Characterization of ETEC-specific Jumbo Phage fPf-Eco01

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Tiivistelmä — Referat — Abstract

Enterotoxigenic *Escherichia coli* (ETEC) infection is the predominant type of colibacillosis in young animals and a significant cause of *E. coli*-related diarrhea worldwide. Urgent solutions are required to address the crisis, and phage therapy stands out as a potential option for treating ETEC-related infections. Jumbo phages possess considerable potential for therapeutic use due to their large genome, although they are still relatively poorly known.

The objective of this thesis was to characterize recently isolated *Escherichia* phage fPf-Eco01, focusing on essential properties for phage therapy, and to determine if the phage is suitable for phage therapy. The phage possesses a genome size of 379 kb classifying it a jumbo phage. Based on its genome sequence, fPf-Eco01 phage is a possible member of the *Asteriusvirus* genus of the *Caudoviricetes* class. The genome analysis did not recognize any harmful genes that encode antibiotic resistance or bacterial toxins. There were also no indications for phage lysogenic lifecycle which would prevent the use of phage in phage therapy. Transmission electron microscopy also revealed that the fPf-Eco01 virion was the size of a jumbo phage, and it was noted to have a contractile tail with visible baseplate and tail fibers. Virion's mean length was 227 nm. Phage had a wide host range by infecting 38 % of the tested clinical *E. coli* strains some of which were Extended-Spectrum Beta-Lactamase (ESBL) strains isolated from Finnish hospital patients. The phage did not infect other ETEC strains than the original host. The phage tolerated low pH conditions and remained infectious in several storage solutions that could be used in phage therapy. In addition, this indicates that the phage may retain its infectious properties when administered orally or possibly as part of an intravenous solution.

As a conclusion, fPf-Eco01 phage is a good candidate for phage therapy use. Due to phage broad-host-spectra, the phage can potentially be used to treat other human *E. coli* infections. Further investigation is required to explore the potential use of the phage against ETEC infections.

Avainsanat — Nyckelord — Keywords

Escherichia coli, phage, bacteriophage, jumbo phage, phage therapy, Enterotoxigenic *Escherichia coli* (ETEC), Extended-Spectrum Beta-Lactamase (ESBL), *Asteriusvirus*, diarrhoea

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Tiivistelmä — Referat — Abstract

Enterotoksigeeninen *Escherichia coli* (ETEC) -infektio on yksi merkittävimmistä kolibasilloosin aiheuttajista nuorilla eläimillä ja on siksi merkittävä syy *E. colin* aiheuttamaan ripuliin maailmanlaajuisesti. Välittömiä ratkaisuja tarvittaisiin tämän kriisin ratkaisemiseksi ja erityisesti faagiterapia olisi yksi mahdollinen vaihtoehto ETEC-infektioiden hoitoon. Jumbofageilla on hyvät ominaisuudet mahdolliseen terapiakäyttöön niiden laajan genomin ansiosta, mutta ne ovat vielä varsin huonosti tunnettuja.
Tämän tutkielman tavoitteena oli karakterisoida äskettäin eristetty *Escherichia coli* faagi fPf-Eco01 keskittyen erityisesti faagiterapian kannalta keskeisiin ominaisuuksiin. Tavoitteena oli myös selvittää, soveltuuko faagi terapiakäyttöön. Faagin genomin koko oli 379 kb, mikä luokittelee sen jumbofageihin. fPf-Eco01 faagi on mahdollisesti *Asteriusvirus*-suvun edustaja kuuluen samalla *Caudoviricetes*-luokkaan. Genomisekvenssin analysoinnissa ei ilmennyt haitallisia geenejä, jotka voisivat koodata antibioottiresistenssiä tai toksiineja. Sekvenssin perusteella faagin elinkierto ei olisi lysogeeninen, mikä voisi estää faagin käytön faagiterapiassa. Läpäisyelektroni-mikroskopia paljasti, että faagi fPf-Eco01 on kooltaan jumbofagin kokoinen ja sillä on supistuva häntä sekä selkeä häntälevy ja häntäsäikeet. Faagi-partikkelin keskipituus on 227 nm. Faagilla on laaja isäntäkirjo, sillä se infektoi jopa 38 % testatuista kliinisistä *E. coli* -kannoista. Osa faagin isäntäkantana toimivista kannoista oli laajakirjoisia beetalaktamaasi (ESBL) kantoja, jotka olivat eristetty suomalaisilta sairaalapotilailta. Faagi ei kuitenkaan infektoinut muita ETEC-kantoja, kuin vain sen alkuperäisen eristysisännän. Faagi sieti matalia pH-olosuhteita ja selvisi infektiokykyisenä useissa säilytysliuoksissa, joita voidaan käyttää faagiterapiassa. Näin ollen faagi pysyisi mahdollisesti infektiokykyisenä, mikäli faagihoitoa annettaisiin suun kautta tai faageja annosteltaisiin suonensisäisen nesteen mukana.
Faagi fPf-Eco01 vaikuttaa olevan hyvä kandidaatti faagiterapiakäyttöön. Faagin laajan isäntäkirjon vuoksi sitä voitaisiin mahdollisesti käyttää ihmisten *E. coli* -infektioiden hoidossa. Lisätutkimuksia tarvitaan, jotta voidaan selvittää faagin mahdollista käyttöä ETEC-infektioita vastaan.

Avainsanat — Nyckelord — Keywords

Escherichia coli, faagi, bakteriofagi, jumbofagi, faagiterapia, enterotoksigeeninen *Escherichia coli* (eng. ETEC), laajakirjoinen beetalaktamaasi (eng. ESBL), Asteriusvirus, ripuli

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INTRODUCTION

The emergence of multidrug resistance in *Escherichia coli* is a cause for concern in both human and veterinary medicine worldwide. While *E. coli* is naturally susceptible to most clinically significant antimicrobial agents, this bacterial species possesses the ability to accumulate resistance genes, primarily through horizontal gene transfer (Poirel et al., 2018). The World Health Organization released a global priority list of multidrug-resistant bacteria (MDR) in 2017. Carbapenem-resistant and extended-spectrum β-lactamases (ESBL) -producing Enterobacteriaceae raking at the top of the first priority group, indicating a critical situation (World Health Organization, 2017). The most challenging mechanisms in *E. coli* are associated with the acquisition of genes that code for, ESBL, resistance to carbapenems, 16S-rRNA methylases, plasmid-mediated quinolone resistance, and polymyxin resistance (Poirel et al., 2018). Extended-Spectrum Beta-Lactamase refers to enzymes that are produced by bacteria that degrade specific antibiotics, such as penicillins and cephalosporins. This feature is acquired by the bacteria and ESBLs are frequently plasmid encoded. ESBL enzymes are produced especially by enterobacterial species *E. coli and Klebsiella* (Paterson & Bonomo, 2005).

E. coli is a gram-negative and facultative anaerobic bacterium, that typically colonizes itself in the gastrointestinal tract of human infants shortly after birth (Tenaillon et al., 2010; Kaper et al., 2004). In most cases, *E. coli* and its human host maintain a symbiotic and mutually beneficial relationship over extended periods and these commensal *E. coli* strains rarely lead to any diseases. While *E. coli* lives in a symbiotic relationship between humans and animals, it has the potential to lead to severe infections when it bypasses the body's immune system (Poirel et al., 2018). One of the concerns lies in the potential transmission of virulent or drug-resistant *E. coli* between animals and humans through various routes, direct contact, exposure to animal excreta, or transmission through the food chain. Pathogenic *E. coli* often carry virulence factors encoded by genetic elements like plasmids, bacteriophages, transposons, and pathogenicity islands, which can be transferred among various bacterial strains, leading to the formation of new combinations of virulence factors (Kaper et al., 2004).

There are seven categories of *E. coli* intestinal pathogens: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DEAC) (Kaper et al., 2004).
Infection with Enterotoxigenic *Escherichia coli* (ETEC) is the prevailing form of colibacillosis found in young animals and a major contributor to *E. coli*-related diarrhea in humans worldwide and it is

often transmitted through contaminated food and water sources (Duan et al., 2012). The prevalence of ETEC infections is high, especially in developing countries, where there is a lack of infrastructure to supply clean drinking water and disposal of excrement (Zhang et al., 2022). Cases caused by ETEC are particularly common among travelers who have been potentially infected by contaminated food or drink (Centers for Disease Control and Prevention, 2014). Prior research has demonstrated that ETEC can endure in fecal matter for over six months and typically exists in water as biofilms, which significantly enhances its survival potential (Ahmed et al., 2013). From a perspective focused on zoonotic factors, EHEC stands out as the primary *E. coli* pathogenicity group of significant concern (Wasteson et al., 2002). The strains that produce Shiga toxin possess the capability to induce severe disease in humans through transmission along the food chain originating from their animal reservoirs. ETEC bacteria do not pose a zoonotic risk, as they are quite species-specific in terms of their binding.

The progress in creating new classes of antibiotics has experienced a slowdown in recent years, mainly due to economic factors and market considerations, underscoring the urgent need for new antimicrobial treatments. The utilization of bacteriophages for therapeutic use has been contemplated for an extended period, accompanied by multiple successful research (Hatful et al., 2022). Novel approaches for treating infections triggered by antibiotic-resistant bacteria are under development, aiming to supplement or replace antibiotics. Phage therapy, a technique that harnesses bacteriophages (bacterial viruses) to target and eliminate specific pathogenic bacteria, offers a promising avenue for addressing the global challenge posed by the proliferation of antimicrobial resistance. (Cisek et al., 2017; Saha et al., 2019)

Antibiotic overuse has become a problem in the animal feed industry, contributing to the emergence of pathogenic bacteria that present both economic and health challenges (Yue et al., 2020). Antibiotic resistance has increased at a tremendous rate in poultry farms (Loponte et al., 2021; Ferriol-González et al., 2021). Phages have emerged as a potential alternative for antibiotics for the treatment of livestock infections due to their numerous advantages as adaptable therapeutic agents, including their capacity to evolve, multiply right at the site of infections, and high specificity to certain bacteria. Gastrointestinal infections in pigs, such as diarrhea, were traditionally treated with antibiotics, but this approach has shown notable repercussions on their gut microbiota, which has contributed significantly to the study of new treatment approaches (Yue et al., 2020). Studies have explored phage therapy in weaning pigs as a promising alternative to other treatment forms, with a notable impact on reducing the number of pathogenic bacteria in the pig’s gut (Zeng et al., 2020; Mao et al., 2023).

The secure and regulated use of phage therapy will require comprehensive knowledge of the characteristics and behavior of the specific phage-bacterium combination, both in controlled laboratory circumstances and more significantly within living organisms (Skurnik & Strauch, 2006) Belgium has taken a practical approach to promote phage therapy by permitting phage products to be prepared in hospital pharmacies (Pirnay et al., 2018) In the rest of the EU there is this a lot of regulations regarding the use of phage therapy. The regulatory authorities are permitting phage therapy exclusively for compassionate use in individual patients who have no other available treatment options (McCallin et al., 2019). In early 2023, the Bacteriophages Working Party as a part of the European Pharmacopeia Commission published the general chapter: “Phage therapy active substances and medicinal products for human and veterinary use”, to establish standardized tools for the research and development of phage therapy (European Pharmacopoeia, 2023).

To be used in phage therapy, bacteriophages must have certain characteristics (Abedon et al., 2011; Hatful et al., 2022; Hyman, 2019) The phage must be virulent i.e., the phage lifecycle must be lytic. In the lytic life cycle, the bacteriophage infects a bacterial cell, after which new phage particles are formed and the bacterial cell dies in cell lysis. The phage typically manages to kill the host cell effectively. Conversely, the lysogenic life cycle allows phages to integrate into the host's genome without causing immediate host cell destruction, which is why the phage may successfully transfer harmful properties to the bacterial cell, which is why these phages are not used in phage therapy. This lysogenic life cycle is found only in temperate phages. Before bacteriophages can be used in phage therapy, the phage genome must be well examined, the phage genome must not contain any antibiotic-resistance genes or bacterial toxin genes that can bring new properties to the bacterial host (Weber-Dąbrowska et al., 2016). The phage genome is also to be examined for properties suggestive of the lysogenic lifecycle, and phages are supposed to have quite low transducing potential. After identifying the characteristics of the phage, the subsequent stage involves evaluating the potential administration of the phage to patients requiring therapy. Phages are commonly applied in the form of phage cocktails, combining multiple phages that target the same bacterial species (Chan et al., 2013). This is attributed to the highly specific nature of the phages, as they selectively act on only one or a few bacterial strains within a species. Phage cocktails are usually administered to the pigs by oral administration in drinking water or food (Zeng et al., 2020; Mao et al., 2023).

Bacteriophages characterized by genomes exceeding 200 kb are categorized as jumbo phages (Yuan & Gao, 2017). These phages earn the label “jumbo” due to their prominent characteristics of having large phage virions and an extensive genome size. Jumbo phages differ from other bacteriophages

not just in size but also in terms of their evolutionary path, orientation of the genetics, virion structure, and the transmission of progeny. The genomes of jumbo phages encompass a multitude of genes related to genome replication, modification, and nucleotide metabolism, facilitating autonomous replication independent of the host (Jo et al., 2023). A groundbreaking moment occurred with the isolation of the first jumbo phage, phage G from *Bacillus megaterium*, boasting a genome length of 497 kb and particle size of 600 nm (capsid size 160 nm) (Nazir et al., 2021). Numerous new jumbo phages have been characterized in recent years, constantly increasing knowledge about their various distinctive capabilities, host ranges, and infection cycles (Hu et al., 2023; Laughlin et al., 2022).

Since the advent of high-throughput sequencing technology, bacteriophage research has been progressing quickly and new bacteriophages can be found more easily (Nazir et al., 2021). The majority of viruses possessing large genomes and isolation from gram-negative microorganisms are affiliated with the following genera: *Aeromonas, Caulobacter, Escherichia, Erwinia, Pseudomonas, Synechococcus* and *Vibrio* (Yuan & Gao, 2017, Wagemans et al., 2020). *Escherichia coli* phages have been isolated from a variety of sources: human and animal fecal, environmental water sources, and sewage systems, from which hospital wastewater is often utilized (Song et al., 2007; Dalmasso et al., 2016; Snyder et al., 2016; Smith et al., 2023). In this thesis, the fPf-Eco01 phage was isolated from pigs' fecal matter earlier in the Skurnik group (Salminen, 2022).

This study aimed to characterize recently isolated and sequenced *E. coli* phage fPf-Eco01. The phage was isolated against the ETEC bacteria that cause diarrhea in weanling pigs. We wanted to ascertain if the phage is safe for treatment use according to the requirements set by European Pharmacopeia. The phage genome was characterized in detail to estimate their suitability for phage therapy purposes. Our research also delved into the phage infection capabilities across various *E. coli* strains and determined the extent host range. Additionally, we examined potential interactions between the phage and porcine serum as well as its possible combined effect with antibiotics. We also investigated the phage resilience under varying pH conditions. The information enabled us to determine if the phage can be used in oral phage therapy, and if it can survive in the gastrointestinal tract with infectious capacity.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The ETEC strains used in this research project were obtained from the Finnish Food Authority and the clinical *E. coli* isolates were obtained from Helsinki and Uusimaa hospital district (HUS) (Supplementary Table 1). All bacterial strains, strain numbers, and isolation origins are listed in Supplementary Table 1. The strain #7163, which was the original isolation host of fPf-Eco01, was used as the standard host for fPf-Eco01. Lysogeny Broth, (LB media) was used as a culture medium (Sambrook & Russell, 2001). LB media supplemented with 1.5% (w/v) agarose was used in the agar plates. In the titrations, LB media was supplemented with 0.3% (w/v) agarose. All bacterial cultures in agar plates were incubated at +37 °C overnight. All bacterial strains in LB liquid media were cultured at +37 °C with shaking. For overnight culture, one colony was picked from an LB agar plate into 5 ml of LB liquid medium and incubated for approximately 22 hours at +37 °C with shaking.

Phage production and plaque assay

Phage was produced by using the liquid growth method, where 400 µl of the host strain in LB liquid media and 40 µl of phage from a deep freezer stock were added to 9.6 ml of the culture medium. The bacteria and phage mixture were cultured at +37 °C with shaking until 5 hours. Phage preparation was then centrifuged for 10 min, 5000 rmp at +4 °C (Thermo Scientific SL 16R TX-400, USA), and the supernatant was sterile filtered with Minisart® 0.22 µm PES filter (Sartorius Stedim Biotech S.A., France). After filtration, 40 % of sucrose was added to a final concentration of 8 % for long-term storage at 4 °C.

The number of plaque forming units (PFU) was determined in all experiments by titration using the double layer method and phage host bacterium unless otherwise stated. The bacteriophage was titrated by using either a whole plate or drop test method (Sambrook & Russell, 2001). In the whole plate method, host strain #7136 was inoculated into 1.3 ml of LB liquid media, and the bacteria was grown until the exponential growth phase at A_{600} values between 0.25-1.00. Absorbance A_{600} values were measured with DSM Cell Density Meter. The volume of the bacterial suspension needed for each titration plate was calculated using the following equation:

$$22{,}5/A600 = X\mu l.$$

Then ten-fold dilution series of the bacteriophage was prepared in LB liquid media. Double-layer plates were prepared by adding bacterial suspension and 50 µl of the phage dilution into tubes, which had 3 ml soft-agar, and then tubes were vortexed briefly and poured on LB plates. LB media was

added in one plate instead of phages and used as a negative control. Soft agar was solidified for 1 hour at room temperature (RT) and then bacteria-phage combinations were incubated at 37 °C overnight. PFU/ml values were calculated using the following equation:

$$Number\ of\ plaques\ x\ Dilution\ factor\ x\ (1000/50)$$

The drop test method was performed like whole plate titrations with the exception that phage dilutions were not added to the soft agar. Instead, 10 µl of each phage suspension was pipetted on top of the solidified soft agar layer containing bacteria. A drop of LB media (10 µl) was used as a negative control. Plates were solidified for 1 hour and droplets were left to dry for 1 hour at RT and then plates were incubated at 37 °C overnight. The plaques were observed and roughly calculated the following day.

Phage genome analysis

The phage genome was sequenced previously with the Illumina platform at Novogene, UK (unpublished data). In this work the phage sequence raw reads were first subsetted to only 50,000 reads per forward and reverse reads files. The reads were then assembled with the A5 pipeline (Tritt et al., 2012), and the raw reads were mapped against the phage genome to confirm the assembly in Geneious Prime (version 2022.1.1) (Coil et al., 2015). The phage genome was aligned with the original reads to find possible mistakes made during the assembly. PhageTerm program (Garneau et al., 2017) was used to predict the phage packaging mechanism and possible terminal repeats. The phage genome was reordered according to the ends of the genome determined by PhageTerm. Phage genome preliminary annotations were performed automatically with RAST 2.0 (Rapid Annotation using Subsystem Technology) (Aziz et al., 2008). VirulenceFinder 2.0 was used to find possible bacterial toxins (Joensen et al., 2014). Antibiotic resistance genes were studied by CARD 3.0 (Alcock et al. 2020) and ResFinder 4.0 (Bortolaia et al., 2020). All bioinformatic tools were applied using standard parameters.

Phylogeny

The BLAST (Nucleotide Basic Local Alignment Search Tool) (Johnson et al., 2008) search was used to identify related phages. BLAST analysis was done in three parts, with the beginning, middle, and end of the fPf-Eco01 genome. Comparing all three BLAST analyses, 11 closest phages were selected for phylogenetic analysis. All 11 selected genomes had query coverage of 80 % and sequence identity of 80 %. VipTree (the Viral Proteomic Tree server) was used to assemble the distance heat map by using the selected phage genomes (Nishimura et al., 2017).

Sample preparation for Transmission electron microscopy

Phage lysate was ultrafiltered into sodium chloride-magnesium sulfate-gelatin buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01% (w/v) gelatin) (SMG buffer) (Sambrook et al., 2001) to remove access proteins and to concentrate the phage lysate. Phage lysate (10 ml) (1,35 x 10^{10} PFU/ml) was purified for transmission electron microscopy (TEM) imaging with 20 ml Vivaspin, (100 kDa) centrifugal PES membrane concentrator (Sartorius) by centrifuge at 3500 rpm, 15 min, +16 °C. First, the sample was concentrated to 1/10 of the initial volume, washed twice with 9 ml of SMG buffer, and adjusted to the final volume of 1 ml. PFU/ml values were determined after each washing step to detect if the phage had been washed through an ultrafiltration tube. The final PFU/ml value was determined for the ultrafiltered fPf-Eco01 sample. After filtration, 40 % of sucrose was added to a final concentration of 8 % for long-term storage at 4 °C. For TEM imaging the pre-purified phage lysate was changed to ammonium acetate buffer (0.1 M) with Vivaspin and ultrafiltration units, as above. The pre-purified sample was concentrated 1/10 of the initial volume and washed twice with 9 ml of ammonium acetate.

For TEM, 5 µl of fPf-Eco01 in ammonium acetate (0.1 M) was added on a copper grid and incubated for 60 sec and the excess liquid was removed with filter paper. Then 5 µl of uranyl acetate was pipetted onto the grid to incubate for 60 sec and then the excess liquid was removed. Transmission electron microscopy was performed using the JEOL JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) at the Electron Microscopy Unit (Institute of Biotechnology, University of Helsinki, Helsinki, Finland). Seven whole phage particle lengths, head lengths, head widths, and tail lengths were measured with Microsoft PowerPoint. The means and standard deviations of the lengths/widths of each particle component were calculated with Microsoft Excel.

Bioscreen condition measurements

Bioscreen FP-1100-C (Oy Growth Curves Ab Ltd) plate reader was used to perform two growth inhibitions tests: porcine serum effect, and antibiotic resistance for three antibiotics. Culture growing conditions were tested for the host *E. coli* strain #7136 and fPf-Eco01. Suitable phage concentration was determined to make it easier to view the results. Measurements were done in liquid culture using 100-well Honeycomb 2 microplates (Oy Growth Curves Ab Ltd). Tests were made by using two phage amounts: 10^9 PFU/ml phage lysate and 10^8 PFU/ml diluted phage. Overnight bacterial culture was diluted in 1:5000 into LB liquid media. Both phage amounts (10 µl each) were pipetted separately with 200 µl of bacterial dilution (Patpatia et al., 2022). All measurements were done in triplicate and

LB liquid medium was used as negative control instead of the phage. Experiments were done at 37 °C for 10 hours, measurements were taken in 30 min intervals and the bacterial turbidity was measured with a wavelength of 600 nm. The plate was shaken with continuous, high amplitude and "normal" speed. The means and standard deviations of each triplicate were calculated, and the growth curves were created with OriginPro.

Porcine serum test

The combined effect of porcine serum and fPf-Eco01 phage was analyzed with Bioscreen using two *E. coli* strains: #7136 phage host bacterium and #2689 DH10b strain as a positive control for porcine serum. Optimized phage and bacterial dilutions were used. Both strains #7136 and #2689 were diluted into 1:2500. Porcine serum was also used as an inactivated form by incubating it for an hour at 56 °C. Both serums were diluted with LB into 40 %, 20 %, and 2 % (v/v), to get a final concentration of 20 %, 10 %, and 1 % serum. Phage or LB liquid medium (10 µl) was added together with either 100 µl of serum and 100 µl of the bacterium or only 200 µl of the bacterium. All serum dilutions were tested with phage and both *E. coli* strains, and all measurement combinations were done in triplicate. All serum concentrations and strains were tested together with LB liquid medium to detect any possible contamination. The assay was performed otherwise like in "Bioscreen condition measurement", but the measurement time was 20 hours. Results were then calculated, and curves were drawn as described in Bioscreen condition measurement.

Antibiotic test

The effect of antibiotics on phage infectivity was tested by using three commonly used antibiotics that are used against *E. coli* infections. Ciprofloxacin was diluted into 160 µg/ml, Piperacillin into 1600 µg/ml, and Ampicillin into 100 µg/ml. All dilutions were made into LB media. Overnight bacterial culture was diluted into 1:2500 for the wells without antibiotics and into 1:5000 for the wells with antibiotics. In each well, 10 µl of phage lysate 10^9 PFU/ml or LB liquid medium, 100 µl of bacterial dilution, and 100 µl of antibiotic dilution were added. All combinations were done in triplicate and measurements were done similarly as described before in the porcine serum test. The means and standard deviations of each triplicate were calculated, and growth curves were then drawn.

Host range screening

The host range of the bacteriophage was determined with the drop test method. Phage lysate (10^9 PFU/ml) was diluted 10^{-7}, 10^{-5} and 10^{-3}. Screening was done with 51 *E. coli* strains (Table S1). fPf-

Eco01 phage infectivity on different strains was then analyzed the next day. A positive result was visible as plaques in 10^{-5} and 10^{-3} dilutions. The result was negative if plaques were not detected. Unclear results were confirmed with the whole plate method. The efficiency of plating was determined for each phage-sensitive strain by using the whole plate method. LB liquid media was used as a negative control. After the overnight incubation, the PFU/ml values for each infected strain were calculated. Titer on the original host was defined as 100 %.

Phage stability determination

Phage stability was tested at different pH values and in various phage storage solutions. Phage lysate was mixed to a final concentration of 10^7 PFU/ml in LB-media which were adjusted with HCl into five pH values of 3, 4, 5, 6, and 7. Phages were incubated for 2 and 24 hours at RT and after that dilution series were prepared in pH 7 LB liquid media. pH 7 LB liquid acted as a control. The pH stability of fPf-Eco01 was determined with the drop test method. The plaques were observed and calculated the following day and the rough PFU/ml values were determined from one drop test titration plate. The phage PFU/ml values of 2 h and 24 h plates were compared to each other.

The phage storage test was performed by using a whole plate plaque assay method. Dilution series were prepared in four different solutions: LB liquid media, PBS (137mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), SMG (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01% (w/v) gelatin), and 0.9% NaCl. LB liquid media was used as a positive control. Phage lysate was diluted 1:1000 into each solution and then the titer was determined for each solution. All phage solutions were stored at 4 °C for a week and then the titer for each solution was determined again.

RESULTS

Genome analysis and phylogeny

Phage double-stranded DNA genome length was 379,266 bp and the GC content of the genome was 35.7 %. PhageTerm was used to determine the physical ends of the pre-assembled genome and it was able to predict the genome packaging mechanism to be similar to T5-type phages. PhageTerm identified direct terminal repeat length to be 19.808 bp. The fPf-Eco01 phage genomic characteristics are listed in Table 1. RAST server detected 651 coding sequences, of which 620 were hypothetical proteins and 23 were non-hypothetical proteins whose functions were unknown. RAST server identified only 8 of the proteins as specific genes. These genes encoded, phage introns, phage DNA

synthesis, ribonuclease H, ribonucleotide reduction, tRNA aminoacylation Val, and ATP-dependent proteolysis. There were no lysogeny-associated genes or integrases identified which indicates the fPf-Eco01 phage to have lytic lifecycle. VirulenceFinder, CARD, and ResFinder did not find any known antibiotic-resistance, virulence-associated or toxic genes from the fPf-Eco01 genome.

Table 1. Statistic of fPf-Eco01 genome analyses

	fPf-Eco01
Genome size (bp)	379.266
Length of direct terminal repeats (bp)	19.808
GC content (%)	35.7
Number of coding sequences	651
Found coding genes	8
Hypothetical proteins (%)	98
Number of RNAs	5
Bacterial toxin genes	none
Antibiotic resistance genes	none
Genes indicating temperate lifecycle	none

BLAST online server was used to find the closest bacteriophage nucleotide sequences against fPf-Eco01. All 11 selected genomes were *Escherichia* phage genomes and had query coverage of 80 % and sequence identity of 80 %. ViPTree was used to assemble the distance heat map (Fig 1.) using those closest bacteriophage whole genome sequences. ViPTree analysis revealed that all selected *Escherichia* phages were 93-97 % similar to the fPf-Eco01 phage. *Escherichia* phage_vB_Ec_M_J was the most similar (97 % identity) to the fPf-Eco01 phage. Currently, only two of the viruses have been classified in the genus *Asteriusvirus*: *Asteriusvirus av121Q* and *Asteriusvirus PBECO4* (Kropinski & Adriaenssens, 2019). *Escherichia* phage_vB_Ec_M_J was the most similar (97 % identity) to fPf-Eco01. *Escherichia* phage_vB_Ec_M_J belongs to the class of *Caudoviricetes* and the possibly in the genus of *Asteriusvirus*. fPf-Eco01 therefore potentially belongs to the same class and genus. All closest relative genome sequence lengths (383.49-347.15 bp) correspond to the genome lengths of jumbo phages.

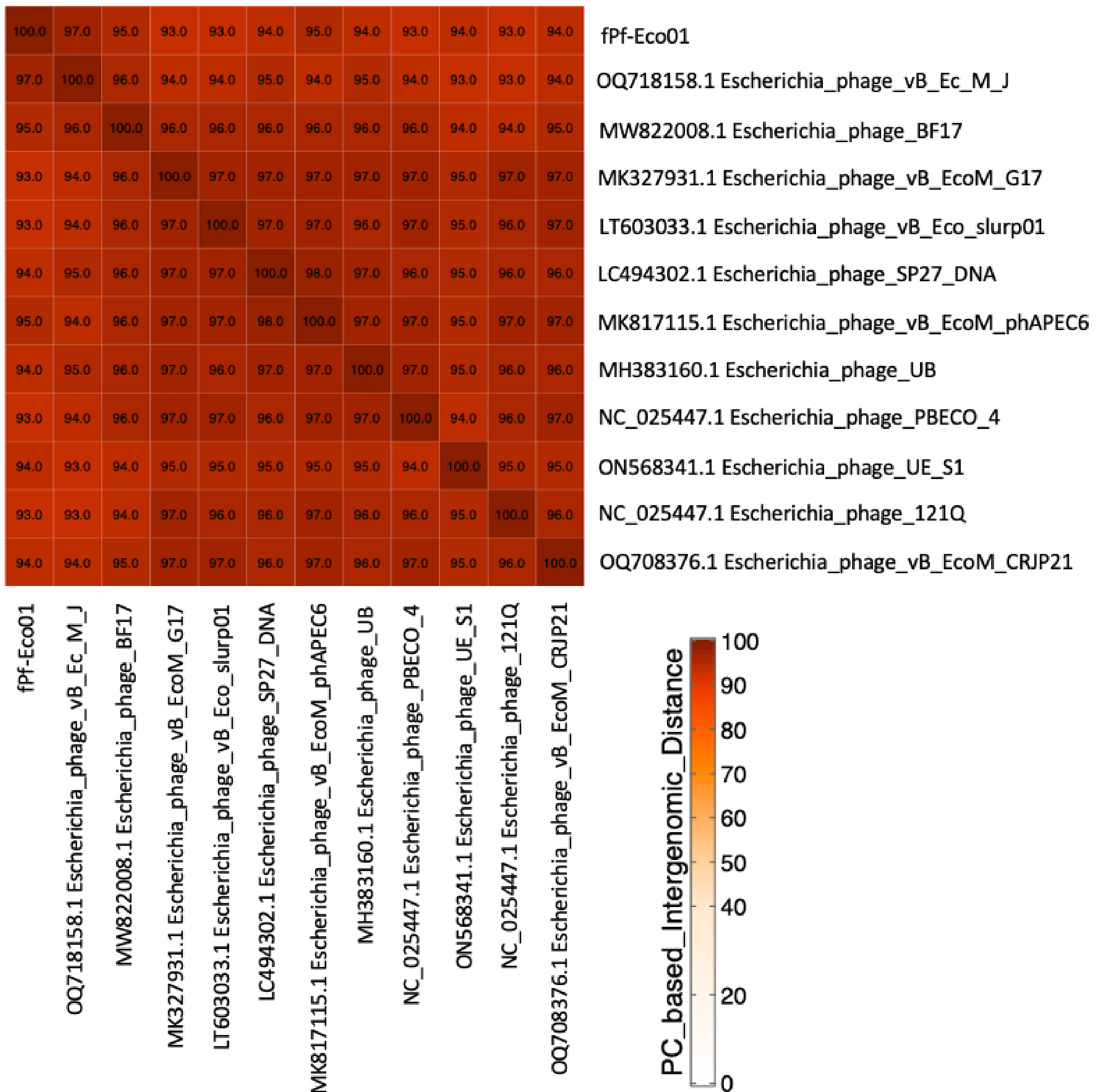

Fig 1. Whole-genome comparison heat map of fPf-Eco01 phage with its closest relatives carried out with VipTree (Viral Proteomic Tree server). In the bottom right side of the heat map, different shades of red indicate the intergenomic distance (%) between the genomes of each pair compared. The intergenomic distance is also shown with numerical values. The darker the red color, the more closely these phages are related.

Phage morphology

Transmission electron microscopy revealed that the negatively stained fPf-Eco01 phage has a big icosahedral head and contractile tail (Fig 2.). Some of the phage particles had also possible base plate and tail fibers attached to the tail. Seven viral particles were measured. The particle's average length was 227 nm, and the standard deviation was 26.21. The head average length was 118 nm, and the standard deviation was 15.94. The Head average width was 99 nm and the standard deviation was

8.60. The tail average length without base plate and tail fibers was 109 nm and the standard deviation was 11.08 (Fig 2a). One phage particle with a contracted tail was found and measured (Fig 2b). When comparing contractile tail length to no-contracted tails, there were no major differences in lengths. These particle sizes suggest that fPf-Eco01 has similar morphological features compared to large virions identified as jumbo phages (Jo et al., 2023).

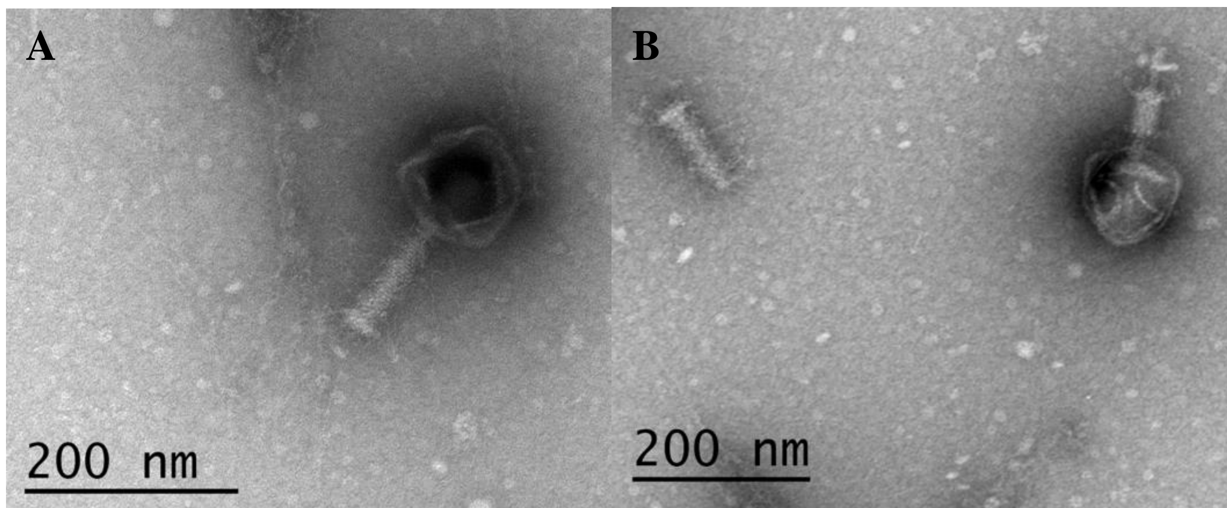

Fig 2. Electron microscopy pictures of negatively stained fPf-Eco01 phage particles. A: Phage with non-contracted tail, B: Phage with contracted tail and one separate tail.

Host range and efficiency of plating

The host range of fPf-Eco01 phage was assessed by using 51 *Escherichia coli* strains (Table S1). Eight of the tested strains were ETEC strains which were obtained from the Finnish Food Authority, and 44 of the strains were clinical strains from HUSLAB hospital patients. Several clinical strains have been identified to be ESBL strains (extended-spectrum beta-lactamases). fPf-Eco01 phage infected its original host and 20 of the clinical strains which represent 38 % of the tested strains. No other ETEC strains were infected. Sixteen of the infected clinical strains had similar or as high EOP as the original host ranging from 1.66-8.6 x 10^9 PFU/ml. Strain #6907 had EOP 1.8 x 10^3 PFU/ml so it could be said that it is sensitive to fPf-Eco01 phage, but the potency of the infection is weak.

The effect of porcine serum on fPf-Eco01 infection

Bacterial growth conditions were optimized in Bioscreen for the host *E. coli* strain #7136 and DH10b. First DH10b strain was used as positive control for porcine serum affection detection. The phage infectivity was not tested with DH10b. Active serum (20 %) inhibited bacterial growth completely (Fig 3a, black line). 10 % active serum (Fig 3a, orange line) inhibited the bacterial growth for 10

hours but then the bacteria was able to reach a density of 0.7 A_{600}. In the 1 % active porcine serum (Fig 3a, blue line), there is a minor difference while comparing the sample without serum (Fig 3a, green line). In the 1 % serum, bacteria were able to reach a density of 1.1 A_{600}, and in the sample without serum, the density was 1.2 A_{600}. By growing the bacteria with the heat-inactivated serum, it can be noticed that when the complement was inactivated the bacteria were able to grow normally (Fig 3b).

An active form of porcine serum (Fig 4) and heat-inactivated serum (Fig 5) were tested together with the fPf-Eco01 phage to determine possible synergist or antagonistic effects on bacterial growth. Active porcine serum (20 %) inhibited bacterial growth slightly, but phage inhibited the bacterial growth better without serum than with serum (Fig 4a). There was less inhibition in the 10 % porcine serum samples (Fig 4b) but still some inhibition when the phage is present. In the 1 % porcine serum samples (Fig 4c) there was no significant difference in growth inhibition. Comparing the active porcine serum results to the heat-inactivated serum (Fig 5), all results are completely similar. It is noticeable that the complement in the active serum does not affect the host bacterial growth. Other component or several components in the serum must have inhibited the bacterial growth since there were no differences in active and heat-inactivated serums. There was almost no phage-serum synergy. Serum, on the other hand, impairs phage's ability to infect but does not prevent it completely.

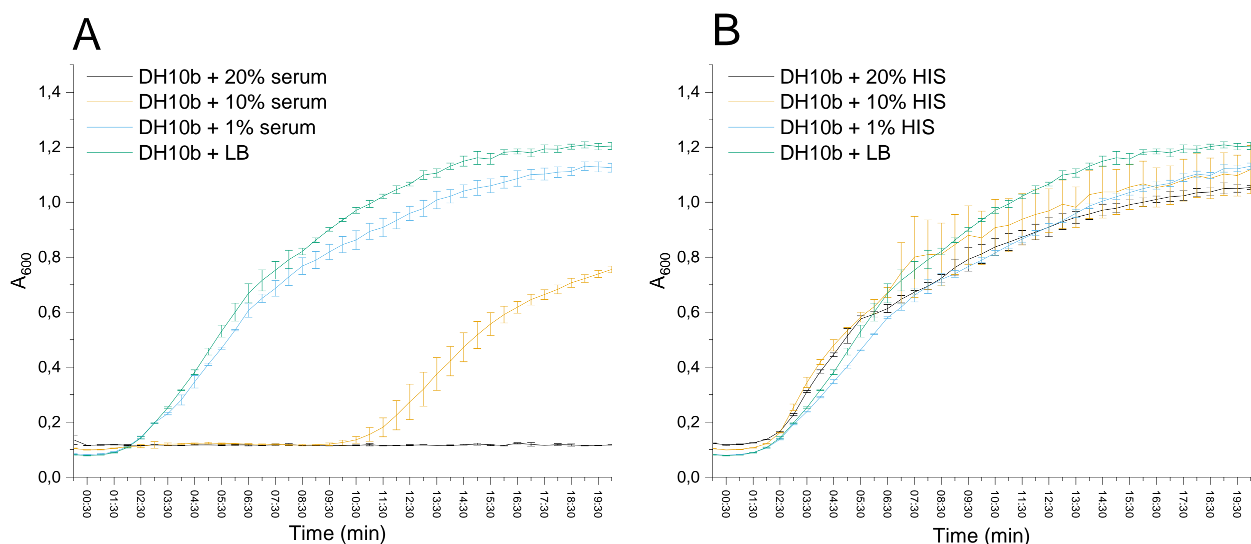

Fig 3. Growth curves of *E. coli* DH10b tested together with porcine serum and heat-inactivated porcine serum (HIS). A: Active porcine serums (20 %, 10 %, 1 %, and positive control), B: Heat-inactivated porcine serums (20 %, 10 %, 1 %, and positive control). LB media together with DH10b was used as positive control. The curves are drawn based on the Bioscreen results. The mean of three parallel wells was calculated. A_{600} stands for bacterial absorbance at 600 nm.

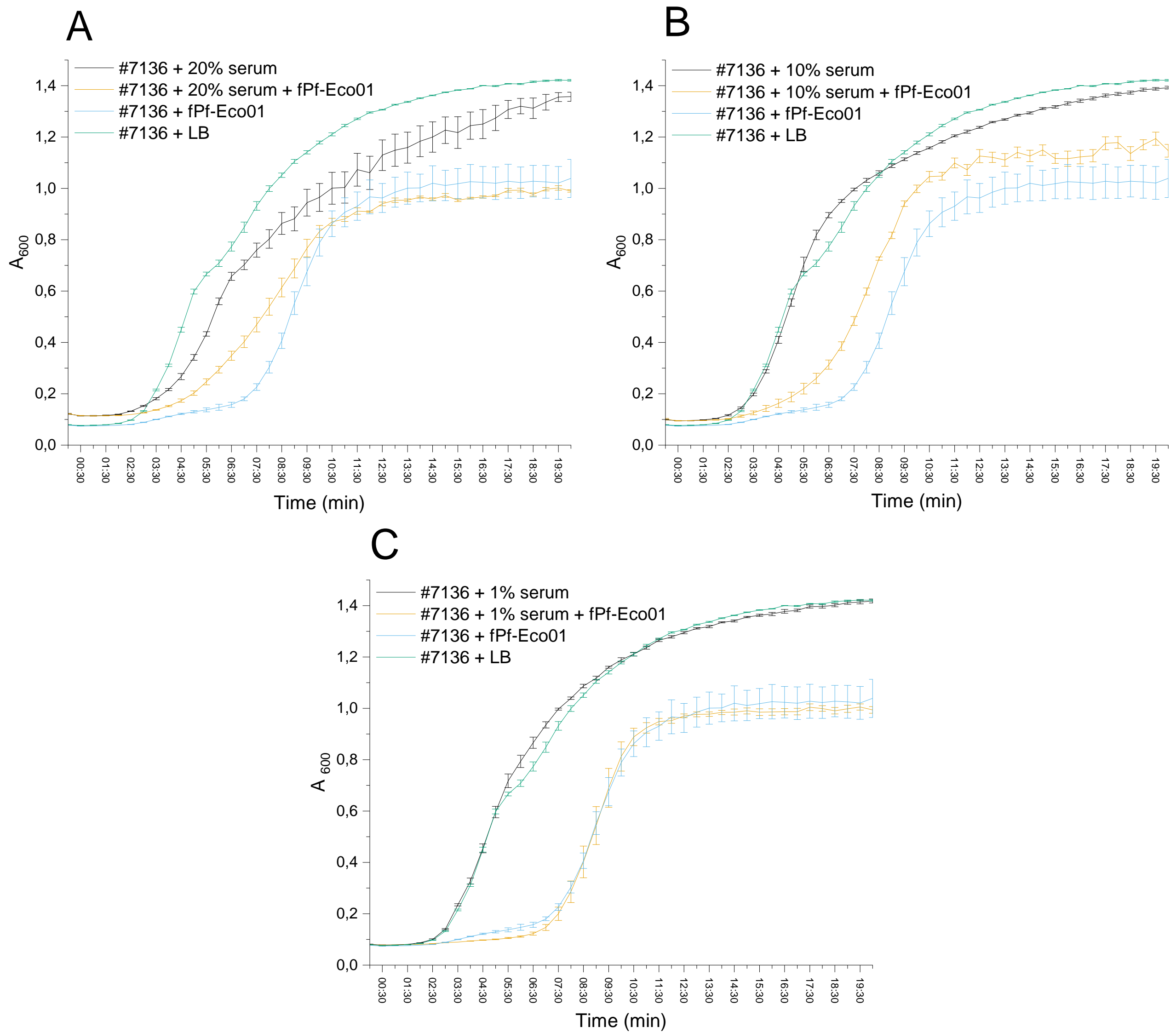

Fig 4. Growth curves of *E. coli* #7136 tested together with fPf-Eco01 phage and the porcine serum. A: Strain #7136 tested together with 20 % porcine serum, #7136 together with 20 % porcine serum and phage, #7136 with only phage, and #7136 with positive control. B: Strain #7136 tested together with 10 % porcine serum, #7136 together with 10 % porcine serum and phage, #7136 with only phage, and #7136 with positive control. C: Strain #7136 tested together with 1 % porcine serum, #7136 together with 1 % porcine serum and phage, #7136 with only phage, and #7136 with positive control. LB media together with strain #7136 was used as a positive control. The curves are drawn based on the Bioscreen results. The mean of three parallel wells was calculated. A_{600} stands for bacterial absorbance at 600 nm.

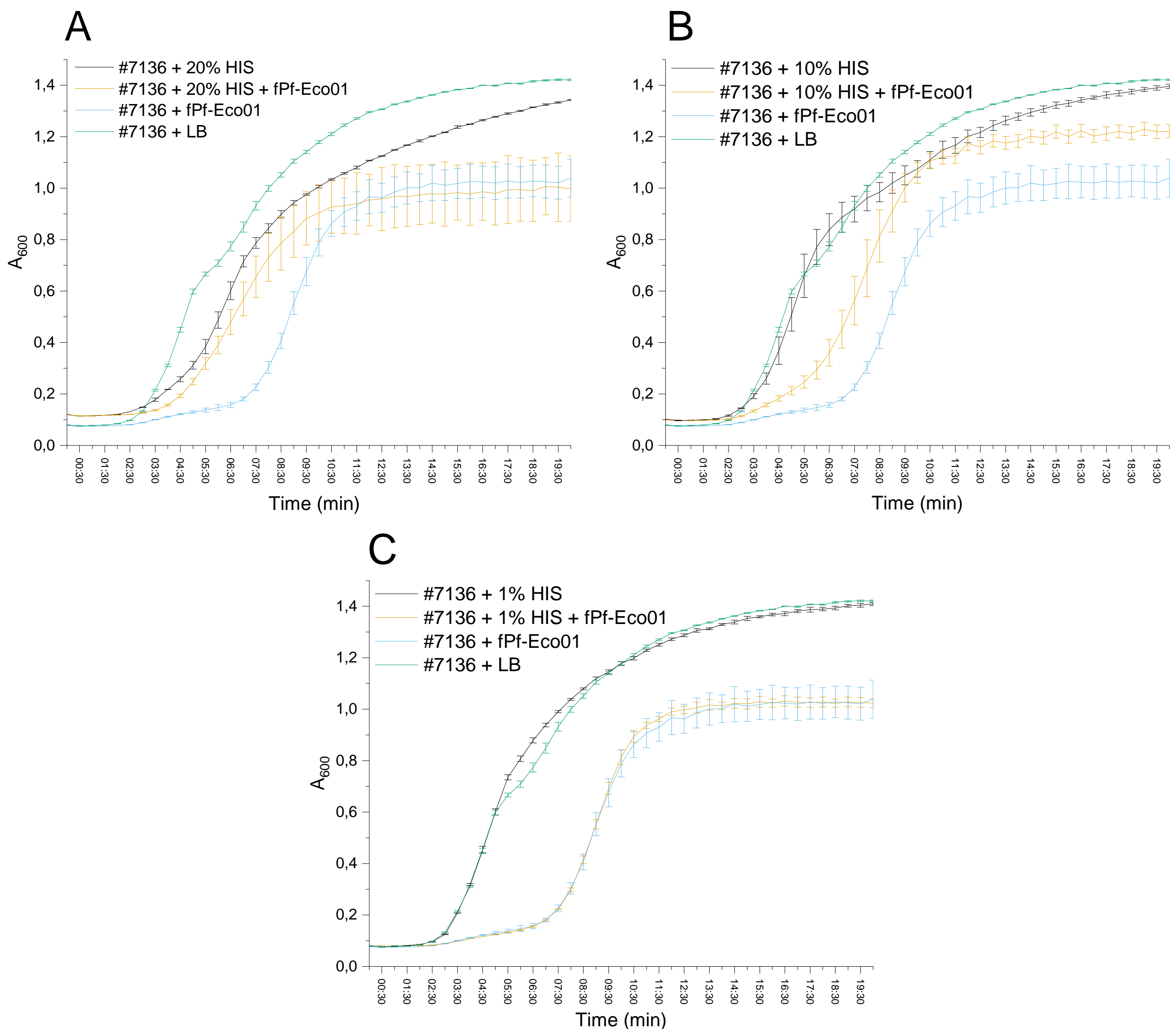

Fig 5. Growth curves of *E. coli* #7136 tested together with fPf-Eco01 phage and heat-inactivated porcine serum (HIS). A: Strain #7136 tested together with 20 % HIS, #7136 together with 20 % HIS and phage, #7136 with only phage, and #7136 with positive control. B: Strain #7136 tested together with 10 % HIS, #7136 together with 10 % HIS and phage, #7136 with only phage, and #7136 with positive control. C: Strain #7136 tested together with 1 % HIS, #7136 together with 1 % HIS and phage, #7136 with only phage, and #7136 with positive control. LB media together with strain #7136 was used as a positive control. The curves are drawn based on the Bioscreen results. The mean of three parallel wells was calculated. A_{600} stands for bacterial absorbance at 600 nm.

Antibiotic effect on the host strain and phage

Ciprofloxacin, piperacillin, and ampicillin were selected to be tested together with the fPf-Eco01 phage to see if there is any combined effect. All three antibiotics are normally used to treat *E. coli* infections in humans (HUS, 2023). All three antibiotics completely inhibited the growth of the host bacteria #7136 (Fig 6), so a possible antibiotic-phage synergy could not be observed. Antibiotic concentrations were possibly so high that the bacteria could not even begin to grow. The antibiotic test confirmed that host bacteria do not have antibiotic resistance genes against these three antibiotics.

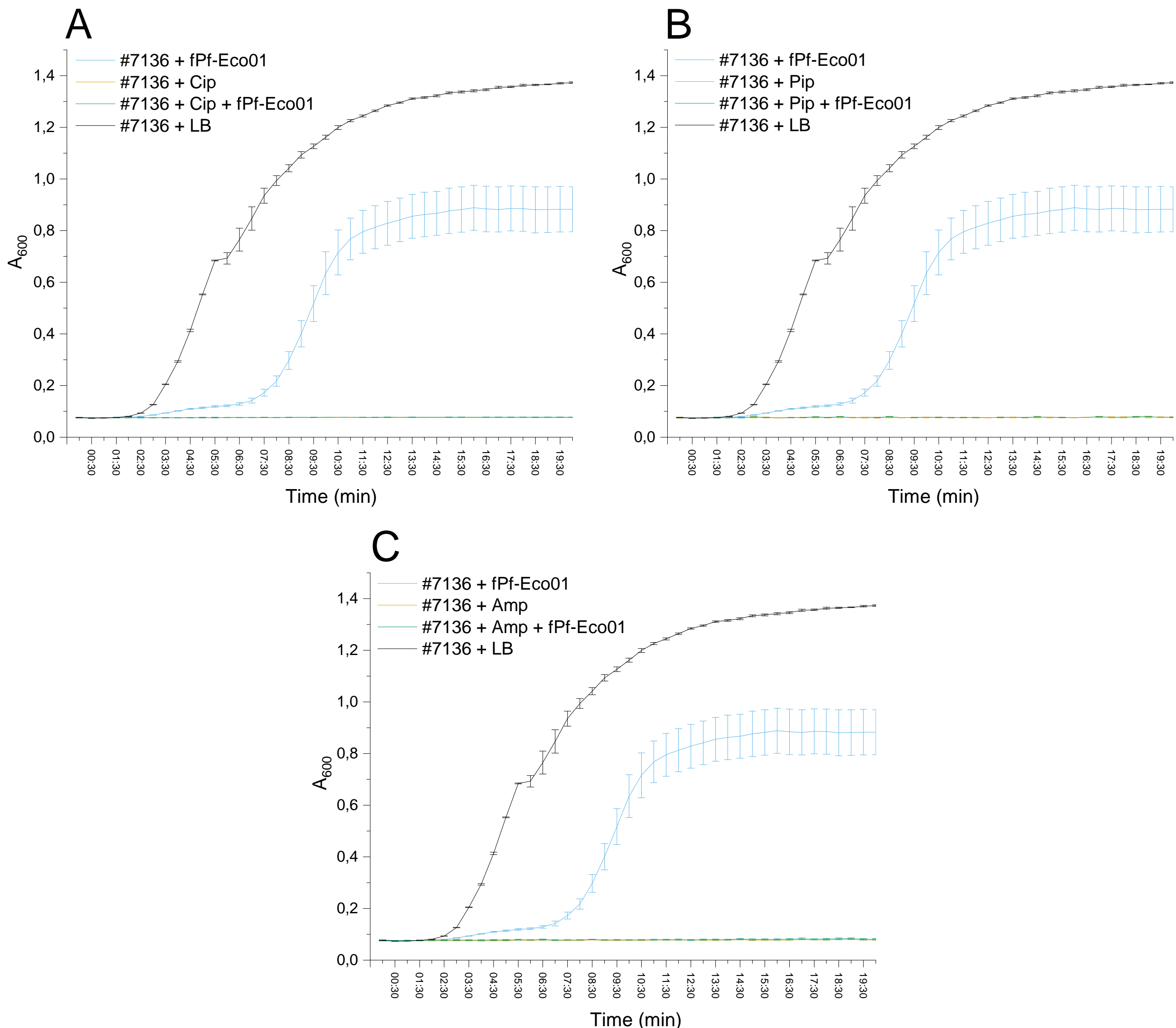

Fig 6. Growth curves of *E. coli* #7136 tested together with fPf-Eco01 phage and three antibiotics. A: Ciprofloxacin (Cip) (160 µg/ml) tested with and without the fPf-Eco01 phage. B: Piperacillin (Pip) (1600 µg/ml), C: Ampicillin (Amp) (100 µg/ml) LB media together with *E. coli* #7136 was used as a negative control. The curves are drawn based on the Bioscreen results. The mean of three parallel wells was calculated to get more accurate results. A_{600} stands for bacterial absorbance at 600 nm.

Phage stability in different solutions

fPf-Eco01 phage pH tolerance was tested at five different pH levels (pH 3-7). Phage titer was roughly 10^9 PFU/ml at the beginning of the experiment and remained at the same level after 2 hours of incubation at RT in all tested pH solutions. The tolerance was tested after 24 hours of incubation to see if the titer might decrease. Titer remained at 10^9 PFU/ml in 5, 6, and 7 pH levels after the 24-hour incubation. Titer was 10^8 PFU/ml in pH 3 and pH 4 conditions after 24-hour incubation. In conclusion, the phage can tolerate low pH conditions and might survive infectious in mammal gut-like environments.

fPf-Eco01 phage stability was determined in four storage solutions (Fig 7). Stability was tested with LB media, PBS, SMG, and 0.9 % NaCl. At the timepoint 0 (0-week), the phage titer was between 10^7 PFU/ml and 10^8 PFU/ml in each solution. The titers were determined after 1-week storage and the phage titer remained in similar values and there were no significant differences between 0-week and 1-week results. Phage maintained its infectivity in all tested solutions. Interestingly plaque sizes were different in different solutions. In plates containing phage preserved in 0.9 % NaCl the plaques were extremely tiny and in PBS plaques were slightly bigger, but in LB and SMG plates the plaque sizes were regular (Fig S1). Sucrose was included to discern the contrast in phage stability between conditions with and without its presence.

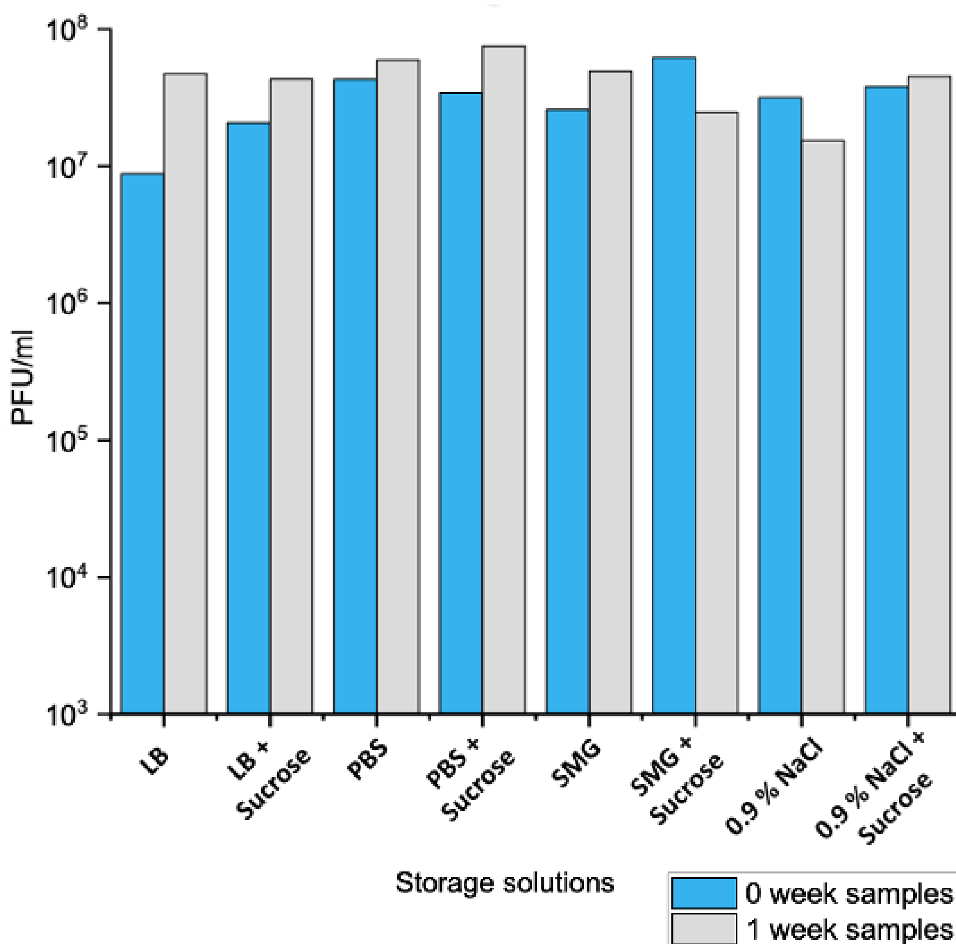

Fig 7. Phage infectivity in LB, PBS, SMG, and 0.9% NaCl with and without sucrose. Infectivity was tested just before storage (0 week) and after 7 days (1 week). PFU/ml values of each storage solution are compared.

DISCUSSION

In this study, the recently isolated *Escherichia* phage fPf-Eco01 genome was characterized, virion morphology was visualized, and host range and infectivity in several ETEC and clinical *E. coli* strains were determined. Phage infectivity was also tested with porcine serum, to detect possible combined effects that might occur in the pig's blood system. Possible antibiotic combined effect together with the phage and antibiotic resistance of the *E. coli* host strain was investigated. Phage was stored at different pH levels to see if the phage survives infectious in a pig cut-like environment and possible oral therapeutic use. Phage stability in different solutions was tested to see if the phage retains its infectivity if the final phage cocktail is stored before usage. Finally, phage suitability in pig's phage therapy was able to be evaluated.

Genomic characteristic

Comparing the fPf-Eco01 genome with the other selected related phages, 97-93 % identity was seen. The highest identity (97 %) was with Escherichia phage vB_Ec_M_J (OQ718158.1) (Fig 1) so it is likely that the fPf-Eco01 phage genomic characteristic is almost similar. Based on heat map results, phage is closely related to other phages. For a phage to be classified as a new species, its genome should be 5 % different, suggesting that the fPf-Eco01 phage does not quite meet the criteria to be classified as a separate species (Grigson et al., 2023). According to the NCBI Nucleotide database, all related phages belong to class *Caudoviricetes* and the genus *Asteriusvirus*. fPf-Eco01 phage 379 kb genome size indicates that the phage can be classified as jumbo phage since phages with a genome size of 200-500 kb are usually classified as jumbo phage (Yuan & Gao, 2017). All related genomes also have genome sizes of 348-383 kb. This correlates that the fPf-Eco01 phage has a lot of predicted genes (620 hypothetical proteins), that code various features.

Based on the fPf-Eco01 genome characters, the phage can be used in phage therapy, since there was no indication of a temperate lifecycle. The phage genome did not contain any known antibiotic resistance genes, that might transfer into new bacterial hosts by transduction. Any bacterial toxin genes were also not found that could bring new properties to the bacteria. The genome contains a gene that is a part of viral protein synthesis: tRNA aminoacylation, Val. This gene, Valyl-tRNA Synthetase is an enzyme involved in the process of aminoacylation, where it specifically attaches the amino acid valine to its corresponding tRNA molecule (Skogman & Nilsson, 1984). This enzyme has a crucial role in the protein synthesis machinery within cells. However, it is still uncertain whether the phage uses self-encoded tRNA or whether the phage utilizes the tRNA molecules of the host cell.

The existence of tRNA and additional translation-related and chaperonin genes, indicates the phage capability to function independently of the host's molecular machinery, providing them with a diverse host range (Nazir et al., 2021). There are a lot of unknown genes in the phage genome that might be part of the phage protein synthesis. For example, certain still unknown genes might have harmful properties that are not beneficial in the context of phage therapy. Jumbo phages are very poorly known, and they are very different from the phages characterized previously.

The plaques of the fPf-Eco01 phages are small and they are very difficult to calculate accurately. To overcome this problem, the plaque assay -method was adapted to better detect plaques. The bacterial amount in each titration plate was divided in half comparing the method that is normally used in the Skurnik laboratory. LB media in titrations was also supplemented with 0.3 % (w/v) agarose rather than the usually used 0.4 % (w/v) agarose. The sizes of the phages can be viewed for the plate images (Fig 1S.). Plaque sizes were quite different in each storage solution. All solutions used affected the infectious capacity of the fPf-Eco01 phage in markedly different ways. When the fPf-Eco01 is stored in NaCl, it might have slowed phage growth in the bacterium, although it does not appear to affect phage PFU/ml values. Large phages with genomes exceeding 200 kb have been documented to produce small plaques (Yuan & Gao, 2017).

Morphology

By fPf-Eco01 morphology the phage reminds typical myovirus morphology. Phages belonging to this group have contractile tails and the phage head structure is bigger compared to other tailed phages (King et al., 2012). fPf-Eco01 phage head width and lengths mean sizes are 99 nm and 118 nm and the tail length is up to 109 nm. Morphologically jumbo phages usually represent head sizes and tail lengths over 100 nm (Nazir et al., 2021). The head of a phage must be large enough to contain its long genome. In TEM images (Fig 2.) several phages seem to have a base plate attached to the tail and some tail fibers. Some phages did not have visible base plate or tail fibers which may be because these structures are not clearly negatively stained, or they have broken down during phage processing before TEM imaging. Tail fibers have a very important role in the phage infectivity. With the tail fibers the phage identifies the host cell wall, penetrates the cell wall structures, and ejects the phage genome into the bacterial host. (Taslem Mourosi et al., 2022)

The host range

The fPf-Eco01 phage has a quite wide host range since it infected 38 % of the tested *E. coli* strains (Table 1S). fPf-Eco01 phage was able to infect its original host, #7136 ETEC stain, and 20 clinical

E. coli strains some of which were ESBL strains. A comprehensive analysis of the phage genome can uncover the reasons why it has such an extensive host range. Studies have also shown that for example, phages that morphologically resemble siphophages and podophages have a narrower host range than phages that resemble myoviruses (Chibani-Chennoufi et al., 2004). *E. coli* infecting phages use several host receptors to attach to the host, such as fimbria, pili, flagella antigens, or other outer membrane proteins of the bacteria (Nobreca et al., 2018). fPf-Eco01 phage possibly uses several receptors to attach to the bacterial membrane which enables phage infections against several strains. The phage's ability to infect is also affected by the structure of its contractile tail, especially the structure of the basal plate's fibrous proteins, which enables the phage to attach to the host cell (Taylor et al., 2018).

The fPf-Eco01 phage did not infect any other ETEC strains than its original host as it was expected. The assumption was that the phage could potentially infect other comparable ETEC strains, given the expectation that they shared similar properties. It may be because a suitable binding site for the phage was not found in those ETEC stains. Previous studies have shown that ETEC-specific phages can have a very narrow or a wider host range and they can infect not only ETEC strains but also other *E. coli* strains (Ferreira et al., 2023; Zhou et al., 2022). It is crucial to determine if the narrow host range of phage against most ETEC strains is a widespread phenomenon. In this case, it becomes essential to investigate the mechanisms that confer immunity to the host and the strategies that phages use, or could potentially use, to counteract these mechanisms. The identification of the phage receptor in the host cell wall would facilitate investigations into how receptor mutations, linked to resistance against the phage, may impact the virulence of the host bacteria. The phage failed to infect more than one ETEC strain, However, it successfully infected several clinical strains. This observation may indicate a high similarity between the phage host strain and the clinical strains, which suggests that the ETEC strain could be zoonotic. All tested ETEC strains have been isolated from pig feces obtained from a farm where pigs are raised. This suggests a potential for bacterial transmission between pigs and humans or vice versa.

Suitability in phage therapy

Based on the several results of this thesis, phage fPf-Eco01 is potential for use in phage therapy in the treatment of infections caused by *E. coli*. The wide host range of phage, especially in human-infecting *E. coli* strains may enable its use in phage therapy in humans. For phage therapy against ETEC, phage seems to be a very narrow spectrum, so studies on phage infectivity, especially against ETEC strains, could be continued. Phages narrow host range in ETEC strains could create the

possibility for customized phage therapy. In the customized phage therapy, a unique therapeutic phage is chosen for each patient based on the bacteria isolated from the patient’s site of infection (Mattila et al., 2015)

The results of porcine serum tests suggest that the complement in the active serum has almost no effect on the host bacterial growth. The bacterium may have developed the ability to defend itself against the serum complement since 20 % serum should have restricted the growth of the bacterium more in the first 10 hours in the porcine serum test. Normally, the serum's complementary system affects bacterial growth by lyzing cells, and by heating the serum the complement system loses the lytic ability (Inglis et al., 2008). There must be other components of several components in the serum that influence bacterial growth. This component or several components have remained similar even after the heat inactivation. The porcine serum seems to have some effect on the ability of phage fPf-Eco01 to infect the host bacterium (Fig 4.). The presence of serum reduced phage infectious ability in the early stages of bacterial growth, but after 20 hours, the differences between phage-only samples and serum together with the phage samples were not significant. Fibrinogen in plasma may act as one inhibitor of phage binding by binding to bacterial cell surface proteins in Staphylococcus (Mutti et al., 2023). It is possible that fibrinogen or some other serum protein or proteins cause fPf-Eco01 phage infection to slow down.

The phage's ability to withstand low pH allows it to survive infectious even in pH 3 which corresponds to similar conditions, such as in the human or porcine gastrointestinal tract. This is important for the potential use of phage in phage therapy since the low pH of the stomach is one of the greatest challenges if phage therapy is to be given orally (Colom et al., 2017). Phages that maintain effectiveness at a pH level of 1-2 are exceptionally infrequent, and the observed ability to endure such conditions is probably due to the buildup of permanent mutations during prolonged exposure to low pH (Nobrega et al., 2016). fPf-Eco01 phage remained its titer in all studied pH levels which indicates good resilience to several pH levels. Similar results on pH resistance have been obtained with other jumbo phages (Alexyuk et al., 2023). Some research studies have indicated that the phages under investigation maintain their titer only within the pH range of 7-8 (Yazdi et al., 2020).

The good preservation of phage in various storage solutions allows a phage cocktail to be produced in any of the tested solutions and the phage remains infectious. These solutions are the ones in which phage can be administered directly to the patient, even intravenously, which is why it was important to test the stability of the phage specifically in these solutions. This feature also facilitates short-term

storage of the phage before phages are administered in the therapy. The phage remains most infectious in a solution with a stable and neutral pH, and low ionic content, but some phages can withstand a wide range of demanding conditions (Duyvejonck et al., 2021). This suggests that determining and optimizing the concentrations of the stabilizer, as well as considering the impact of pH and osmolality are crucial parameters for each specific phage.

Future prospects

This thesis provided basic genome analysis for the fPf-Eco01 phage as well as some knowledge of this phage infectivity. Complete characterization of fPf-Eco01 phage proved to be difficult, which is why the studies could still be continued. The genome was so large that considerably more time could be spent on genome annotating and protein analysis. More specific genome analysis could provide more information about this phage infection procedure in the host cell, and how the phage possibly uses the host protein production machinery. Knowledge of the phage structure proteins could visualize how the virion head and tail structures are composed.

In the future, host range analysis could be continued with more *E. coli* strains, for example, by complementing the study with more ETEC strains. The research could be expanded further across species boundaries by studying the host range in other nearby genera or families. This would provide even more comprehensive research results on this phage host range. All three antibiotics could be performed with several antibiotic concentration and bacterial amount combinations to detect the possible synergy of antagonism with the fPf-Eco01 phage. Even other antibiotics could be included in the antibiotic combined effect -test to get even more information. The research could be continued, especially concerning jumbo phages, as their utility in phage therapy is relatively poorly understood.

CONCLUSION

In conclusion, fPf-Eco01 phage appears to be a promising candidate to be used in phage therapy. The phage genome has no identifications of a temperate lifecycle, there were no antibiotic-resistant or bacterial toxin genes found in the genome. With its relatively broad host range, this phage stands as a potential candidate for treating diverse *E. coli* infections in humans. Furthermore, the phage has the potential to remain infectious in the gastrointestinal tract of piglets or humans and does not lose its infectious capacity when stored in various solutions before usage in phage therapy.

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SUPPLEMENTARY MATERIAL

Table 1S. *Escherichia coli* strains used in this thesis

Figure 1S. Plaque sizes when preserved in each storage solutions supplemented with sucrose

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SUPPLEMENTARY MATERIAL

Table 1. *Escherichia coli* strains used in this thesis

Bacterial species	Strain number	Origin	Isolation laboratory	Notes	Sensitivity to fPf-Eco01	Efficiency of plating
Escherichia coli	#7136	Pig isolate	Finnish Food Authority	ETEC	+	1.00E+00
	#5506	Blood isolate	HUSLAB			
	#5507	Blood isolate	HUSLAB			
	#5509	Blood isolate	HUSLAB			
	#5510	Blood isolate	HUSLAB			
	#5520	Blood isolate	HUSLAB		+	1.40E-02
	#5521	Blood isolate	HUSLAB		+	1.63E-02
	#5522	Blood isolate	HUSLAB		+	9.25E-01
	#5759	Unknown	HUSLAB	ESBL	+	8.00E-01
	#5760	Unknown	HUSLAB	ESBL	+	2.83E-01
	#5761	Unknown	HUSLAB	ESBL	+	4.18E-01
	#5762	Unknown	HUSLAB	ESBL		
	#5763	Unknown	HUSLAB	ESBL		
	#5764	Unknown	HUSLAB	ESBL		
	#5765	Unknown	HUSLAB	ESBL		
	#5768	Unknown	HUSLAB	ESBL	+	1.08E+00
	#5769	Unknown	HUSLAB	ESBL		
	#5770	Unknown	HUSLAB	ESBL	+	2.08E-01
	#6040	Human stool	HUSLAB	ESBL		
	#6041	Human stool	HUSLAB	ESBL		
	#6042	Human stool	HUSLAB	ESBL		
	#6043	Human stool	HUSLAB	ESBL		
	#6044	Human stool	HUSLAB	ESBL		
	#6045	Human stool	HUSLAB	ESBL		
	#6046	Human stool	HUSLAB	ESBL		
	#6047	Human stool	HUSLAB	ESBL		
	#6048	Human stool	HUSLAB	ESBL		
	#6049	Human stool	HUSLAB	ESBL		
	#6460	Unknown	HUSLAB			
	#6471	Unknown	HUSLAB		+	9.75E-03
	#6500	Unknown	HUSLAB			
	#6729	Unknown	HUSLAB		+	1.05E+00
	#6730	Unknown	HUSLAB			
	#6731	Unknown	HUSLAB			
	#6741	Urine isolate	HUSLAB	ESBL	+	5.30E-01
	#6742	Blood isolate	HUSLAB	ESBL	+	5.50E-01
	#6907	Unknown	HUSLAB		+-	2.25E-07
	#6929	Unknown	HUSLAB	ESBL		
	#6954	Urologist sample	Seinäjoki central hospital	ESBL	+	9.50E-01
	#7012	Urologist sample	Seinäjoki central hospital	ESBL	+	8.25E-01
	#7035	Urologist sample	Seinäjoki central hospital	ESBL	+	7.00E-01
	#7074	Urologist sample	Seinäjoki central hospital	ESBL	+	5.00E-01
	#7093	Urologist sample	Seinäjoki central hospital	ESBL	+	6.75E-01

#7104	Urologist sample	Seinäjoki central hospital	ESBL	+	4.45E-01
#7135	Pig isolate	Finnish Food Authority	ETEC		
#7137	Pig isolate	Finnish Food Authority	ETEC		
#7138	Pig isolate	Finnish Food Authority	ETEC		
#7141	Urologist sample	Seinäjoki central hospital	ESBL	+	7.25E-01
#7206	Pig isolate	Finnish Food Authority	ETEC		
#7207	Pig isolate	Finnish Food Authority	ETEC		
#7208	Pig isolate	Finnish Food Authority	ETEC		
#7209	Pig isolate	Finnish Food Authority	ETEC		

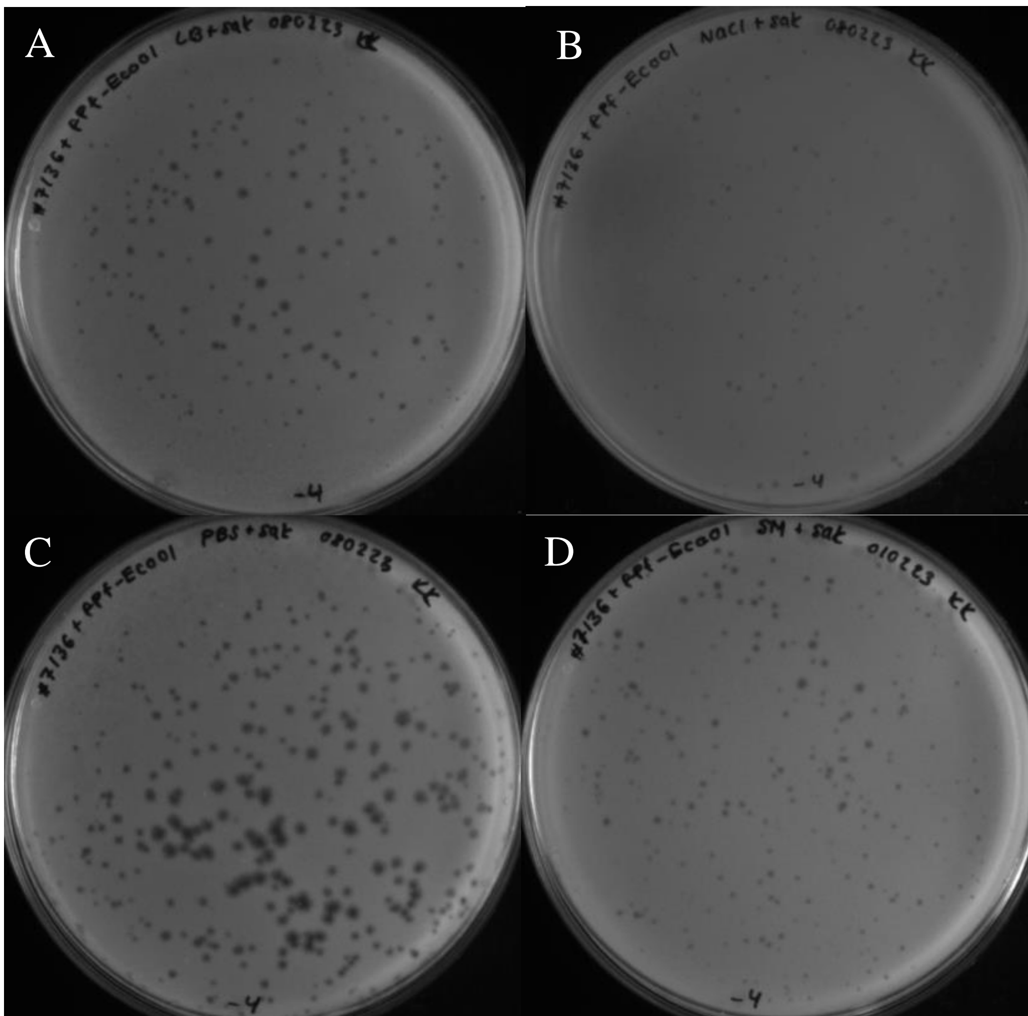

Fig 1S. Plaque sizes when preserved in each storage solutions supplemented with sucrose. A. Regular fPf-Eco01 plaque size when phage was preserved in LB, B. Plaque size when preserved in 0.9 % NaCl, C. Plaque size when preserved in PBS, D. Plaque size when preserved in SMG.