display *Virol.* 293, (1): 182–191. doi:10.1006/viro.2001.1254.

Monk A.B., C.D. Rees, P. Barrow, S. Hagens and D.R. Harper. 2010. Bacteriophage applications: where are we now? *Lett Appl Microbiol.* 51, (4): 363-369. doi: 10.1111/j.1472-765X.2010.02916.x.

Navidinia M. 2016. The clinical importance of emerging ESKAPE pathogens in nosocomial infections. *J Paramed Sci*. 7, (3): 43-57. ISSN: 2008-4978.

Obradovic A, J.B. Jones, M.T. Momol, B. Balogh and S.M. Olson. 2004. Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. *Plant Dis.* 88, (7): 736–740. doi: 10.1094/PDIS.2004.88.7.736.

Oliveira A., R. Sereno and J. Azeredo, 2010. In vivo efficiency evaluation of a phage cocktail in controlling severe *colibacillosis* in confined conditions and experimental poultry houses. *Vet. Microbiol.* 146. 303–308. doi: 10.1016/j.vetmic.2010.05.015.

O'Neill J. 2016. Review on Antimicrobial Resistance. Tackling drug-resistant Infections Globally: Final report and recommendations. (https://amr-review.org/) Accessed: 19.1.2017.

Oppenheim A.B., O. Kobiler, J. Stavans, D.L. Court and S. Adhya. 2005. Switches in Bacteriophage Lambda Development. *Annu Rev Genet.* 39, (1): 409-429. doi: 10.1146/annurev.genet.39.073003.113656.

Overbeek R., R. Olson, G.D. Pusch, G.J. Olsen, J.J. Davis, T. Disz, R.A. Edwards, S. Gerdes, B. Parrello, M. Shukla, V. Vonstein, A.R. Wattam, F. Xia, R. Stevens. 2013. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res*. 42: D206-D214. doi: 10.1093/nar/gkt1226.

Parracho H.M., B.H. Burrowes, M.C. Enright, M.L. McConville and D.R. Harper. 2012. The role of regulated clinical trials in the development of bacteriophage therapeutics. *J Mol Genet Med.* 6: 279-286. PMCID: PMC3410379.

Poranen M.M., D.H. Bamford and H.M. Oksanen. 2015. Membrane-Containing Bacteriophages. In Encyclopedia of Life Sciences (eLS). (pp. 1-11). John Wiley & Sons Ltd. doi: 10.1002/9780470015902.a0000779.pub3.

Reese J.B., L.A. Urry, M.L. Cain, S.T. Wasserman, P.V. Minorsky and R.B. Jackson. 2011. Campbel Biology 9th edition. Pearson education Inc. 1301 Sansome St., San Francisco, CA 941111. 613-619. ISBN: 10: 0321558235.

Sambrook J. and D.W. Russel. 2001 Commonly Used Techniques in Molecular Cloning. Vol. 3, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA. 631-632. ISBN: 978-1-936113-42-2.

Santiago-Rodriguez T.M., M. Naidu, M.B. Jones, M. Ly and D.T. Pride. 2015. Identification of *staphylococcal* phage with reduced transcription in human blood through transcriptome sequencing. *Front. Microbiol.* 6: 216. doi: 10.3389/fmicb.2015.00216.

Sarker S.A., B. Berger, Y. Deng, S. Kieser, F. Foata, D. Moine, P. Descombes, S. Sultana, S. Huq, P.K. Bardhan , V. Vuillet , F. Praplan , H. Brüssow. 2016. Oral Application of *Escherichia coli* Bacteriophage: Safety Tests in Healthy and Diarrheal Children from Bangladesh. *Environ Microbiol.* 19, (1): 237-250. Accepted article. doi: 10.1111/1462-2920.13574.

Schmerer M., I. Molineux and J. Bull. 2014. Synergy as a rationale for phage therapy using phage cocktails. *Peer J.* 2: e590. doi: 10.7717/peerj.590.

Schindelin J., I, Arganda-Carreras and E. Frise. 2012, "Fiji: an open-source platform for biological-image analysis". *Nat meth.* 9, (7): 676-682. PMID 22743772.

Schook L.B., T.V. Collares, K.A. Darfour-Oduro, A.K. De, L.A. Rund, K.M. Schachtschneider, and F.K. Seixas. 2015. Unraveling the swine genome: implications for human health. *Annu Rev Anim Biosci.* 3: 219-244. doi: 10.1146/annurev-animal-022114-110815.

Somerville G.A. and R. A. Proctor. 2009. *Staphylococci* in Human Disease. Editors: K.B. Crossley, K.K. Jefferson, G. Archer and V.G. Fowler Jr. 2nd Edition. Blackwell Publishing Ltd. UK. 3-30. ISBN: 978-14051-6332-3.

Stover C.K., X.O. Pham, A.L. Erwin, S.D. Mizoguchi, P. Warrener, M.J. Hickey, F.S. Brinkman, W.O. Hufnagle, D.J. Kowalik, M. Lagrou, R.L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L.L. Brody, S.N. coulter, K.R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G.K-S. Wong, Z. Wu, I.T. Pulsen, J. Reizer, M.H. Saier, R.E.W. Hancock, S. Lory and M.V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nat.* 406, (31): 959-964. doi: 10.1038/35023079.

Swoboda J.G., J. Campbell, T.C. Meredith and S. Walker. 2010. Wall teichoic acid function, biosynthesis, and inhibition. *Chembiochem.* 11, (1): 35-45. doi: 10.1002/cbic.200900557.

Šmarda J. and O. Benada. 2005. Phage Tail-Like (High-Molecular-Weight) Bacteriocins of *Budvicia aquatica* and *Pragia fontium* (*Enterobacteriaceae*). *Appl Environ Microbiol.* 71, (12): 8970-8973. doi: 10.1128/AEM.71.12.8970-8973.2005.

Tenover F.C. and R.V. Goering. 2009. Methicillin-resistant *Staphylococcus aureus* strain USA300: origin and epidemiology. *J. Antimic Chemother.* 64: 441-446. doi: 10.1093/jac/dkp241.

Tuomala H. 2016. Isolation and Host Specificity Determination of Bacteriophages for Phage Therapy. (Thesis) Retrieved from Theseus Open Repository of the Universities of Applied Sciences. http://urn.fi/URN:NBN:fi:amk-2016112517423.

Tritt A., J.A. Eisen, M.T. Facciotti, A.E. Darling. 2012. An Integrated Pipeline for de Novo Assembly of Microbial Genomes. *PLoS ONE*. 7, (9): e42304. doi:10.1371/journal.pone.0042304.

Kung V.L., E.A. Ozer and A.R. Hauser. 2010. The Accessory Genome of *Pseudomonas aeruginosa*. *Microbiol Mol Biol Rev*. 74, (4): 621-641. doi: 10.1128/MMBR.00027-10.

Verbeken G., I. Huys, D. De Vos, A. De Coninck, D. Roseeuw, E. Kets, A. Vanderkelen, J.P. Draye, T. Rose, S. Jennes, C. Ceulemans and J.P. Pirnay. 2016. Access to bacteriophage therapy: discouraging experiences from the human cell and tissue legal framework. *FEMS Microbiol Lett.* 363, (4): fnv241. doi: 10.1093/femsle/fnv241.

Watson B.B. and W.C. Eveland. 1965. The Application of the Phage-Fluorescent Antiphage Staining System in the Specific Identification of Listeria monocytogenes: I. Species Specificity and Immunofluorescent Sensitivity of Listeria monocytogenes Phage Observed in Smear Preparations. *J. Infect. Dis.* 115, (4): 363-369. PMID: 4953784.

Weber-Dąbrowska B., E. Jończyk-Matysiak, M. Żaczek, M. Łobocka, M. Łusiak-Szelachowska and A. Górski. 2016. Bacteriophage Procurement for Therapeutic Purposes. *Front Microbiol.* 7: 1177. doi: 10.3389/fmicb.2016.01177.

Whitman W.B., D.C. Coleman and W.J. Wiebe. 1998. Prokaryotes: The unseen majority. *Proc Natl Acad Sci USA*. 95, (12): 6578-6583. PMCID: PMC33863.

World Health Organization (WHO). 2014. Antimicrobial Resistance: Global Report on Surveillance. http://www.who.int/drugresistance/documents/surveillancereport/en/. Accessed: 20.10.2016.

Wittebole X., S. De Roock and S.M. Opal. 2014. A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virul.* 5:226-235. doi: 10.4161/viru.25991.

APPENDICES

Appendix 1: Solutions and reagents

Buffer A	20 mM Tris-Cl pH 7.5 dH_2O
Buffer B	20 mM Tris-Cl 1 M NaCl pH 7.5 dH_2O
LB medium	10 g/l Bacto-Tryptone 10 g/l NaCl 5 g/l Bacto Yeast Extract pH 7.5 dH_2O
SM-buffer	100 mM NaCl 8mM $MgSO_4 \cdot 6H_20$ 50 mM Tris-Cl pH 7.5
TE-buffer	10 mM Tris-HCl (pH8.0) 1 mM EDTA dH_2O
TSA	15 g/l Tryptone 5 g/l NaCl 5 g/l soytone dH_2O

TSB + Mg^{+}	17 g/l Tryptone
	3 g/l Phytone
	5 g/l NaCl
	2.5 g/l glucose
	2.5 g/l K_2HPO_4
	20 mM Mg^{2+}
	dH_2O
6X loading dye buffer	30 % glycerol
	0.25 % bromophenol
	0.25 % xylene cyanol FF

Appendix 2: Phages used in the experiments.

Phage identifier	Host strain	Plaque size	Description	Reference
SA14P1	SA14	small	*Siphoviridae*	Mattila et al.
fRu-Sau02*	5676	large	*Myoviridae*	**
PA1P1	PA1	small	-	Mattila et al.
PA5P1	PA5	medium	-	Mattila et al.
PA8P1	PA8	small	-	Mattila et al.
PA10P2	PA10	small	-	Mattila et al.
PA11P1	PA11	small	-	Mattila et al.
PA12P1	PA12	small/medium	-	Mattila et al.
PA14P1	PA14	very small	-	Mattila et al.

* Isolated from phage therapy product for *Staphylococcus*. **Microgen, Russia; http://en.microgen.ru/

Appendix 3: *Staphylococcus aureus* strains used in the experiments.

Strain identifier(s)	Origin	Description	Spa type	Reference
SA14, 60881	Hospital sample*	Host for SA14P1	-	Mattila et al.
5676, 13KP	Hospital sample**	Host for fRu-Sau02	-	HUSLAB
7879_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
7879_6_10P	Carcass swab	Sensitivity test	t2741	Heikinheimo et al.
7081_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
7605_16_20	Nasal swab	Sensitivity test	t108	Heikinheimo et al.
7605_6_10P	Carcass swab	Sensitivity test	t091	Heikinheimo et al.
7936_6_10	Nasal swab	Sensitivity test	t034	Heikinheimo et al.
7936_11_15	Nasal swab	Sensitivity test	t011	Heikinheimo et al.
7936_16_20	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
4507_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
4507_6_10	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
1333_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
1333_6_10	Nasal swab	Sensitivity test	-	Heikinheimo et al.
1333_11_15	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
6277_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
7594_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
7594_6_10	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
7594_11_15	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
7594_16_20	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
1057_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
1057_6_10	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
1057_11_15	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
1057_16_20	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
7502_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
7502_6_10	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
7502_11_15	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
7502_1_5P	Carcass swab	Sensitivity test	t091	Heikinheimo et al.
6161_1_5	Nasal swab	Sensitivity test	t034	Heikinheimo et al.
6161_6_10	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
6161_11_15	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
6161_16_20	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
6161_6_10P	Carcass swab	Sensitivity test	t091	Heikinheimo et al.
3582_6_10	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
3582_11_15	Nasal swab	Sensitivity test	t034	Heikinheimo et al.
0812_1_5	Nasal swab	Sensitivity test	t108	Heikinheimo et al.
0812_6_10	Nasal swab	Sensitivity test	t108	Heikinheimo et al.
0812_11_15	Nasal swab	Sensitivity test	t108	Heikinheimo et al.
0812_16_20	Nasal swab	Sensitivity test	t108	Heikinheimo et al.
0250_1_5	Nasal swab	Sensitivity test	t1381	Heikinheimo et al.

0250_6_10	Nasal swab	Sensitivity test	t127	Heikinheimo et al.
0250_11_15	Nasal swab	Sensitivity test	t1381	Heikinheimo et al.
0250_16_20	Nasal swab	Sensitivity test	t1381	Heikinheimo et al.
5105_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
5105_6_10	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
5105_11_15	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
5105_16_20	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
0186_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
0186_6_10	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
0186_11_15	Nasal swab	Sensitivity test	t034	Heikinheimo et al.
6672_1_5	Nasal swab	Sensitivity test	t034	Heikinheimo et al.
6672_6_10	Nasal swab	Sensitivity test	t034	Heikinheimo et al.
6672_11_15	Nasal swab	Sensitivity test	t034	Heikinheimo et al.
1724_1_5	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
1724_6_10	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.
1724_11_15	Nasal swab	Sensitivity test	t2741	Heikinheimo et al.

*Human derived sample provided by Turku University hospital. **provided by HUSLAB.

Appendix 4: *Pseudomonas aeruginosa* strains used in the experiments.

Strain identifier(s)	Origin	Description	Reference
PA1, 61841	Hospital sample*	Host strain for PA1P1	Mattila et al.
PA5, 11AN03663	Medix**	Host strain for PA5P1	Mattila et al.
PA8, 62263	Hospital sample*	Host strain for PA8P1	Mattila et al.
PA10, 62206	Hospital sample*	Host strain for PA10P2	Mattila et al.
PA11, 62180	Hospital sample*	Host strain for PA11P1	Mattila et al.
PA12, 62181	Hospital sample*	Host strain for PA12P1	Mattila et al.
PA14, 62109	Hospital sample*	Host strain for PA14P1	Mattila et al.
5513, 123768	Hospital sample***	-	HUSLAB
5514, 123754	Hospital sample***	-	HUSLAB
5525, 123702	Hospital sample***	Host strain for fHoPae01	HUSLAB
5537, 123650	Hospital sample***	-	HUSLAB
5538, 123612	Hospital sample***	MDR	HUSLAB
5539, 123610	Hospital sample***	MDR	HUSLAB
5548, 123448	Hospital sample***	-	HUSLAB
5550, 123411	Hospital sample***	-	HUSLAB
5551, 123379	Hospital sample***	-	HUSLAB
5553, 123358	Hospital sample***	-	HUSLAB
5826, 14TS0007	Hospital sample***	-	HUSLAB
5847, 14KK1140	Hospital sample***	-	HUSLAB
5827, 14KK0007	Hospital sample***	MDR	HUSLAB
5828, 13ET07332	Hospital sample***	-	HUSLAB
5668, KP11051	Hospital sample***	-	HUSLAB
5829, 13KP11089	Hospital sample***	-	HUSLAB
5831, 13TS07995	Hospital sample***	-	HUSLAB
5669, 13KP11107	Hospital sample***	-	HUSLAB
5832, 13KP11109	Hospital sample***	-	HUSLAB
5833, 13KP11076	Hospital sample***	MDR	HUSLAB
5834, 14UB0073	Hospital sample***	MDR	HUSLAB
5670, 14US152	Hospital sample***	-	HUSLAB
5835, 14KP00198	Hospital sample***	-	HUSLAB
5671, 14TS0093	Hospital sample***	-	HUSLAB
5672, 14TS0253	Hospital sample***	-	HUSLAB
5740, 14UB00438	Hospital sample***	-	HUSLAB
5836, 14TS00365	Hospital sample***	-	HUSLAB
5837, TS644	Hospital sample***	-	HUSLAB
5838, TS630	Hospital sample***	-	HUSLAB
5741, ME 654.2	Hospital sample***	-	HUSLAB
5742, KK 784	Hospital sample***	-	HUSLAB
5839, TS649_1	Hospital sample***	-	HUSLAB
5848, 14ME966_2	Hospital sample***	-	HUSLAB
5840, TS562	Hospital sample***	-	HUSLAB
5743, ME 604	Hospital sample***	-	HUSLAB

5841, ME618	Hospital sample***	-	HUSLAB
5842, TK659	Hospital sample***	MDR	HUSLAB
5844, HA760	Hospital sample***	MDR	HUSLAB
5845, ME854_1	Hospital sample***	-	HUSLAB
5745, KK 831	Hospital sample***	-	HUSLAB
5746, KK 858	Hospital sample***	-	HUSLAB
5747, TE 132	Hospital sample***	-	HUSLAB
5846, ME733_2	Hospital sample***	-	HUSLAB

*Provided by Turku University Hospital. **Purchased from Medix Laboratories. ***Provided by HUSLAB

Appendix 5: Phage DNA isolation protocol

1.3 µl of DNaseI (Promega 1 U/µl, RNase free) and 4 µl of RNseA is added to 400 µl of raw phage lysate. Incubate 30 min at 37 °C to break down the bacterial DNA and RNA.

EDTA is added to a final concentration of 20 mM with 50 µg/ml protease K and 0.5 % SDS concentration. Incubate at 56 °C for 1 h.

Cool to RT and extract once with 1 volume of phenol 1:1, once in 1 volume of phenol-chloroform-isoamylalcohol (25:24:1). Repeat until no white sedimentation is visible in the intermediate phase. Finally extract with 1 volume of chloroform (24:1).

Extract the DNA by adding final concentration of 0.3 M of NaOAc, pH 7.0. Stir and add 2 volume Abs EtOH and stir. Let the DNA strand extracts. Transfer the DNA strand to new Eppendorf containing 70 % EtOH. Centrifuge with 12 000 g for 2 min at 4 °C. Then remove EtOH and dry the DNA. Finally dissolve DNA to 100 µl of TE-buffer and store at 4 °C.

Appendix 6*: Pseudomonas aeruginosa* phage host specificity test results.

Strain ID	***Pseudomonas aeruginosa* phages**							
	PA1P1	PA5P1	PA8P1	PA10P2	PA11P1	PA12P1	PA14P1	fHoPae01
5513	+++	---	+++++	+++	+++	+++++	+++	-+
5514	-+	-+	-+	-+	-+	-+	-+	-+
5525	++++	---	++++	++++	++++	++++	++++	++
5537	+++	-+	+++++	+++++	-+	+++++	--	---
5538	---	---	---	---	---	++	---	---
5539	+++	--	+++	+++	+++	+++	+++	-+
5548	--	--	--	--	--	--	--	--
5550	-+	---	++	+++++	++++	+++++	+++++	++++
5551	---	-+	-+	-+	-+	---	---	---
5553	+++	++	+++	+++	++++	++	++	++
5826	++++	---	+++	++++	-+	++	+++	++
5847	++	---	++++	-+	-+	++	++	-+
5827	+++	---	++++	+++	+++	++++	+++	++
5828	+++++	-+	+++++	+++++	+++	+++++	+++	+++
5668	+++	--	++	+++	-+	-+	---	-+
5829	-+	---	-+	-+	-+	--	--	--
5831	+++	---	-+	++	---	---	++	--
5669	+++	++	+++++	+++++	++++	+++	++++	+++
5832	++	-+	+++	+++	-+	-+	++++	-+
5833	+++	---	+++	++	++	++	++	++
5834	+++	++	++++	+++++	+	+	++++	+
5670	+	---	+++	+++++	+++	+++	+++	++
5835	+++	-+	++	+++	++	+++	+++	++++
5671	++++	---	++	++++	+++	++	++	-+
5672	++	---	+++	-+	-+	++++	+++	++
5740	-+	---	---	---	--	---	---	---
5836	+++	--	+	++++	+++	++++	---	++
5837	+++	++	++	++	++	++	++	-+
5838	+++	++	+++++	+++	+++	++++	--	--
5741	+++	--	+++	+++	+++	--	+++	+++
5742	+++	--	+++++	+++	+++	--	--	+++
5839	+++	---	+++	+++	+++	++++	--	++
5848	+++	---	+++	+++	--	--	+++++	--
5840	+++	---	++++	+++	++	---	++	--
5743	+++++	---	+++++	+++++	+++	++	+++++	++
5841	---	-+	--	---	---	--	--	++
5842	-+	-+	++	++	+++	++	-+	++
5844	++	++	++	+++	+++	++	---	+++
5845	--	++	---	-+	++	--	--	--
5745	++	++	++	+++	+++	+++	++++	++++
5746	-+	--	+++++	+++	+++	+++	+++	-+
5747	-+	--	+++	-+	+++	+++	+++	+++
5846	++++	+++	++++	+++	+++	+++	+++	+++

+++++ = complete lysis, +++ = lysis, ++ = partial lysis, + = poor lysis -+ = result not clear, -- = some reaction, --- = no reaction

Appendix 7: Hospital waste water samples

Raw sewage samples were prepared by centrifuging at 7500 rpm for 15 min at 4 °C (RC-513, refrigerated Superspeed centrifuge, Sorvall) using GSA-rotor. The supernatants were filtered through Stericup and seritop Vacuum driven filtration system with Millipore Express™ PLUS 0.22 µm bottle top filter. The samples were stored at 4 °C for 133 days before isolation attempt. Samples were prepared by Henni Tuomala.

Table 4. **Hospital sewage samples used in *Pseudomonas aeruginosa* phage extraction**.

Sample	Location in Finland	Type	Collector	Date
1	Clinic of Cancerous diseases	Sewage	Ismo Pukkinen	15.4.2016
2	Peijas (OS)	Sewage	Heikki Hakomäki	14.4.2016
3	Lohja Hospital (OS)	Sewage	Pekka Eloranta	13.4.2016
4	Porvoo Hospital (PS)	Sewage	Niklas Andersson	13.4.2016

OS=outside sewer, PS = pump sewer