Isolation and Genomic Characterization of Bacteriophages Targeting Extended-Spectrum Cephalosporin Resistant *E. coli*

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Murdoch
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Honours Thesis

School of Veterinary and Life Sciences

Murdoch University

October 2016

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Declaration

This thesis was presented as part of the requirements for the degree of

Bachelor of Science (Honours) – Veterinary Biology.

I declare this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary education institution.

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Abstract

Overuse of antibiotics has resulted in the emergence of antibiotic resistant bacteria resulting in bacterial infections in livestock and humans, that can no longer be controlled by these drugs [2]. Third generation cephalosporins are an antibiotic class used in critical situations as the last line of defence, however bacteria have now developed resistance to these drugs [3].

Bacteriophages are viruses which can infect and destroy bacteria, and are being developed as a new therapeutic method for the control and management of bacterial infections in swine. This method offers a highly specific therapy with minimal side effects on the gut microflora [4]. Administration of phages in animal feed has resulted in a reduction of the severity of bacterial infections in addition to a reduction in the shedding of bacteria in faecal matter [2, 5]. This shedding is a major human health concern as it has the potential to transfer antibiotic resistant bacteria and plasmids carrying resistant genes to humans through the faecal to oral route.

This project isolated 21 bacteriophages, from three separate sources, that are capable of lysing extended-spectrum cephalosporin (ESC) resistant *E. coli*. Characterisation of these phages, through electron microscopy and genome sequencing, identified phages belonging to the three different families within the order *Caudovirales*; *Siphoviridae, Myoviridae and Podoviridae.* Analysis of the phage genomes resulted in the identification of two clusters within the phages belonging to the *Siphoviridae* family, named Cluster 1 and 2. Comparison of the specificity of phages sourced from pig farms with (South Australia) and without (Murdoch University) ESC resistant bacteria suggests that highly specific phages can be sourced from locations infected and uninfected by the target bacterial isolate. Three of the phages isolated from Murdoch University have a broad host range of the target ESC resistant *E. coli* isolates,

highlighting these phages for further studies and potential development into therapeutic products.

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List of Abbreviations

°C	degree Celsius
mg	milligram
g	gram
kg	kilogram
µL	microliter
mL	milliliter
L	Litre
µm	micrometer
nm	nanometer
BHI	Brain heart infusion
CFU	Colony forming units
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTR	Direct terminal repeats
EM	Electron microscopy
EPEC	Enteropathogenic *Escherichia coli*
ESC	Extended-spectrum cephalosporins
ETEC	Enterotoxigenic *Escherichia coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HDP	Host defence peptides
ICTV	International Committee on Taxonomy of Viruses
LB	Luria bertani
LPS	Lipopolysaccharides
MDR	Multi-drug resistant
MH	Mueller-hinton

MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MOI	Multiplicity of infection
NGS	Next generation sequencing
OMP	Outer membrane protein
PCR	Polymerase chain reaction
PFU	Plaque forming units
RBP	Recognition binding protein
SNP	Single nucleotide polymorphism
ssDNA	Single stranded deoxyribonucleic acid
ssRNA	Single stranded ribonucleic acid
STEC	Shiga toxin-producing *Escherichia coli*
SUMO	Small ubiquitin-like modifier
TEM	Transmission electron microscopy

Acknowledgements

I would first like to express my deepest gratitude to my principal supervisor Dr. Mark O'Dea for your unwavering support and guidance throughout the project. You have helped overcome every hurdle, generously shared your knowledge with your door always open for any questions. Thank you to my supervisor Dr. Sam Abraham for your assistance, enthusiasm and passion. Your devotion towards your work has been truly inspirational. Together you have provided me with many opportunities that have allowed me to learn and experience more than I could have ever expected this year.

I would like to thank Michael Platten for his contribution of the electron micrographs. The morphological characterization of the phages was only possible due to your help. To Dr. Stanley Pang, thank you for your contribution to the sequencing and aid in the genetic analysis. I would like to acknowledge Lina Lee for preparing the runs for the sequencing and Terence Lee for teaching me how to perform broth microdilutions and the continuous advice about the Honours program.

I am forever indebted to my parents for the unwavering encouragement they have provided throughout my study and for the person they have shaped me to become. To my partner Craig Cherrington, thank you for your continuous support, your patience during the heavy workload and always relieving my stress. I could never have enjoyed this year so much without you by my side. Lastly, I would like to deeply thank my sister Jodie Laird for dealing with the stresses and celebrating the highs, you have never stopped believing in me.

1. Introduction

In the swine industry, diarrhoea-inducing bacterial infections cause reduced growth and can result in increased piglet mortalities, greatly decreasing meat production and profitability [6]. For a prolonged period following their discovery, the use of antibiotics led to the control and management of the majority of significant bacterial infections. However recently bacteria have evolved resistance to various groups of antibiotics due to selective pressures, and with this resistance spreading rapidly throughout farms and across countries, antibiotics may no longer be depended upon for the control of certain infections in both veterinary and human medicine [2].

Bacteriophages (phages) are one alternative method for the management of bacterial infections. A phage is a virus that infects bacteria. A certain class of phage, known as lytic phages, causes the host cell to burst and thus has a bactericidal effect. The high specificity, low incidence of side effects and natural abundant occurrence makes phages an attractive alternative to conventional antibiotics [4]. Due to this, studies have developed methods to isolate phages and hijack their lytic activity for use in therapeutics, an intervention termed phage therapy [7].

1.1 Antibiotic Resistant Bacteria

Antibiotics have been developed and utilised as the main therapeutic method against bacterial infection since the discovery of penicillin in 1929 [8], and as outlined in Figure 1, discovery of new antibiotic classes continued through to the late 1980s [1], however following this period there was a void in the discovery of new antibiotics. Recently a new method for discovery of antibiotic compounds has led to the discovery of teixobactin [9]. This new antibiotic is the first new antibiotic class in over 30 years, however further studies and clinical trials need to be conducted before the drug is cleared as safe and available for animal and human use [10].

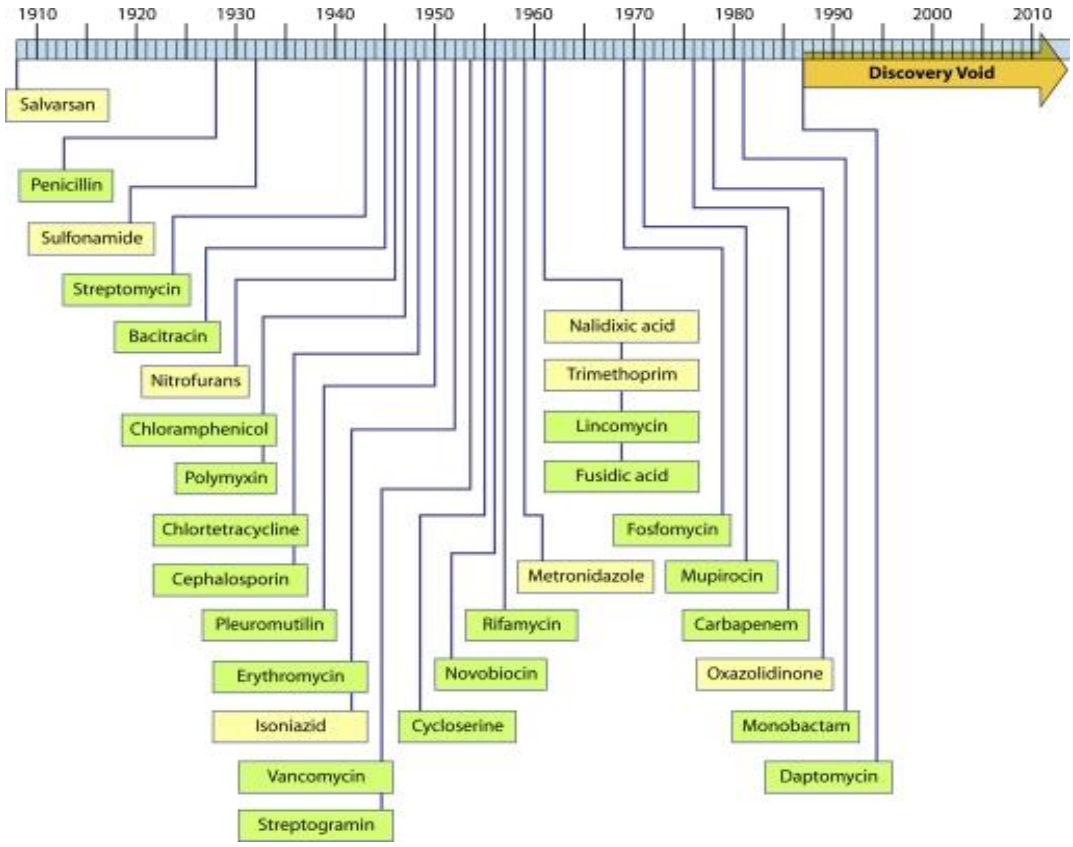

Figure 1. Timeline of antibiotic class discovery from 1908-2010. Adapted from Silver (2011) [1].

In the food animal industry, antibiotics may be added to the animal's diet in order to prevent disease and increase the animal's growth rate [11]. This has resulted in the overuse of various classes of antibiotics, including macrolides, penicillins and tetracyclines, exerting a selective pressure on the bacteria and resulting in the emergence and rapid spread of antibiotic resistant genes [2, 12]. These genes can be transferred amongst bacteria via horizontal transfer (including

plasmids and mobile genetic elements) or via vertical transfer (parent to offspring) [13]. Humans are at a risk of transfer of antibiotic resistance through the direct transfer of bacterial isolates or mobilization of plasmids by direct contact with animals, with animal faeces or contamination of carcasses at slaughter [14, 15]. It should be noted that although this review is predominantly livestock focused, inappropriate use of various antimicrobial classes in humans has also contributed greatly to the development of resistance [12].

Bacterial isolates can be resistant to single antibiotics or to multiple classes of antibiotics and are then termed multidrug resistant (MDR) bacteria [16]. This is due to bacterial isolates accumulating various genes that confer resistance to different classes of antibiotics (such as critically important cephalosporins and fluoroquinolones) [3]. In some cases, these antibiotic genetic cassettes mobilise together on elements termed integrons, such that selecting for resistance using one drug will co-select for another. The class 1 integron with the *dfrA12*-orfF-*aadA*27 genetic cassette array is an example, resulting in resistance to trimethoprim and aminoglycosides [17].

Antibiotic resistant bacteria have emerged and spread rapidly [2]. Comparison of antimicrobial resistance between the periods 1950-1959 to 2000-2002 showed an increase in resistance to 11 out of 15 agents in animal isolates. Resistance against ampicillin, sulphonamide and tetracycline increased from 0% to 69.4%, 73.7% and 85.5% respectively in animals (cattle, chicken and pigs). This pattern was repeated in human isolates with an increase of ampicillin, sulphonamide and tetracycline resistance from 0% to 66.7%, 50% and 58% respectively. The number of multidrug resistant *Escherichia coli (E. coli)* isolates from humans and food production animals in the US has also increased over this timeframe from 7.2% of all isolates in 1950-1959 to 63.6% in 2000-2002. The 285 pigs tested between 1950 and 2002 from across America were tested for the presence of multidrug resistant bacteria with 53.7% of isolated bacteria showing resistance to three or more drug classes [16].

Not only are bacteria showing an increase in multidrug resistance, an increasing number of bacterial isolates have demonstrated resistance to the critical antibiotic class of ESCs [18]. This

is a major health issue as cephalosporin antibiotics are relied upon as a last line of defence, both in human health and in tightly controlled cases in Australian food animals [3]. The enteric bacteria carrying genes encoding resistance to ESCs (found on plasmids) can potentially infect humans via the faecal-oral route. One example is the IncA/C-type plasmids which have been shown to carry bla_{CMY-2} [19]. These plasmid carrying bacteria may colonise the human host or transfer the plasmids carrying resistant genes into commensal human gut microflora [20]. The IncA/C-type plasmids have been attributed as the cause of the rapid spread of the cephalosporinase resistant genes [19].

Ceftiofur and ceftriaxone are extended-spectrum cephalosporins (ESC) and the use of these antibiotics is tightly controlled in an attempt to prevent the emergence and spread of resistance in populations. This tight control of antibiotics and the geographic and genetic isolation of Australian livestock have resulted in no ceftiofur resistance being detected in 117 *E. coli* isolates from 1999-2005 [21] with ESC resistance only first being detected in 2015 [15]. This is in comparison to countries such as Canada, where 13% of ETEC isolates from 2001-2003 were ceftiofur resistant, and Denmark where the prevalence of ESC *E. coli* isolates from slaughter pigs was 11% in 2009 [22, 23].

In 2006 a survey was conducted on the use of ceftiofur in large Australian pig herds. 25% of these herds had used ceftiofur within the preceding year [24]. Studies have highlighted that an increased antimicrobial usage on-farm results in a higher frequency of resistant bacteria compared to low antimicrobial usage. Agerso *et al.* (2012) described this pattern in the use of third generation cephalosporins with an increase of resistance from 10.8% to 26.3% in farms that had used cephalosporins within the prior 12 months. With a growing dependence on ceftiofur for managing multi-drug resistant bacterial isolates, there is concern of an increased emergence and spread of ESC resistant bacteria amongst livestock. Global cephalosporin use in humans has also increased from approximately 8.5 billion standard units in 2000 to 16.5 billion standard units in 2010 [12].

With *E. coli* being a major component species of an animal's normal gut microflora, these commensal isolates can act as a reservoir of plasmids carrying antimicrobial resistant genes, resulting in the spread of resistant genes and the potential to transfer horizontally into pathogenic species. Kheiri *et al.* (2016) recently discovered 136 of 200 commensal *E. coli* isolates from humans and animals were multi-drug resistant, showing the significance of commensal bacteria with antibiotic resistance. Decolonising animal species of commensal bacterial species carrying these genes, such as commensal ESC resistant *E. coli*, can aid in the prevention of the spread of these genes into humans [25]. An estimated 25,000 deaths occur in Europe alone each year due to antibiotic resistant bacterial infections in humans due to the spread of resistance markedly reducing the number of last line antibiotics available to the human health system [26]. Given the discovery void in antibiotic development (Figure 1), it has certainly become clear that alternatives to conventional antibiotic therapies are already required for control of infection. Development of these therapies and continued antibiotic discovery are both needed to prevent the spread and overcome continued emergence of bacterial resistance.

1.2 Alternatives to Antibiotics

Immune-modulating agents, host defence peptides (HDP) and phages are only a few strategies considered to reduce or potentially replace the use of antibiotics in livestock. The vast numbers of livestock to be managed requires that any adopted strategy be low cost, practical and stable to transport whilst also having minimal side effects, no/minimal effect on the animal's normal microflora and a low rate of bacterial resistance [27].

Immune-modulating agents are used to induce or enhance the animal's immune response against pathogenic bacteria, without directly killing the bacteria. These agents can be in the form of vaccines (both live-attenuated and inactivated forms) or immunostimulants (including thymosin and probiotics) [27]. Recently, two studies have tested the effects of using the prebiotic, sodium butyrate, as an addition to livestock feed. The first study looked at the diarrhoea incidence rate in weaned pigs comparing a negative control group, a group receiving a combination of butyrate and antibiotics and another receiving only antibiotics. Both groups showed a reduction in the percentage of piglets with diarrhoea compared to the control group, from 17% in the negative control group to 11.3% and 12.4% for the combination therapy and antibiotics respectively [28]. Comparison of these groups to a group receiving sodium butyrate with no antibiotics would show a clearer result to the effects of sodium butyrate itself. This recent study demonstrates the potential of immune-modulating agents however further studies are needed to confirm results before development for commercial use.

HDPs are naturally released by the host's immune system in the presence of an infection [29]. These peptides can either directly kill bacteria or modulate the host's immune response [30]. Synthetic HDPs have been produced that have greater stimulatory and enhancement effect on the host's immune response, resulting in higher efficacy against bacterial infections [29]. The incidence of diarrhoea in piglets was reduced by 47.6% for piglets treated with the HDP cecropin, when compared to piglets without treatment [30]. HDPs have also been shown to be effective in killing antibiotic resistant bacteria including methicillin resistant *Staphylococcus aureus (S. aureus*; MRSA) [31]. Despite this potential, in the past the high manufacturing costs

associated with the production of HDPs have reduced their use. In addition to this the toxicity towards the bacterial host producing the antimicrobial peptides has resulted in limited output. Recently a new method has been developed that conquers both of these issues, with multiple studies demonstrating the reduced cost and higher output of HDPs. This method uses a protein to fuse the peptide and a small ubiquitin-like modifier (SUMO) gene and after expression of the HDP, a sumolase protease is used to cleave the HDP peptide. The HDP has a reduced toxicity towards the host cell whilst attached to the SUMO, resulting in a higher output of HDP peptides [29, 30].

Perhaps the most promising and economically viable of the listed methods are phages. An estimated 10^{31} phages inhabit the earth and many isolated phages have shown antimicrobial activity against antibiotic resistant bacteria [4, 11]. Studies on phages have shown no general side effects, minimal effects on the normal microflora and the ability for practical application for treatment of livestock through addition to feed [2, 32, 33]. These attributes have resulted in phages being considered as potential alternatives for management of bacterial infections in swine.

1.3 Brief History of Phages

Bacteriophages were co-discovered by Frederick William Twort and Felix Hubert d'Hérelle in the early 20th century. Twort described the phage phenomenon as a virus in 1915 however discontinued further research [34]. In 1917, d'Hérelle 'officially' discovered phages when he confirmed the phenomenon was a virus. He named these bacteria eating viruses, bacteriophages. d'Hérelle isolated phages from samples collected at site and successfully treated soldiers suffering from bacillary dysentery in World War I through ingestion of the phage preparation. In 1921 the use of phages for clinical treatment continued when Richard Bruynoghe and Joseph Maisin injected *Staphylococcus* specific phages into and around opened lesions, successfully treating *Staphylococcal* skin disease in human patients [35].

In the 1930's pharmacological companies began to develop commercially available phage preparations to treat infections against bacterial pathogens including *E. coli* and *S. aureus* [36]. However, there was controversy over the use of phages for clinical therapy. This controversy arose from the lack of knowledge of phage biology and underdeveloped scientific methods of the times resulting in inconsistent results, inappropriate controls and a lack of reproducibility [34]. The discovery of penicillin in the 1940's began the antibiotic era with many countries ceasing development of phage therapy and turning to antibiotics for treatment of bacterial infections [8, 34]. Only some countries, including the former USSR and Poland, continued to use phage therapy [34]. The recent emergence of antibiotic resistant bacteria has led to a re-evaluation of the therapeutic use of phages [36].

1.4 Characteristics of Phages

Phages are bacteria-infecting viruses and are classed as virulent or temperate [37]. They occur naturally in abundance and can be isolated from sewerage and faecal material [38], and potentially from any environment in which bacteria are present. The benefits of phages include their ability to self-amplify in the presence of host bacteria and their high specificity towards bacteria, leading to fewer side effects and limited cross-resistance occurring in bacteria other than the target species [2, 32]. To harness the lytic capability of these viruses they must first be isolated and demonstrate lysis of the intended bacterial isolate. When purified, the phages can be characterised using electron microscopy and genome sequencing [39]. After the initial isolation and characterisation steps, further studies can be conducted on their physical characteristics including host range and burst size and the phage's *in vivo* activity. The burst size of a phage is the number of progeny virion released by the infected bacterial cell from one cycle of phage growth, with larger burst sizes being beneficial for therapy [40].

1.4.1 Virulent vs Temperate

There are two types of phages; virulent (lytic) and temperate (lysogenic) (Figure 2) [37]. Virulent phages are ideal for phage therapy as they follow a strictly lytic cycle [37]. The life cycle of virulent phages begins with the adsorption of the phage to the bacterium [42]. This occurs through interaction between the tail fibers of the phage and specific receptors found on the surface of the bacterium. The phage then injects its genetic material into the cytoplasm where the host cell machinery is hijacked and used to synthesise new phage progeny. The release of the new phages occurs via phage-encoded enzymes which break the bacterium cell wall and cause lysis of the host cell [7].

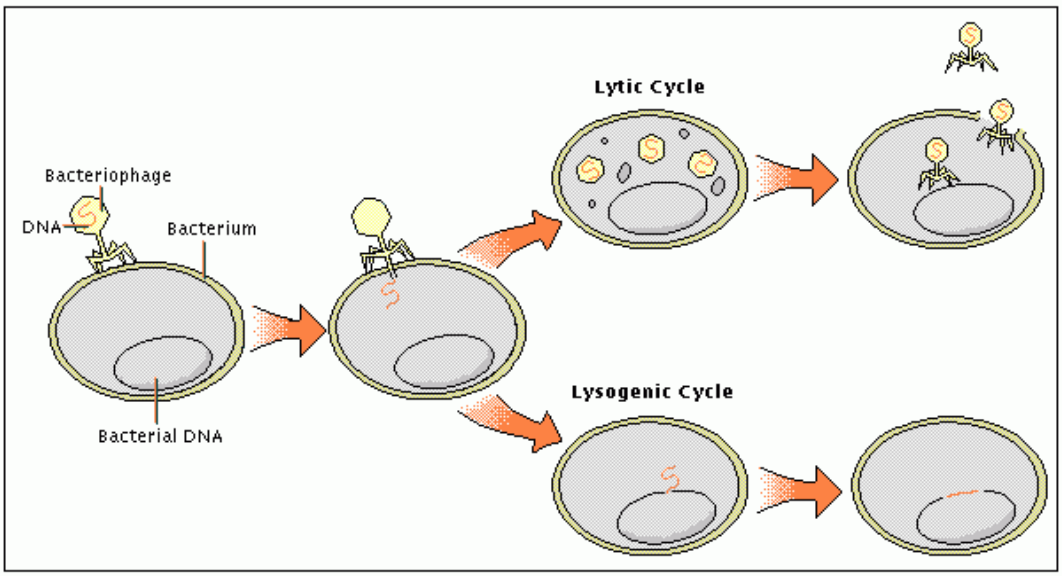

Figure 2. Lytic vs lysogenic phage life cycle. Adapted from https://blogs.unimelb.edu.au/sciencecommunication/2010/11/07/viruses-used-for-good-gene-therapy/

Temperate phages can interact with the host cell in a lytic or lysogenic manner, depending on the surrounding conditions of the phage [43]. The lysogenic cycle involves integration of the phage genome into the host genome. There is some hesitation towards using temperate phages in phage therapy, with the potential for the host cell to increase in virulence due to the horizontal transfer of genes, including antibiotic resistant genes and toxin producing genes through the cycle of integration [7]. A study conducted by Moon *et al.* (2015) demonstrated the ability of a temperate *staphylococcal* specific phage, φSaBov, to horizontally transfer the genomic island υSaβ, which carries varying sequences of toxins, superantigens and bacteriocins, into human and animal isolates of *S. aureus*. Temperate phages are still being researched to further evaluate their suitability to be used for phage therapy [37].

1.4.2 Phage Therapy

Development of phages as therapeutic agents requires *in vivo* testing. In these trials, the ratio of phage to bacteria, termed the multiplicity of infection (MOI), that maximizes the lytic activity of the phage therapy is determined. This ratio differs between phages and the target isolate with a MOI of between 0.01 and 100 normally optimizing phage lytic activity [41]. The phage is formulated for administration (in SM buffer or encapsulated in liposomes) and administered

systemically to animals through feed and to humans through drinking water [5, 32]. Attachment of phages occurs through the recognition of the bacterial host isolate, with the phage then creating a pore in the host cell's wall and injecting the phage DNA inside. After the phage DNA has been expressed and proteins synthesized, phage enzymes break down the cell wall lysing the host cell and releasing virions. Phage therapy utilises this lytic activity to reduce the number of bacterial cells resulting in a high level of control of bacterial infections and reduction of the shedding of harmful of resistant carrying commensal and pathogenic bacterial isolates by livestock [34].

1.5 Phage Taxonomy

The International Committee on Taxonomy of Viruses (ICTV) was formed to categorise viruses. This system was created for easy management in identifying new viruses and comparing the previously recorded viruses [45]. The hierarchical system classifies the bacterial viruses into families based on the nucleic acid genome (dsDNA, ssDNA, dsRNA and ssRNA), morphology (tailed, polyhedral, filamentous and pleomorphic) and physical characteristics (including host range and resistance to organic solvents) [45, 46]. Tailed phages account for 96% of all phages and form the three families belonging to the order *Caudovirales*; *Siphoviridae*, *Myoviridae* and *Podoviridae*. These three families all contain double stranded DNA genomes [17]. *Siphoviridae* are the most common of the tailed phages with 61%, 24.5% and 14% of tailed phages belonging to *Siphoviridae*, *Myoviridae* and *Podoviridae* respectively [39].

There is controversy as to whether this historical classification system is the most appropriate method for classification of phages [17, 46]. A newly suggested method of classifying phages used the genetic similarity of genes coding for capsid proteins, however not all bacteriophages carried a homologous gene [17]. Rohwer and Edwards (2002) analysed the whole genome of 105 phages isolated from various bacterial species. These isolates again did not share a common single gene due to the high rate of lateral and horizontal transfer of genetic material between phages [46]. Comparison between phages within families can sometimes be achieved using genes including portal protein, lysin and major capsid genes. Without a common gene amongst all phages to base the hierarchical classification on, the classification of phages using whole genome comparison becomes difficult [47].

1.6 Phage Characterisation

1.6.1 Morphological Characterisation

Bacteriophages show great variability in their morphological structure and this can be used to distinguish which family a phage belongs to (Figure 3). The primary classification tool and standard technique to produce images capturing viral structure is transmission electron microscopy (TEM), first used in 1940 [4, 39]. These images allow preliminary classification of phages and are mandatory for the acceptance of the classification of a phage by the ICTV [39]. TEM offers a relatively cheap, fast and simple method of the morphological classification of phages. The phages are negatively stained using stains including phosphotungstic acid or ammonium molybdite. The electron micrograph shows the shape and diameter of the phage head, the presence, length and contractibility of tails and the presence of tail fibers, base plates and tail spikes [39].

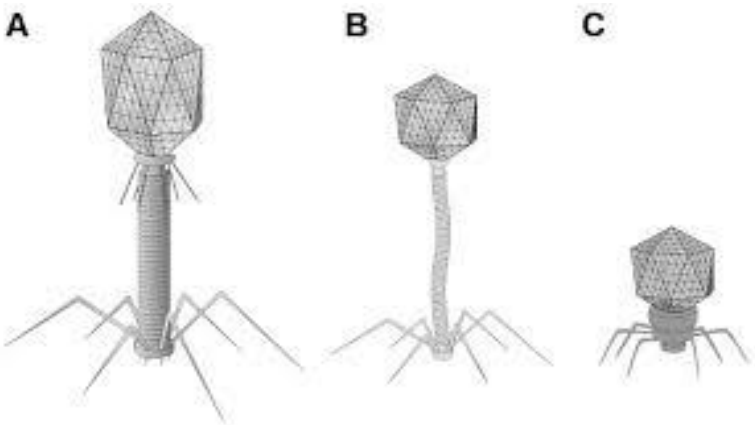

Figure 3. Virion morphological structure of tailed bacteriophages. Adapted from https://www.diva-portal.org/smash/get/diva2:807580/FULLTEXT01.pdf.

A: *Myoviridae*, B: *Siphoviridae* and C: *Podoviridae.*

Once the morphology of the phage is captured using TEM, it can be classified into a family. The shape of the head can be differentiated between elongated and icosahedral with further differentiation of the head determined by the diameter. Families can be differentiated due to the length and contractile ability of the tail. *Siphoviridae* have long, non-contractile tails of length 79-539 nm and head diameters of 40-97 nm. *Myoviridae* have long contractile tails of length 80-

485 nm and head diameters of 53-160 nm whilst *Podoviridae* have short tails of length 3-40 nm and head diameters of 38-75 nm [48]. Classification of phages to lower taxonomic levels can't be performed using electron microscopy and therefore other techniques including whole genome sequencing are employed [39].

1.6.2 Genetic Characterisation

Further characterisation of phages into lower taxonomic levels can be performed using genome sequencing [39]. Analysis of the genome sequence allows an increased understanding of the biology of phages and their bacterial hosts, through the identification of proteins and mutations involved in this interaction [49]. Despite this, various complications have resulted in the number of sequenced phage genomes being minimal in comparison to the sequencing of bacterial genomes [49].

The first issue is the overabundance of host DNA in the sample due to the inability of the virus to self-replicate. To minimize this problem, phage DNA needs to be purified by methods including the use of chloroform, or sequenced reads belonging to the host need to be removed before analysing the sequencing output data. A second issue is the high levels of methylation and repeats of phage DNA required for successful protection from the host. These can affect the success of polymerase chain reactions (PCR) and sequencing of phages, however can be overcome through the use of a combination Next Generation Sequencing (NGS) technologies [50], such as scaffolding using long read technology and assembling using short technology. Lastly issues with cloning the DNA of lytic phages into *E. coli* hosts, required for Sanger sequencing, reduces the success of sequencing phage genomes due to the potential lytic effects of the phage DNA on the host [49, 50]. The development and technological advances in NGS have resulted in faster, cheaper and more efficient whole genome sequencing of phages [49, 50]. Presently the sequencing of the entire genome of each phage is mandatory for the phage to be recognised by the ICTV [50]. Before 2010 this requirement was only desirable of phages for use in phage therapy, leading to concerns of toxic or potential viral transduction genes being missed and thus introduced into a mammalian species [51, 52]. This has since been modified with

whole genome sequencing currently being a requirement for all phages used for phage therapy [50].

1.7 Phage Specificity

Phages have narrow host ranges due to their high specificity with most phages being species or even strain specific [5]. This level of specificity has both advantages and disadvantages for phage therapy.

Host ranges of phages differ due to the ability of various phage recognition binding proteins (RBP) to attach to the different surface receptors of bacterial cells [53]. The recognition binding proteins are often contained in the phage tail fiber [54]. They attach to surface receptors such as the hydrophilic component of specific lipopolysaccharides (LPS) and/or certain outer membrane proteins (OMPs) referred to as porins [53]. The Sf6 phage uses both receptors with LPS as a primary receptor and OmpA as a secondary receptor. Furthermore, when grown on *ompA*$^-$, *ompC*$^-$ and *ompA*$^-$*C*$^-$ mutated bacterial isolates the phage can still infect the cells, demonstrating a switch of the secondary receptor. This switch of the surface receptors in the absence of the preferred surface receptor demonstrates a phages ability to mutate and overcome bacterial resistance, a strong advantage in therapeutic products. Another example of this switch in receptor preference is the ability of phage Lambda to recognise OmpF when the preferred receptor LamB is absent [55]. The natural host range of a phage depends on the recognition binding proteins of that phage, the presence of the appropriate surface receptors and the ability of the recognition binding proteins to switch between receptors [53, 55]. An increased understanding of these interactions can lead to a more targeted and effective phage therapy.

An advantage of phage specificity is the reduced risk of phages having lytic activity against commensal microflora. Denis *et al.* (2009) recovered 80 bacterial isolates from the faecal matter of 20 healthy humans. From here, increasing concentrations of four virulent *E. coli* specific phages were tested as to whether they had lytic activity against these gut microflora isolates. 81% of isolates showed no lysis by phages with 19% of isolates showing lysis by one or more of the four phages (under extremely high concentrations of phages of 10^8 plaque-forming units(PFU)/mL) [33]. However, it should be noted that this study was conducted *in vitro* with a high ratio of phage to bacteria. Bruttin *et al.* (2005) conducted a safety trial of the T4 phage *in*

vivo on 15 healthy humans, in which all individuals had no phages present in faecal matter before the experiments. The T4 phage lysed diarrhoea inducing *E. coli* and had demonstrated a broad host range in previous animal studies, therefore safety trials in humans were undertaken. There was no significant difference in adverse effects, listed as stomach pain, nausea and a sore throat, or *E. coli* titres between the treatment groups and the placebo group. This study demonstrates the specificity of the T4 phage having no effect on microflora. During the experiment the control group still had no phages present while T4 phages were isolated from the treatment groups. The concentration of phage in faecal matter of the high concentration treatment group was 34,000 PFU/g. The peak titre of phage in the faecal matter of the low concentration treatment group was 68 PFU/g, 500-fold lower than the high concentration treatment group. The lower concentration treatment group also showed a sharp decrease in the concentration of excreted phages when treatment was stopped compared to the high concentration treatment group, with approximately only 10% of individuals having phages present 24 hours and 96 hours after treatment was stopped for the low and high concentration treatment groups respectively [32]. This demonstrates that the phages transit through the gastrointestinal tract quickly and have no prolonged impact following treatment. Denou *et al.* (2009) conducted a study measuring the effect of phages on gut microflora. There was no decrease in the 10^{11} colony forming units (CFU) of microflora bacteria per gram of faeces recorded with the addition of no adverse effects, normal weight gain, normal behavior and no changes in phage or antibody levels after the experiment [5]. These studies demonstrate that the specificity of phage treatment results in minimal side effects on gut microflora, highlighting the attractiveness of using phage therapy to treat and manage bacterial infections.

The disadvantage of phage therapy is the limited range of pathogens that each phage can infect. A therapy that is highly specific may result in an ineffective coverage of the target pathogen after spending limited time, resources and trials to produce and test it [32]. This limiting specificity has been demonstrated by various studies. Bruttin and Brüssow (2005) [32] tested the lytic activity of an *E. coli* specific phage T4 against 42 *E. coli* isolates. Only two of the 42 isolates were lysed by the phage [32]. Another study conducted by Denou *et al.* (2009) tested a

number of phages in their ability to lyse 25 enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* isolates. Phage RB49 had the largest host range at 8 of 25 isolates, with the other phages infecting four or less isolates [5]. In contrast, a study testing phages GJ1-GJ6 against 85 ETEC isolates demonstrated a broad host range. Four of the phages lysed 99% of the isolates with the other two phages lysing 100% of the isolates suggesting variation in phage recognition proteins [56]. These studies show the varying host ranges of the phages from highly specific to broad spectrum. Selection of broad spectrum phages for production as phage therapy can result in effective management of bacterial infections, with the caveat that extensive *in vivo* testing would be required to ensure commensal bacteria are not adversely affected. An increased knowledge of factors and phage recognition proteins resulting in broad spectrum phages will minimize the number of phages needing to be developed for management and control of bacterial infections.

1.8 Phage Therapy

1.8.1 Therapy in Livestock

Studies observing the therapeutic effect of phages against pathogenic *E. coli* continue to be conducted. One of the earliest studies highlighted the potential for phages to reduce the impact of bacterial infections and the concentration of bacteria excreted in faeces in livestock. In this study, 14 pigs were challenged with *E. coli* P433, with seven receiving a mix of phage P433/1 and P433/2 (10^{10} viable particles of each), at the onset of diarrhoea. The duration of diarrhoea for the untreated group was recorded to be between 26-84 hours with the duration of the treated group reduced to 7-13 hours. Four of the untreated pigs died as compared to survival of all treated pigs. In addition to reduced diarrhoea, duration and mortality in the phage treated group, there was also a reduction in the excreted *E. coli* count from $10^{9.4}$ CFU/g of *E. coli* P433 in the untreated group compared to $10^{4.6}$ CFU/g in the treated group [57]. These results have continued to be supported through various studies focusing on different phages [2, 58].

Pathogenic bacterial infections have a significant financial impact on the swine industry due to the death and reduced growth of swine. As such, phages are being isolated from pig farms to find an alternative to antibiotics. Jamalludeen *et al.* (2009) isolated phages GJ1-GJ6 and studied their ability to reduce the duration and severity of diarrhoea in pigs infected with ETEC JG280. All phages reduced the average group weight changes from the control showing a decrease in weight of approximately 1 kg compared to phage treatment groups increasing in weight of approximately 0.2kg to 1.2kg. The phages also reduced the group's average duration of diarrhoea with the control group having a duration of four days compared to two and a half days for treatment groups [58]. Further studies supported these results [2, 59]. The phage CJ12 was mixed with pig feed at a ratio of 1:1,000 (0.1% w/w) for two groups of pigs using a concentration of the phage of 10^6 and 10^8 PFU/g. This was continued for the duration of the experiment. One week later the pigs, along with two control groups, were orally challenged with ETEC at a concentration of 10^{11} CFU/mL. The faecal matter was scored (diarrhoea score) from 0 (firm and normal shape) to 3 (frequent passage of watery faeces) to determine the percentage

of infected pigs. In this study the highest diarrhoea score of the pigs reached 2 compared to a maximum of 3, meaning diarrhoea scores consistent with severe bacterial infections were not reached. This may explain the lack of a significant difference in the weight changes between the control and treatment groups. Despite this, the phage treated groups showed a decrease of 60.73% and 63.92% in the concentration of ETEC in faeces showing that the phage treated group had a reduced severity of infection following ETEC challenge [2]. Another study with EPEC *E. coli* isolates conducted on calves did induce severe bacterial infection and demonstrated reduction of the severity and the duration of diarrhoea in the phage treatment group. 12 calves were inoculated with *E. coli* 85, with six calves pre-inoculated with phage B85/1. The calves with no treatment developed severe diarrhoea scores with diarrhoea lasting until death compared to moderate to mild diarrhoea scores and duration of 12-40 hours of the phage treated group [59]. These results support the therapeutic effects of bacteriophages in the treatment of bacterial diarrhoea in livestock.

Phage therapy is also being utilized to reduce the excretion of bacterial isolates and therefore the risk of transfer of resistance genes to humans, with these isolates potentially carrying plasmids coding for antibiotic resistance. Studies have observed a beneficial impact of phage therapy on bacterial load of faecal matter. These studies have shown a reduction of excreted pathogenic bacterial load after phage treatment. A study on the impact of phage CJ12 on the ETEC bacterial load of faeces demonstrated that groups treated with phages showed a large reduction of excreted bacterial load, reducing the bacterial load in faeces by 63.92% and 60.73 respectively compared to the positive control [2]. This demonstrates that phage therapy may reduce the risk of humans acquiring antibiotic resistance via faecal-oral contamination, by reducing the pathogenic bacterial load within contaminated faeces.

1.8.2 Phage Cocktails

Single treatments containing multiple phages are termed phage cocktails. The interest in phage cocktails is the potential of an enhanced effect and host range of the phages. Phage cocktails have demonstrated an increase in the lytic activity against bacteria, an increase in the host range

of the therapy and a reduction in the development of bacterial resistance to phages [58, 60, 61]. Nale *et al.* (2016) compared the effect of five different phages, as either single phages or as mixtures, on bacterial infection. The single phages lysed the bacteria by between 2-6 log units within five hours however bacterial regrowth occurred within 24 hours. Various combinations of phage mixtures were tested with a cocktail of two phages and a cocktail of three phages completely lysing the infection within two hours with no regrowth occurring [60]. Furthermore a study by Denou *et al.* (2009) demonstrated a cocktail of six phages (lysing between four and eight isolates as single phages) were capable of fully lysing 13 out of 25 (52%) pathogenic *E. coli* isolates [5]. Another study conducted by Khan *et al.* (2015), determined lysis of 76% of isolates from the *E. coli* reference collection using a cocktail of six phages. The study selected these six phages as they had the broadest host range, varying from phage SU57 lysing 15% of all isolates compared to phage SU16 causing lysis of 60% of isolates [62]. It is evident that mixtures of phages can have a greater impact on the bacterial infection than a single phage alone.

These studies suggest that by selecting phages with different host ranges and combining them in the one mixture, an increased host range is created. However, there are some studies showing a decrease of the expected host range of the phage cocktails. Bourdin *et al.* (2014) conducted a study on various phages selecting the three phages with the broadest host range to be combined as a cocktail. Using the host range determined for each single phage, the three phages should have been able to lyse 87% of the bacterial isolates however only 54% were lysed. They then used six phages to create a cocktail which could only lyse 69% of isolates and a cocktail of nine phages had no increase in the host range [63]. This shows that there can be a limit on the efficacy of the number of phages used in a cocktail. The overlapping of host ranges and decreased concentration of each individual phage with the addition of more phages to a cocktail, may interfere with the effectiveness of the cocktail, possibly through non-specific blocking of receptor sites by phage species. Despite this, the advantages of phage cocktails are apparent. Careful selection of the phages incorporated into a cocktail and studies on the effectiveness of

the cocktail *in vitro* and *in vivo* is required when producing phage cocktails with the highest potential for phage therapy.

1.8.3 Phage Resistance

The evolution of antibiotic resistance in bacteria has led to the re-emergence of studies into phage therapy, but could bacteria further evolve resistance towards phage therapy? The presence of the phages with lytic activity selects for mutated bacteria that have evolved resistance. However, phages can also adapt and evolve, unlike antibiotics and other alternative strategies. For the phages to survive they must adapt to gain lytic activity against the mutated bacteria, leading to phage-susceptible bacteria, as seen with the switching of the host receptor preference [61]. This co-evolution decreases the ability of bacterial cells to become permanently resistant to phage therapy compared to the current resistance towards antibiotics.

In comparison to antibiotic resistance, resistance developed against phages has limited spread between bacterial genera. This is due to genes for antibiotic resistance being present on mobile genetic elements, the integrons and gene cassettes as outlined above, resulting in the transfer of multiple resistance genes across bacterial genera. The transfer of resistance against phages are unlikely to spread across in a similar pattern as the modified genes are not on mobile genetic elements.

Resistance against phages has occurred and the mechanisms of resistance studied include prevention of phage adsorption, blocking of phage DNA entry and the nicking of phage nucleic acids. Phages infect bacterial cells through the recognition of host surface molecules referred to as phage adsorption to the host cell. Some resistant bacterial isolates have edited the surface structure preventing phage infection. An example of this is the production of immunoglobulin G-binding protein A by *S. aureus* which masks the phage receptor. Another method to prevent phage adsorption is the production of competitive inhibitors [61]. Microcin J25 out-competes phages T1 and T5 for the *E. coli* FhuA (iron transporter) adsorption site, reducing the adsorption of phages and therefore phage infection [64].

Pseudomonas aeruginosa isolates have evolved resistance to the phage OMK01 by changing the multi-drug efflux (Mex) system. This system increases resistance to multiple classes of antibiotics including quinolones, macrolides and tetracyclines [65], as it transports the antibiotics out of the cell. The phage OMK01 binds to the outer membrane protein OprM, one of the three components of the Mex system. A study performed demonstrated that the resistance developed by the bacteria against the phage was coupled to an increased susceptibility of the bacteria to all four antibiotics tested (ciprofloxacin, ceftazidime, erythromycin and tetracycline). The decreased expression of the OprM protein results in reduced activity of the Mex system increasing the sensitivity towards antibiotics showing an advanced phage therapy [66]. Overall the phage treatment increases the bacteria's susceptibility to antibiotic treatment. Further studies on the potential of phage therapy to be used in this manner need to be conducted after discovery of the phage's recognition binding protein.

1.8.4 Complications of Phage Therapy

Studies have reported reduced anti-bacterial activity of phages *in vivo* compared to *in vitro* systems. One to 1000 PFU/CFU concentrations of phages e11/2 and e4/1c both showed successful reduction of *E. coli* 0157:H7 within four hours in an *ex vivo* rumen model system. This success was not repeated *in vivo,* with a cocktail of the two phages having no effect on reducing the number of cattle infected with *E. coli* 0157:H7 and no reduction on the excretion levels of the bacteria. The proposed reason behind the decrease in phage infectivity was the 1 to 1,000 ratio of the phage and bacteria used in the study, resulting in the lack of exposure of the phage to the *E. coli*. With the potential of phages being inactivated *in vivo*, this ratio of bacteria to active phages would become even more significant. Studies with varying systems using higher concentrations of phages than bacterial cells have shown success in reducing bacterial infection in cattle [67], therefore analysis of the methods and concentrations used in this study need to be conducted.

Another factor influencing the effectiveness of phage therapy is the highly acidic levels of the gastrointestinal tract. Kerby *et al.* (1949) determined phage T7 to have optimum stability at a

pH range of 6-8. At pH 4, the majority of lytic activity was lost after 96 hours and at pH 3 all lytic activity was lost after one hour [68]. Jamalludeen *et al.* (2007) also conducted tests studying stability in acidic conditions with optimum stability being at pH 5-9. Incubation for 16 hours at pH 1 and 2 denatured all the phages to the point of no detection with pH 3 denaturing five out of nine phages [56]. With acidity of the GI tract of swine ranging from pH 1-2 before a meal to pH 4-5 after a meal, the administration of phages in animal feed helps to reduce the acidic impact on the phages resulting in a higher therapeutic effect. This study also highlighted the variability of acidic resistance amongst various phages, with four out of nine phages tolerating acidic levels of pH 3 [56]. Bruttin *et al.* (2005) also demonstrated the stability of phages in *in vivo* studies with the dose of phages received in participant's drinking water being $9x10^7$ PFU/mL with only $1x10^7$ PFU/g excreted in the faeces. The small reduction of phage concentration shows stability of the phage through the gastrointestinal tract. The acidity of the gastrointestinal tract can reduce the success of phage therapy however with the correct selection of acid stable phages and the application method of therapy this issue can be greatly minimised [56].

Another strategy to protect phages from acidic conditions is the use of liposomes to encapsulate the phage. Survival of liposome-encapsulated phages was compared to non-encapsulated phages in gastric fluid of pH 2.8. The decrease in phage titre was 3.7-5.4 log units and 5.7-7.8 log units for encapsulated and non-encapsulated respectively. This *in vitro* experiment shows the advantage of providing adequate protection for the phage until it reaches its site of action. Similar results of improved stability of encapsulated phages were demonstrated with phages being detected in 38.1% of animals compared to detection of non-encapsulated phages in 9.5% of animals. The protection level of the phage against bacterial infection was similar for both groups when tested *in vivo*, however the encapsulated phages provided a longer time of protection when treatment was stopped with non-encapsulated phages disappearing within 72 hours and encapsulated phages remaining for one week [69]. For these reasons, phages with high acidic resistance should be selected for phage therapy in the gastrointestinal tract or

methods to protect the phages against acidic conditions should be implemented or a combination of these methods.

1.8.5 Phage-Antibiotic Synergy

Studies over the past decade have demonstrated the potential of using bacteriophages as phage therapy in order to replace the current method of antibiotics. Instead of complete replacement of antibiotics, Comeau *et al.* (2007) performed a study determining the effect of a combined therapy of both phage and antibiotic. This study demonstrated an increased phage titre and increased rate of lytic activity with the combined therapy. The phenomenon was repeated in different bacterial species using various phages combined with antibiotics. This included *E. coli* infected with phage MFP and cephalosporins, and *Yersinia pseudotuberculosis* infected with phage PST and quinolone. The combined treatment of T4 phage and 30 mg/L ceftotaxime demonstrated an 11-fold increase in the total phage titre. The theory behind this synergistic relationship is the filament-induced state of the bacterial cells by the antibiotics, leads to an enhanced rate of phage assembly and increased sensitivity of the bacterial cell to the phage lysin. This filamentous state of bacteria is stress-induced; therefore from an ecological view the phages may have developed to identify weak and easily infected cells for fast production of viral progeny [70]. The synergistic effect was termed phage-antibiotic synergy (PAS). Torres-Barcelo *et al.* (2016) demonstrated the synergistic effect of phage LKD16 and varying antibiotics on *P. aeruginosa*. The difference in the average final optical density of bacterial growth was decreased to 0.92 +/- 0.07 in the combined therapy compared to the antibiotic therapy of 1.32 +/- 0.05. The observed difference in optical density was compared to the expected difference of the combined therapy. This was repeated using various antibiotics with a threefold increase in the observed reaction of phage and carbenicillin and a twofold increase in the observed reaction of phage and gentamicin and trimethoprim, demonstrating the synergistic, not additive, relationship [71]. Further studies need to be conducted to ensure resistance towards phages and antibiotics are not selected for when using a combination of therapies. The highly specific lytic activity of phages has been demonstrated to be successfully harnessed to control bacterial infection, however with the combined therapy of phage and antibiotics a higher

efficiency therapeutic method may be developed.

1.9 Project Aim

The emergence and spread of critically important antimicrobial resistance such as ESC resistant *E. coli* urgently requires a novel strategy for the control and management of these bacterial isolates. The significant financial impact on meat production and the risk of direct transfer or mobilization of plasmids carrying ESC resistant genes along the food chain, from livestock to humans [6, 14], shows the need for a phage therapeutic agent targeting ESC resistant *E. coli* to be developed. Phages offer a highly specific control of infections in livestock, however phages that target ESC resistant *E. coli* need to be isolated, characterised using electron microscopy and whole genome sequencing, and the host range determined. These host specificity tests will determine if the lytic phages target ESC resistant *E. coli* isolates with no lysis of commensal *E. coli* and other gut microflora. Analysis of the phage genomes, with concentration on tail proteins, may determine the phage recognition protein allowing development of a more valuable phage therapy.

Hypotheses of this study

1) Lytic phages that can specifically lyse ESC resistant *E. coli* can be isolated from faecal samples from pig farms that have detected ESC resistant *E. coli* after ESC (ceftiofur) use.
2) Lytic phages isolated from faecal samples from pigs treated with ESCs and target ESC resistant *E. coli* present will have greater host specificity than phages isolated from faecal samples from untreated pigs or environmental samples without ESC resistant *E. coli*, making them more suitable as phage therapy agents

Aims of this study

1) Isolate lytic bacteriophages that target ESC resistant *E. coli* from different sources including
    a) Faecal material from pig farm where ceftiofur was used in the past
    b) Faecal material from pigs that did not receive ceftiofur treatment in the past
    c) Environmental water samples

2) Identify the host range of the bacteriophages that are active against ESC resistant *E. coli* by testing lytic activity against a range of bacteria including ESC resistant *E. coli*, commensal and pathogenic *E. coli* isolates from pigs and wide range of bacterial genera
3) Characterisation of bacteriophages specific to ESC resistant *E. coli* using transmission electron microscopy and next generation sequencing
4) Identify differences in tail segments of lytic bacteriophages which may account for different host ranges

2. Methods

2.1 Sample Background

Samples for isolation of *E. coli* specific phages were sourced from three locations; a piggery in South Australia, grower pigs housed at Murdoch University and swampland on the south side of Murdoch University, which was a runoff area for paddocks holding sheep, cattle and horses.

Samples were collected and isolated on different dates (Table 1), with faecal samples collected from the freshly defecated samples on the ground. The three pooled faecal samples from South Australia (S1, S2 and S3) were analyzed separately due to the potential that the three samples were collected from three different pens from around the piggery. The six faecal samples from Murdoch University (M1) grower pigs were pooled together for testing due to all samples being collected from a single house of grower pigs on the same day. The water samples from the Murdoch University swampland (M2) were analysed separately due to collection at different geographical locations within the swampland.

Table 1. Collection details of source samples used for phage isolation.

Sample type	Number of samples	Location collected from	Date collected
Pooled Faeces (S1, S2 and S3)	3	Piggery, South Australia	March 2015
Faeces (M1)	6	Grower pigs, Murdoch	May 2016
Water (M2)	6	Swampland, Murdoch	June 2016

2.2 Bacterial Isolates

All bacterial isolates used in this study are listed in Table 2. Bacterial isolates were provided by Dr Sam Abraham from a reference strain collection (Table 2). Isolation of phages was conducted using bacterial isolates SA35-46, 72-73 and ATCC 25922 *E. coli* strain. Isolates SA35-46 and 72-73 were isolated from healthy livestock whilst 107 is the ATCC 25922 *E. coli* strain (Table 2).

Cross-reactivity within *E. coli* was tested using commensal and pathogenic *E. coli* isolates from pigs and other *E. coli* isolates from seagulls and dogs. Phage lysis was also tested against multiple bacterial genera, including S*almonella spp*, *Streptococcus spp, Enterococcus spp* and *Staphylococcus spp*, to determine the potential effect of the lytic phages on gut microflora.

Table 2. List of all bacterial isolates used in this project for isolation of phages and host range determination.

ID Number	Species	Source
SA 35*	*Escherichia coli*	Pig – healthy
SA 36*	*Escherichia coli*	Pig – healthy
SA 37*	*Escherichia coli*	Pig – healthy
SA 38*	*Escherichia coli*	Pig – healthy
SA 39*	*Escherichia coli*	Pig – healthy
SA 40*	*Escherichia coli*	Pig – healthy
SA 41*	*Escherichia coli*	Pig – healthy
SA 42*	*Escherichia coli*	Pig – healthy
SA 43*	*Escherichia coli*	Pig – healthy
SA 44*	*Escherichia coli*	Pig – healthy
SA 45*	*Escherichia coli*	Pig – healthy
SA 46*	*Escherichia coli*	Pig – healthy
SA 72*	*Escherichia coli*	Pig – healthy
SA 73*	*Escherichia coli*	Pig – healthy
SA 18	*Escherichia coli*	Pig – diarrhoea
SA 20	*Escherichia coli*	Cow – diarrhoea
SA 22	*Escherichia coli*	Cow – diarrhoea
SA 25	*Escherichia coli*	Pig – diarrhoea
SA 26	*Escherichia coli*	Pig – diarrhoea
SA 27	*Escherichia coli*	Pig – diarrhoea
SA 28	*Escherichia coli*	Human – Sepsis
SA 34	*Escherichia coli*	Dog – Sepsis
SA 58	*Escherichia coli*	Human – Sepsis
SA 63	*Escherichia coli*	Lab strain J53
SA 102	*Escherichia coli*	Pig – diarrhoea
SA 103	*Escherichia coli*	Pig – diarrhoea
SA 104	*Escherichia coli*	Pig – diarrhoea
SA 107	*Escherichia coli*	ATCC 25922
SA 118	*Escherichia coli*	Cattle – carriage STEC
SA 120	*Escherichia coli*	Cattle – carriage STEC
ETEC 24	*Escherichia coli*	Pig – diarrhoea
ETEC 28	*Escherichia coli*	Pig – diarrhoea
ETEC M10	*Escherichia coli*	Pig – diarrhoea
Com 20	*Escherichia coli*	Pig – healthy
Com 78	*Escherichia coli*	Pig – healthy
Com 119	*Escherichia coli*	Pig – healthy
Com 132	*Escherichia coli*	Pig – healthy
Com 151	*Escherichia coli*	Pig – healthy
APLMAR WA3	*Escherichia coli*	Pig – healthy
APLMAR WA7	*Escherichia coli*	Pig – healthy
APLMAR WA13	*Escherichia coli*	Pig – healthy
APLMAR WA16	*Escherichia coli*	Pig – healthy
APLMAR WA20	*Escherichia coli*	Pig – healthy
SG 84	*Escherichia coli*	Seagull – healthy
SA 2	*Salmonella enterica*	Cat – diarrhoea
SA 89	*Staphylococcus aureus*	Pig – Healthy
SA 90	*Staphylococcus aureus*	Pig – Healthy
SA 108	*Staphylococcus aureus*	ATCC 25923
SA 101	*Staphylococcus epidermis*	Dog – skin infection

SA 84	*Streptococcus suis*	Pig – Meningitis
SA 86	*Streptococcus suis*	Pig – Meningitis
SA 105	*Enterococcus faecalis*	ATCC 29212
SG 28	*Enterococcus gallinarum*	Seagull – healthy
E104	*Enterococcus hirae*	Pig – healthy
E112	*Enterococcus durans*	Pig – healthy
E107	*Enterococcus faecium*	Pig – healthy
E115	*Enterococcus faecium*	Pig – healthy
E119	*Enterococcus faecium*	Pig – healthy
SA 6	*Enterbacter cloacae*	Human – healthy
SA 109	*Pseudomonas aeruginosa*	ATCC 27853
SA 1	*Citrobacter frendii*	Human – healthy
SG 77	*Aeromonas veronii*	Seagull – healthy
SG 40	*Proteus mirabillis*	Seagull – healthy

*Extended-spectrum cephalosporin resistant isolate

Prior to this project all isolates were stored at -80 °C in 1 mL of brain heart infusion broth (BHI) (Thermo Fisher Scientific, Australia) with 20% glycerol (Ajax finechem, Australia). ESC resistant *E. coli* isolates were subcultured onto blood agar plates (Micromedia, Australia) and incubated at 37 °C for 24 hours. To ensure pure colonies were used for experiments, a single colony was subcultured onto another blood agar plate and incubated at 37 °C for 24 hours. Bacterial cultures were harvested from the subcultured plate using a 10 µL loop taken and suspended in BHI broth with 20% glycerol in a 2 mL cryotube (Sarstedt, Germany). The suspension was vortexed and stored at -80 °C. This stock was used for the project. All other isolates were cultured from original stocks stored at -80 °C. Cultures used in all experiments were recultured from these -80 °C stocks onto Luria-Bertani (LB) (Thermo Fisher Scientific, Australia) agar plates using a 10 µL loop. The plates were incubated overnight at 37 °C.

2.3 Broth Microdilution

Broth microdilution was performed using ceftriaxone (Sigma Aldrich, Australia) to reconfirm ESC resistance of previously isolated ceftiofur (ESC) resistant bacterial isolates that were stored at -80 °C. The reason for this is because ESC resistance encoding plasmids can be lost after freezing at -80 °C. The minimum inhibitory concentration (MIC) was determined by the lowest concentration of antibiotics which inhibited bacterial growth. To ensure sterility all preparation work was conducted inside a Class II Biological Safety Cabinet and microtitre plates were only opened inside the cabinet. The 96-well polystyrene round bottom microtitre plates (Thermo Fisher, Australia) were labelled with column 1 used as a negative control to confirm the sterility of the Mueller Hinton II broth (MH) (Thermo Fisher, Australia) (Figure 4). Column 2 was a positive control to confirm growth of bacterial isolates with only MH broth and inoculum added to this column (Figure 4). All other columns received broth, inoculant and varying concentrations of antibiotics with two-fold dilutions of antibiotics across the columns (Figure 4).

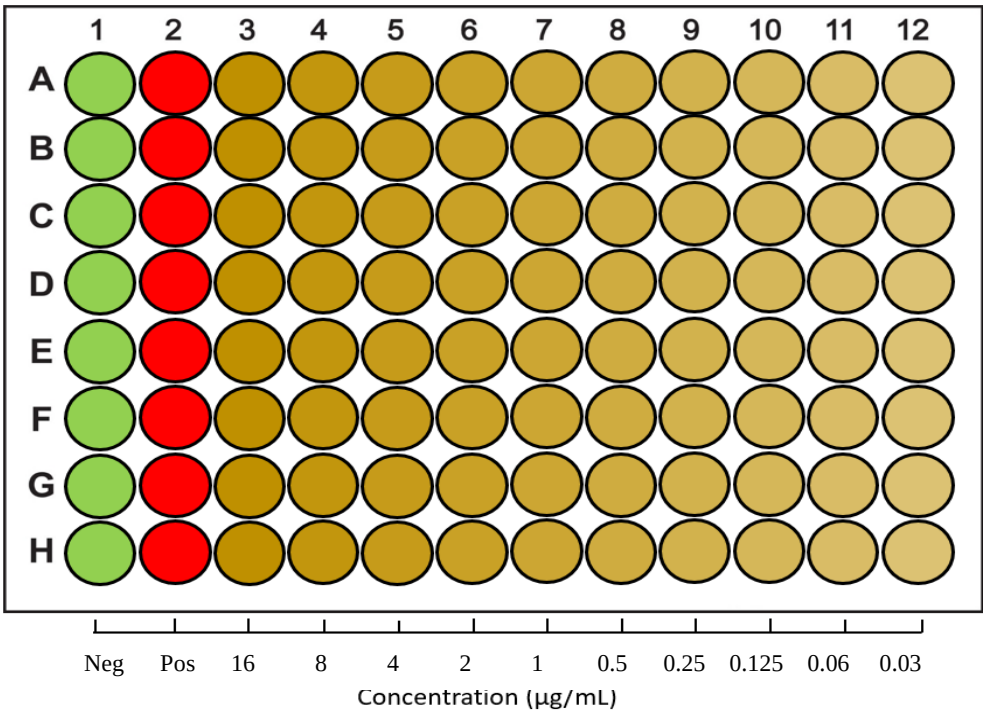

Figure 4. Schematic diagram of broth microdilution plates for susceptibility testing with labelled ceftriaxone concentrations.

Neg: Negative control. Pos; Positive control.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline concentrations for broth microdilution resistance testing were used to calculate the concentration of ceftriaxone required. The MIC breakpoints from EUCAST are 2 µg/mL for ceftriaxone.

For serial dilutions, 90 µL MH II broth was aliquoted into all columns using an electronic multichannel pipette. All wells in column 3 received 90 µL of 16 µg/mL ceftriaxone (Sigma Aldrich, Australia), the solution was pipetted up and down to mix and 90 µL transferred to the next column. This serial dilution step was repeated to columns 3-12 with the 90 µL of the last column discarded. This resulted in each column being diluted in a two-fold dilution series.

The inoculation stage standardizes the bacterial concentration used to inoculate the plates. The required concentration following the EUCAST guidelines is 5 x 10^5 CFU/mL. The acceptable range is 2 - 8 x 10^5 CFU/mL. Bacterial isolates were recultured from frozen stocks on blood agar plates 48 hours prior to the broth microdilution. The plates were incubated at 37 °C for 24 hours. To ensure pure colonies a single colony was re-plated onto blood agar and incubated at 37 °C for 24 hours. Single colonies (1-3) of similar morphology were suspended from the plate into 2 mL of saline (0.9% w/v NaCl). The turbidity of this solution was compared to the turbidity of the calibrated McFarland 0.5 standard (Thermo Fisher, United States). The turbidity was adjusted through suspension of more colonies or by dilution with 0.9% w/v saline. The solution was used within 15 minutes to prevent bacterial growth occurring before incubation, as per EUCAST guidelines. The solution was then diluted 1:20 in MH II broth to reach a bacterial concentration of 5 x 10^6 CFU/mL. This was achieved via addition of an aliquot of 25 µL of bacterial suspension into 475 µL of MH II broth. Each well was inoculated with 10 µL of the diluted bacteria in MH II broth, with the exception of column 1 (negative control). When pipetting, the solution was mixed thoroughly by pipetting up and down multiple times. After inoculation the plates were incubated for 24 hours at 37 °C in an ambient air incubator. The results were read by eye with comparison against the control as per EUCAST guidelines. Results were recorded as growth or no growth observed on the bottom of each well.

2.4 Chemicals, Equipment and Media

All chemicals, reagents and equipment used in this project are listed in Appendix I. The method for preparation of all media used in this project are listed in Appendix II.

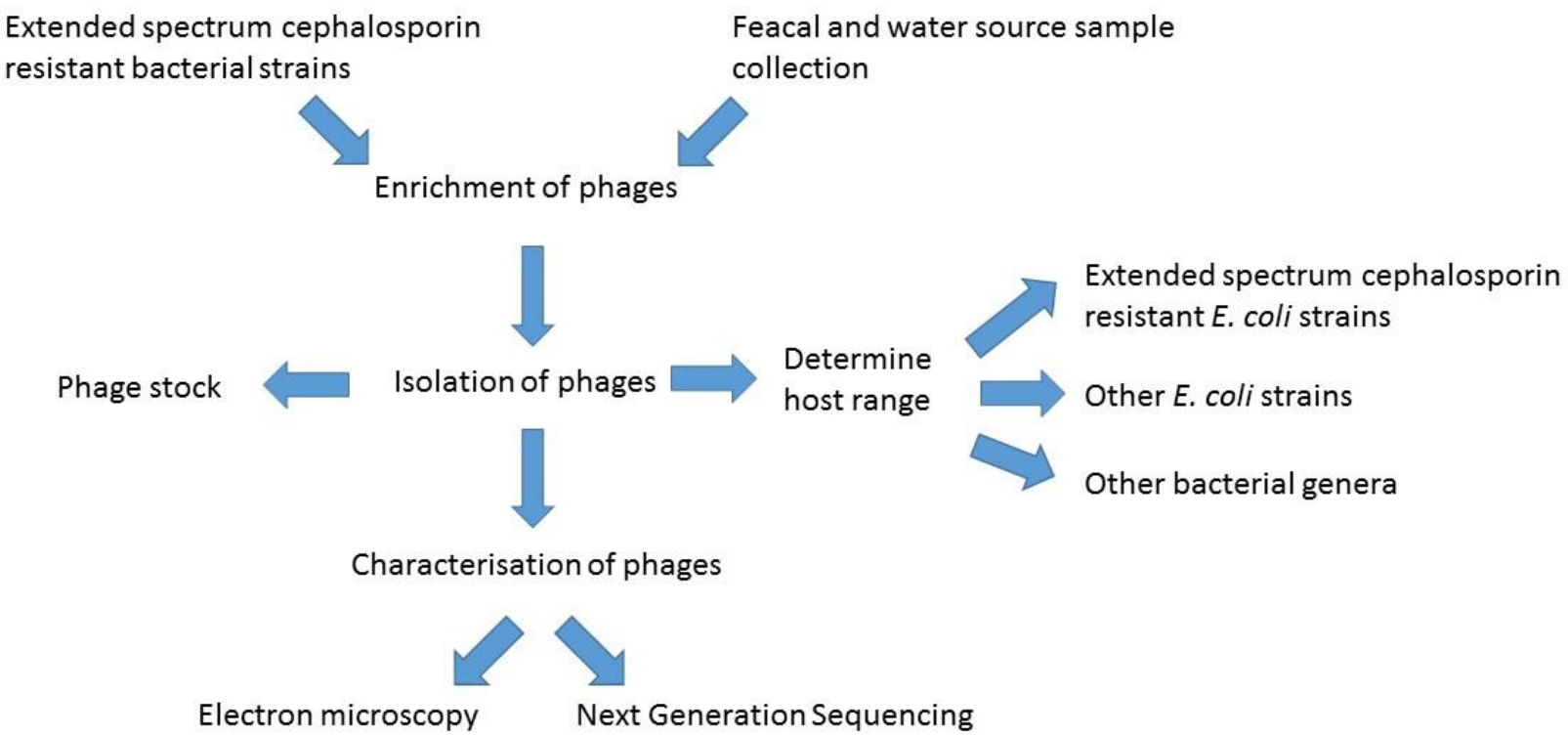

Figure 5. Flow chart describing the experimental design for the isolation and characterisation of phages with lytic activity against ESC resistant *E. coli* isolates.

2.5 Isolation of Phages

Phages with lytic activity against ESC resistant *E. coli* isolates were isolated from source samples using the following method. The first step was enrichment of the phages through the incubation of source samples and the target bacterial isolates in broth. Phages were subcultured and serial dilutions of the phage preparation were conducted in order to isolate a single phage plaque. The lytic activity of all phages was tested against ESC resistant, commensal and pathogenic *E. coli*, as well as other bacterial genera to determine host specificity. Electron microscopy and NGS were conducted to characterise the phages (Figure 5).

2.5.1 Phage Enrichment

Faecal samples were suspended in SM buffer at a ratio of 1:10 and stirred using a magnetic stirrer for 24 hours at 4 °C. The suspensions were centrifuged at 4000 g for 10 mins and then filtered using a 0.45 µm syringe driven membrane filter unit. An equal volume (50 mL) of the filtrate and 2x LB broth were aliquoted into conical flasks. The solution was inoculated with a single colony of the different ESC resistant *E. coli* isolate selected for isolation of phages. Samples were incubated at 37 °C for 18 hours on an orbital shaker at 80 rpm. The solution was centrifuged at 4000 g for 10 mins then filtered through a 0.45 µm membrane filter. The collected lysate was immediately used for phage isolation (as outlined below) with the remainder stored as stock at 4°C.

2.5.2 Phage Isolation

Phage lysates were spot tested onto lawn plates of their host bacterial isolation isolates. Bacterial isolates for isolation of phages and host range tests were prepared from frozen stocks with a 10 µL loop of frozen stock suspended in 3 mL of LB broth. This was incubated on an orbital shaker at 220 rpm for 5 hours at 37 °C. After incubation, 1 mL of the bacterial broth was dispensed onto a LB agar plate and the plate swirled to ensure even coverage, followed by removal of excess broth. The plates were allowed to dry, then four 20 µL drops of phage lysate were applied to a lawn plate of the target bacterial isolate. The plates were allowed to dry and were then incubated for 24 hours at 37 °C to determine the lytic activity of phages.

Phage growth was indicated by the formation of phage plaques (areas of bacterial lysis), with a section of the plaques formed harvested using a sterile pasteur pipette and suspended into a solution containing 1 mL of SM buffer and 25 µL of chloroform (Sigma Aldrich, Australia). The samples were held at room temperature for several hours.

Ten-fold dilution series were conducted to ensure isolation of a single phage. This was achieved using a 96-well polystyrene flat bottom plate (Thermo Fisher, United States). For each plaque suspension 90 µL of Luria-Bertani broth was added to seven wells in a row with 10 µL of plaque buffer solution added to the first well. From here the solution was mixed by pipetting up and down and 10 µL transferred to the next well. This was repeated for all wells containing LB broth with the 10 µL of the last well transferred to a waste bottle. LB agar plates were divided into eight sections and a lawn plate of the corresponding bacterial isolate were prepared as above. The phage solutions were dispensed onto the plate in 15 µL volumes, in order of decreasing concentrations and the plate allowed to air dry. The plates were incubated for 24 hours at 37 °C. Single plaques were harvested using a sterile pasteur pipette as described above.

2.5.3 Phage Stock Preparation

A bacterial broth was incubated for each isolate as described (Section 2.5.2). Aliquots of 100 µL of the corresponding bacterial isolate to each phage was added into a 0.5 mL microcentrifuge tube. To this an aliquot of 100 µL of the harvested phage in buffer was added. These were incubated together for 20 mins at 37 °C. After incubation the 200 µL of bacteria and phage was aliquoted into 3 mL of soft agar. The agar was mixed and poured onto a LB agar plate covering the surface. The agar was allowed to harden and the plates were incubated for 16-18 hours at 37 °C.

A solution of 10 mL SM buffer and 200 µL chloroform was prepared for each plate that was incubated overnight. This was mixed and poured on top of the soft agar. The plates were then stored at 4 °C for several hours and agitated manually every hour. The supernatant was then extracted and aliquoted into 15 mL centrifuge tubes. The samples were centrifuged at 4000 g for 10 mins and the supernatant was filtered through a 0.45 µm membrane filter syringe. Phages

were stored by dispensing 1 mL of phage into multiple 2 mL screw cap micro tubes. One micro tube was stored at 4 °C for electron microscopy and DNA extraction whilst the remainder were stored at -80 °C.

To ensure the phage preparation contained a sufficient concentration of phages for electron microscopy and DNA sequencing, 1 mL of the preparation was concentrated tenfold and stored at 4 °C. This was prepared using 500 µL Vivaspin 10 kDa cutoff protein concentrator spin columns (GE Healthcare Life Sciences, Australia). A 500 µL aliquot of the phage preparation was added to the column and centrifuged at 10,000 g for 10 mins. The membrane was washed repeatedly with 50 µL SM buffer and wash was stored at 4 °C.

2.6 Electron Microscopy

Sample particles were fixed onto a formvar grid and allowed to air dry for 5 minutes. Grids were negatively stained using ammonium molydbate. Electron micrographs were captured using a Tecnai G^2 D1237 electron microscope (FEI, United States).

2.7 Host Range

The host range of the phages was determined using spot tests on all bacterial isolates listed in Table 2. Cross-reactivity between the different ESC resistant *E. coli* isolates from the South Australian piggery was conducted first. The isolates were incubated as previously described (2.5.2). The lawn plate was prepared as described in section 2.5.2 using LB agar plates. The lawn plate was divided into a 4x4 grid structure with 10 µL of each phage spotted onto each square. The last square on each plate was used as a negative control with 10 µL of SM buffer dispensed on it. The plates were incubated at 37 °C for 16-20 hours before examination for lysis. This was repeated for all ESC resistant *E. coli* isolates.

Following this the specificity of the phages amongst multiple *E. coli* isolates were tested using the procedure explained above. To determine the potential of the phages to be used in phage therapy the lytic activity of each phage was also tested on various bacterial genera including

commensal and enterotoxigenic *E. coli, Salmonella spp, Streptococcus spp* (on blood agar) and E*nterococcus spp*.

2.8 DNA Extraction

DNA extractions of phages was conducted using two methods. DNA extractions of a small set of samples were conducted using a DNeasy Blood and Tissue Kit (Qiagen, Australia) [72]. The manufacturer recommended protocol was followed for cultured animal cells with the following modifications; the digestion of protein contaminations step was conducted at 55 °C instead of 70 °C and 50 µL of Buffer AE was used for the final illusion instead of 200 µL.

DNA extractions for a large set of samples (greater than 12) were conducted using a MagMAX Viral Isolation Kit (Ambion, Australia), according to the manufacturer's instructions.

2.9 DNA Quantification

DNA extracts were quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Australia) with a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Australia). The concentrations were diluted to the required concentration (0.3 ng/µL, with an accepted range of 0.2-0.4 ng/µL) for the DNA library preparation for NGS. The diluted DNA extracts were used immediately for sequencing preparation.

2.10 MiSeq DNA Library Preparation

The DNA library preparation for sequencing was conducted using the Nextera XT DNA library preparation kit (Illumina, United States) according to the manufacturer's protocol with the exception that the incubation of the tagmentation reaction was extended from 5 minutes to 7 minutes at 55 °C [72]. Library quality was assessed on a LabChip GXII (Perkin Elmer, Australia), before samples were normalised and loaded onto an Illumina MiSeq V3 2x300 flowcell and sequencing performed on the Illumina MiSeq platform [72].

2.11 Bioinformatics

Analysis and annotation of whole genome sequences was conducted using CLC Genomics Workbench V7.7.64 (QIAGEN, Australia), Geneious V9.1.6 [73], Mauve V2.4.0 [74] and Mega7 V7.0.18 [75]. *De novo* assembly of paired reads was performed using CLC Genomics Workbench. Contigs larger than 10,000 base pairs in length were retained for further analysis. Contig sequence homology to known viral sequences was characterized by searching against the BLASTn database. The accession with the highest similarity and query cover was recorded.

Phages were annotated using Phantome (www.phantome.org) and Geneious [73]. Manual annotation was conducted by BLASTp interrogation of translated open reading frames of the unrecognized proteins against all protein sequences on the NCBI database.

Phylogenetic analysis was conducted in Mega7 [75]. The genes were first aligned, selection of the best DNA model for each data set were conducted and used for creation of trees. For the lysin gene, the evolutionary history was inferred by using the maximum likelihood method based on the Whelan and Goldman model [76]. For the DNA polymerase gene, the evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model [77].

Mauve [74] and Mega7 [75] were used to build alignments of both whole genomes and particular protein sequences between the different phages for visual comparison and identification of nucleotide changes.

Sections of the genomes accounting for tail genes were concatenated and aligned for analysis using Geneious [73]. Alignments of each of the major tail protein sequences were also conducted using Mega7 [75].

3. Results

3.1 MIC Value

Antimicrobial susceptibility was performed via broth microdilution to confirm extended-spectrum cephalosporin resistance of *E. coli* isolates SA35-46 and 72-73. All tested *E. coli* isolates demonstrated resistance to ESC with the minimum inhibitory concentration of all isolates being >8 µg/mL (Table 3).

In all plates the negative and positive controls worked successfully with no growth present in the negative control and growth in all positive controls. This demonstrated the broth sterility and viability of all isolates. The EUCAST MIC breakpoint for ceftriaxone is 2 µg/mL. The ATCC *E. coli* control 25922 should be susceptible to ceftriaxone at this concentration as confirmed in Table 3, with the MIC being 0.5 µg/mL.

Table 3. Minimum inhibitory concentration values of ESC resistant *E. coli* isolates against ceftriaxone.

Isolate number	**Ceftriaxone (µg/mL)**
SA35	>8
SA36	>8
SA37	>8
SA38	>8
SA39	>8
SA40	>8
SA41	>8
SA42	>8
SA43	>8
SA44	>8
SA45	>8
SA46	>8
SA72	>8
SA73	>8
ATCC 25922	0.5

3.2 Phage Enrichment

In order to determine the presence of phages specific to ESC resistant *E. coli* bacterial isolates, phages were isolated and purified through enrichment using these bacterial isolates. The spot tests of the phage lysates onto target microorganisms did display strong lytic activity against the ESC resistant *E. coli* host isolates (Figure 6). Faecal samples S1, S2 and S3 were enriched in bacterial isolates SA35, 36, 72 and 73. The presence of phages were detected in S1, S2 and S3 enriched in isolate SA35, 72 and 73 and S2 and S3 enriched in isolate SA36 (Table 4). There was no phage lysis in three of the source sample and bacterial isolate combinations; faecal samples S1 enriched in isolate SA73 and S2 and S3 in isolate 72.

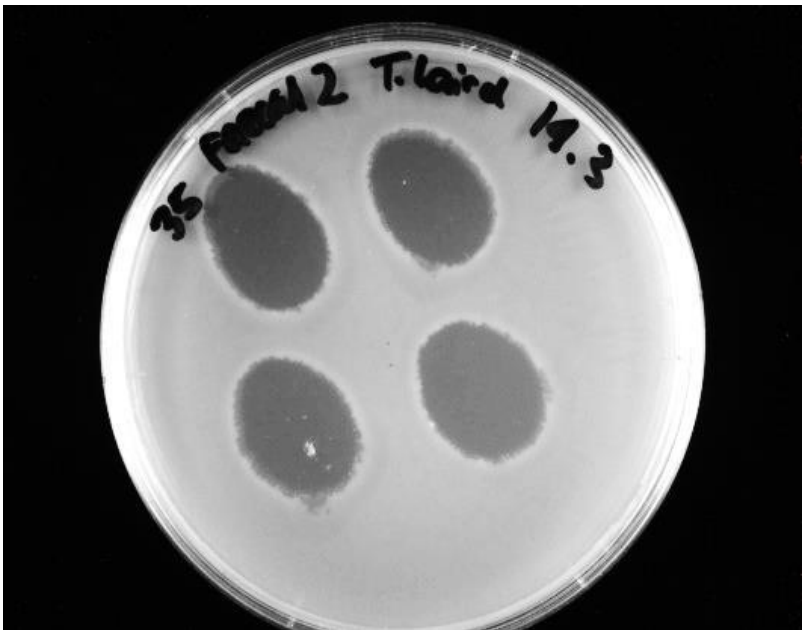

Figure 6. Spot test of phage enrichment from faecal sample S2 and isolate SA35 onto lawn plate of ESC *E. coli* isolate SA35 demonstrating four plaques of strong lytic activity.

A larger bacterial collection range was used for isolation of phages from faecal samples from pigs at Murdoch University with faeces enriched in *E. coli* isolates SA10, 18, 25, 26, 35, 36, 72, 73, 102, 103 and 104. Phages were only isolated from enrichments with isolate SA35, showing the presence of only phages with lytic activity against the ESC resistant *E. coli* (Table 1). The water samples sourced from Murdoch University swampland were enriched in broths with the

ESC resistant *E. coli* isolate SA35 and ATCC *E. coli* strain 25922. Five phages were isolated from the water samples. All phages were isolate using the ATCC *E. coli* strain 25922, with no phages isolated with lytic activity against *E. coli* SA35 (Table 4).

Table 4. Isolated phage IDs showing the bacterial host strain and source sample used in the phage enrichment step.

Phage ID	**Bacterial isolate**	**Sample**
1-4	SA35	S1
5-8	SA35	S2
9-12	SA35	S3
13	SA36	S2
14, 15	SA72	S1
16-23	SA72	S2
24, 25	SA73	S3
26-33	SA35	M1
34-38	ATCC 25922 *E. coli* strain	M2

S1: faecal matter 1 from South Australia, S2: faecal matter 2 from South Australia, S3: faecal matter 3 from South Australia, M1: faecal matter from Murdoch University, M2: water samples from Murdoch University swampland.

3.3 Phage Isolation

After phage enrichment, individual phage plaques were isolated for purification of the phage preparation. The ten-fold dilution series showed a decrease in the size of bacterial plaques in reducing phage concentrations (Figure 7). The dilutions resulted in single plaque formation in the 10^{-5}, 10^{-6} and 10^{-7} dilutions (see section -5, -6 and -7 Figure 7), with these single plaques being used for phage harvesting. Some dilution series were repeated due to single colonies not being evident or separated. These were successful following repetition, with a total of 38 phage plaques isolated from the three source samples against *E. coli* isolates SA35, 36, 72, 73 and ATCC *E. coli* strain 25922.

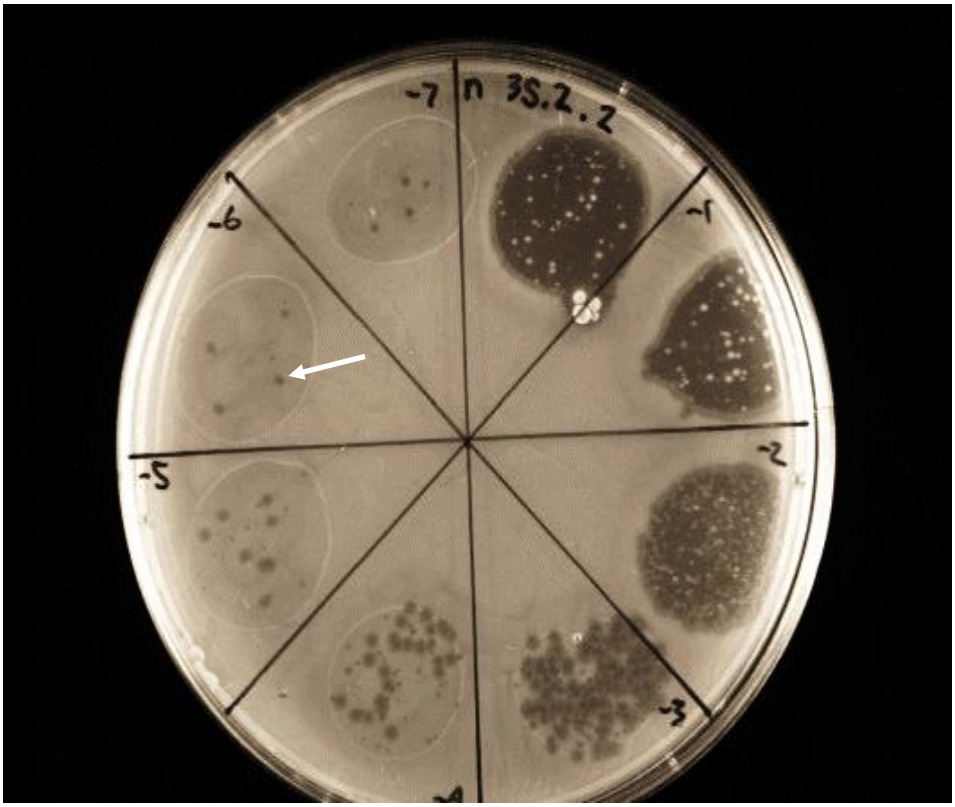

Figure 7. Ten-fold dilution series of phage lysate 6 on lawn plate of ESC *E. coli* isolate SA35 for phage plaque purification, with decreasing concentration of phage in a clockwise direction from n being the original concentration PFU/mL to section -7 being 10^{-7} PFU /mL.

The diameter of plaques from the serial dilutions varied significantly between phages (see in Figure 8). Figure 8 shows a six-fold larger burst size over phage 40 (1100µm) compared to phage 35 (180µm). Picking of the phage plaques was difficult for the phages with smaller burst size, in some cases requiring a dissection microscope, and may have accounted for the loss of lytic phages in some samples.

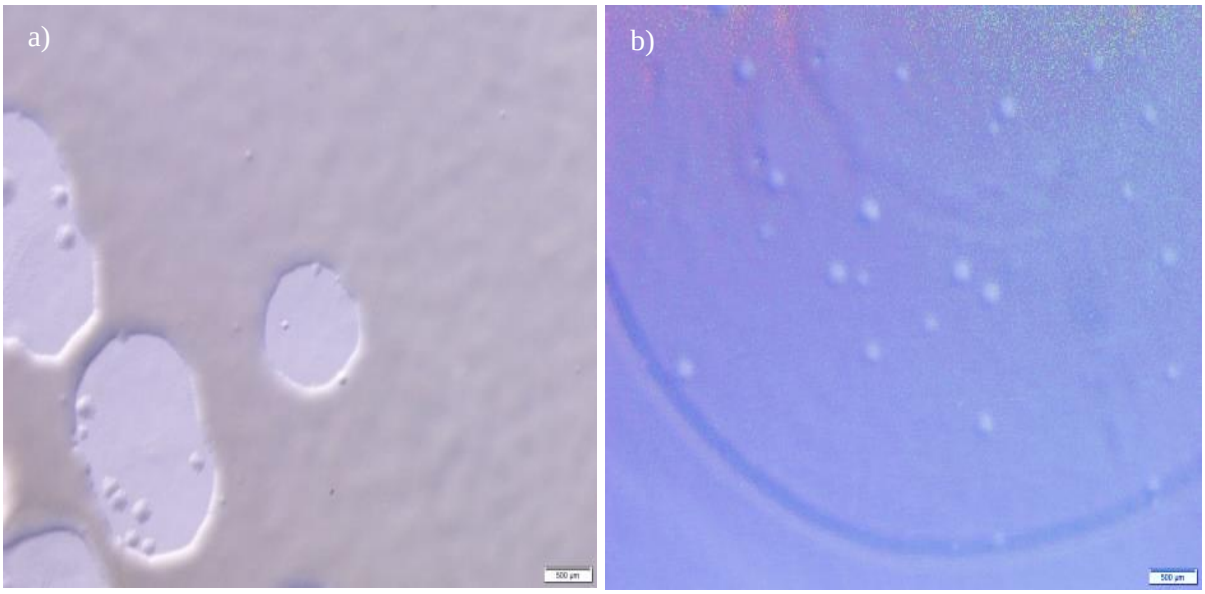

Figure 8. Comparison of bacteriophage single plaque size variation on lawn plate of the host strain ATCC *E. coli* 25922 between phage 40 (a) and phage 35 (b) using light microscopy

Scale bar = 500 µm. a) Burst size = 1100µm b) Burst size = 180µm.

The phages were then purified and harvested for stock using the soft agar method (Section 2.5.3). The phage lysis differed between phages in this soft agar process, with some showing strong lysis of the bacterial lawn plate and therefore a high amount of phage for harvesting, and others demonstrating no lysis of the bacterial lawn plate preventing these phages from being harvested, this may be due to the small burst size as mentioned in the above paragraph. Repetition of this process didn't affect the results with phages 16, 18, 19, 20, 21 and 22 still showing no phage growth. Plaques of these phages from the first spot test were taken and the serial dilution and soft agar processes repeated with no success of phage plaque formation.

Phage purification was successfully achieved from 32 of the 38 plaques isolated. Six of the plaques did not show bacterial lysis during the harvesting of the phage and could not be

isolated. To confirm that the harvested phages remained active and were taken up into the SM buffer, spot tests on the host isolates were conducted. Figure 9 demonstrates the lytic activity of phages 1-12 on the host isolate SA35. Phages 13-15 were isolated on a different isolate and therefore were not expected to lysis SA35. Phages 14-15, 17, 23 and 35-36 lost lytic activity against the host isolate which may be due to an issue with the small size of the single plaques from the dilution series.

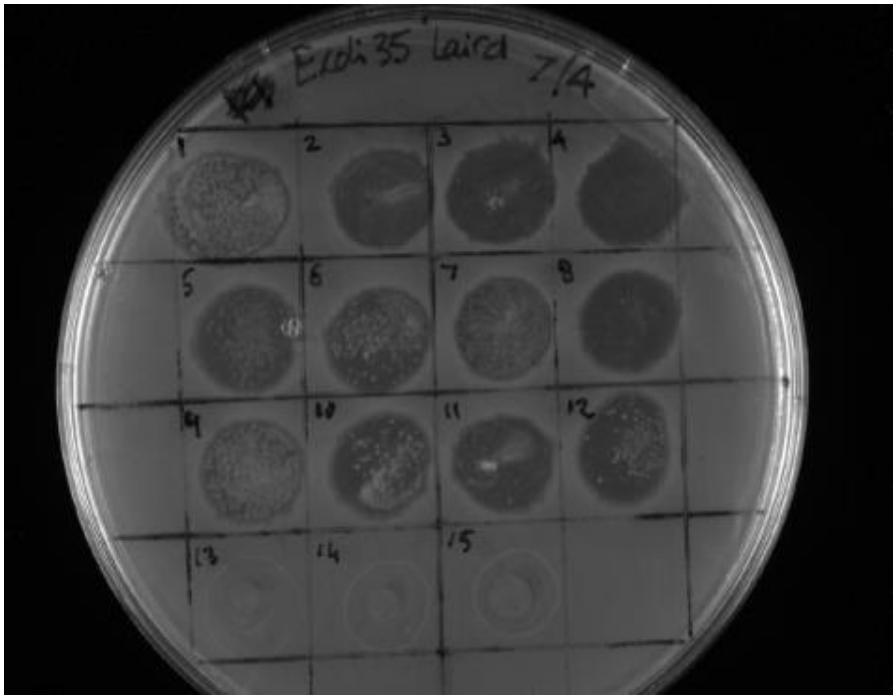

Figure 9. Spot test of phages 1-15 on lawn plate of ESC *E. coli* isolate SA35 with lysis of the host isolate for phages 1-12 and no lysis of SA35 from phages 13-15 which were isolated on SA36.

3.4 Host Range Determination

The host range of each phage was determined against all the ESC resistant *E. coli* isolates (Table 5). This was determined in order to see the potential coverage of single and combined phages against the ESC resistant isolates. The majority of phages had a small host range with 20 (74%) phages lysing only one bacterial isolate, two (7%) phages lysing two bacterial isolates and two (7%) lysing three bacterial isolates. Three (11%) phages showed a larger host range with phage 30 lysing five ESC resistant *E. coli* isolates and phage 26 and 27 lysing nine ESC resistant *E. coli* isolates.

Further host range tests were completed on all phages against a large collection of bacteria compromising of commensal *E. coli* from pigs, diarrhoea inducing *E. coli* and other bacterial genera (Table 6). These tests were conducted in order to determine whether the isolated phages had lytic activity against common gut microflora species. Of all bacterial isolates tested, only three *E. coli* isolates were lysed by the phages, with one isolated from swine with diarrhoea (SA25), one isolated from healthy swine from Western Australia (APLMAR-WA16) and the other strain being a sodium azide resistant laboratory strain.

Table 5. Host range of all isolated bacteriophages against ESC resistant *E. coli.*

Phage Number	**Bacterial Isolate Identification**													
	SA35	SA36	SA37	SA38	SA39	SA40	SA41	SA42	SA43	SA44	SA45	SA46	SA72	SA73
1	+	-	-	-	-	-	-	-	-	-	-	-	-	-
2	+	-	-	-	-	-	-	-	-	-	-	-	-	-
3	+	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	-	-	-	-	-	-	-	-	-	-	-	-	-
5	+	-	-	-	-	-	-	-	-	-	-	-	-	-
6	+	-	-	-	-	-	-	-	-	-	-	-	-	-
7	+	-	-	-	-	-	-	-	-	-	-	-	-	-
8	+	-	-	-	-	-	-	-	-	-	-	-	-	-
9	+	-	-	-	-	-	-	-	-	-	-	-	-	-
10	+	-	-	-	-	-	-	-	-	-	-	-	-	-
11	+	-	-	-	-	-	-	-	-	-	-	-	-	-
12	+	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	+	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	+	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	+	-	-	-	-	-	-	+	-	+
25	-	-	-	-	+	-	-	-	-	-	-	+	-	+
26	+	+	-	+	-	-	+	+	+	+	+	+	-	-
27	+	+	-	+	-	-	+	+	+	+	+	+	-	-
28	+	-	-	-	-	-	-	-	-	-	-	-	-	-
29	+	-	-	-	-	-	-	-	-	-	-	-	-	-
30	+	-	-	-	+	+	-	+	-	-	-	+	-	-
31	+	+	-	-	-	-	-	-	-	-	-	-	-	-
32	+	-	-	-	-	-	-	-	-	-	-	-	-	-
33	+	-	-	-	-	-	-	-	-	-	-	-	-	-
34	-	-	-	-	+	-	-	-	-	-	-	-	-	-
37	-	-	-	-	+	-	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	+	-	-	-	-	+	-	-

+ = lysis of bacteria

- = no lysis of bacteria

Table 6. Host specificity of all isolated phages tested against multiple bacterial genera.

Bacterial species	**Number of isolates tested**	**Number of lysed isolates**	**Isolate lysed (phage ID)**
E. coli	44	3	SA25 (24), SA63 (1-13, 26, 27, 31), APLMAR-WA16 (1)
S. enterica	1	0	
S. aureus	3	0	
S. epidermis	1	0	
S. suis	2	0	
E. faecalis	1	0	
E. gallinarum	1	0	
E. hirae	1	0	
E. durans	1	0	
E. faecium	3	0	
E. cloacae	1	0	
P. aeruginosa	1	0	
C. frendii	1	0	
A. veronii	1	0	

3.5 Phage Characterisation

3.5.1 Electron Microscopy

Morphological characterization of phages 1, 2, 3 and 4 was performed using electron microscopy (EM). Later in the project, morphological characterization of phages 23 and 26 was performed using electron microscopy, in order to capture the structural differences between each family of phages. Electron micrographs of these phages are shown in Figure 10. Phage tails were present in all samples therefore all belong to the *Caudovirales* order with representations of all three tailed phage families captured.

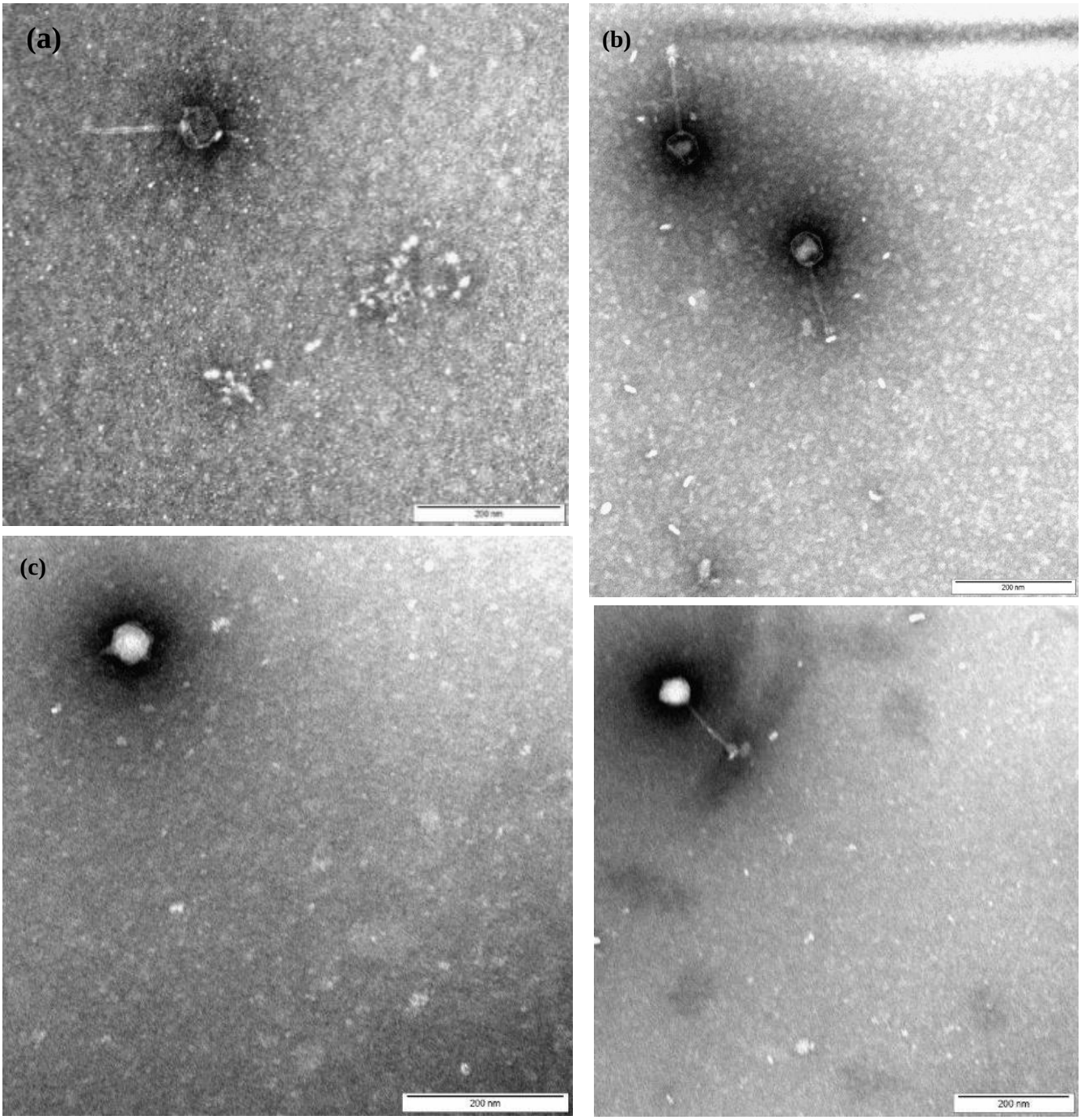

Figure 10. Transmission electron micrographs of bacteriophages against ESC resistant *E. coli* representing all tailed phage family – *Myoviridae, Siphoviridae and Podoviridae*.

Micrographs captured using a Tecnai G^2 D1237 electron microscope. a) Phage 1 – *Myoviridae*. Scale bar = 200nm. b) Phage 4 – *Siphoviridae*. Scale bar = 200nm. c) Phage 23 – *Podoviridae.* Scale bar = 500nm. d) Phage 26 – *Siphoviridae.* Scale bar = 200nm.

The diameter of the head and length of the tail were determined using the measuring tool from the program ImageJ V1.6.0 [78] and additional structures identified. Differentiation into families was determined based on the characteristics as outlined in Table 7. The tail length of phage 1 and the presence of the contractile tail sheath inferred that phage 1 was from the family *Myoviridae.* Phages 4 and 26 tails were non-contractile however of length 121nm and 126nm respectively, placing these phages within the *Siphoviridae* family. The short tail length of 18nm of phage 23 is consistent with the phage belonging to the *Podoviridae* family. The morphology of each phage showed an icosahedral shaped head with a diameter range of 50-56nm.

Table 7. Morphological characteristics captured using transmission electron microscopy of bacteriophages isolated against ESC resistant *E. coli*.

Phage number	Head diameter (nm)	Tail length (nm)	Extra features	Virus Family
1	56	127	Tail sheath and baseplate	*Myoviridae*
4	50	121	Baseplate	*Siphoviridae*
23	50	18		*Podoviridae*
26	52	126	Baseplate	*Siphoviridae*

3.5.2 Whole Genome Sequencing

All 28 phages that were active against ESC resistant *E. coli* were prepared for whole genome sequencing in order to confirm characterization of phages into families and potentially further characterization into genera. Successful sequencing of twenty one phages with contigs over 10,000 base pairs were used for molecular analysis. Comparison of the isolated phages against the NCBI database was performed by using the BLASTn tool from NCBI and confirmed phage 1 belonged to the family *Myoviridae*, with phages 13, 17 and 23 belonging to the *Podoviridae* family and all other phages being *Siphoviridae* (Table 8**)**.

Table 8. Analysis of contig sequences from whole genome sequencing of isolated phages against ESC resistant *E. coli* using BLASTn.

Phage	**Contig length (bp)**	**Top BLAST result**	**Accession Number**	**Genome length (bp)**	**Query (%)**	**Ident**	**Phage family**	**Phage Genus**
1	42413	vB EcoM	KM360178.1	42351	76	93	*Myoviridae*	Unclassified
2	44518	JK06	DQ121662.1	46072	65	83	*Siphoviridae*	Tunalikevirus
3	44518	phiEB49	JF770475.1	47180	54	84	*Siphoviridae*	Tunalikevirus
3	42462	K1ind2	GU196280.1	42765	67	89	*Siphoviridae*	Unclassified
4	42462	K1ind2	GU196280.1	42765	68	89	*Siphoviridae*	Unclassified
4	44518	phiEB49	JF770475.1	47180	54	84	*Siphoviridae*	Tunalikevirus
5	26129	phiEB49	JF770475.1	47180	53	83	*Siphoviridae*	Tunalikevirus
6	44518	JK06	DQ121662.1	46072	64	83	*Siphoviridae*	Tunalikevirus
7	44518	phiEB49	JF770475.1	47180	54	84	*Siphoviridae*	Tunalikevirus
8	44518	phiEB49	JF770475.1	47180	54	84	*Siphoviridae*	Tunalikevirus
9	44519	phiEB49	JF770475.1	47180	54	84	*Siphoviridae*	Tunalikevirus
10	11509	phiEB49	JF770475.1	47180	88	84	*Siphoviridae*	Tunalikevirus
11	44532	phiEB49	JF770475.1	47180	54	84	*Siphoviridae*	Tunalikevirus
13	38842	YpsP-G	JQ965703.1	38288	88	93	*Podoviridae*	T7likevirus
17	38749	YpsP-G	JQ965703.1	38288	88	93	*Podoviridae*	T7likevirus
23	39036	YpsP-G	JQ965703.1	38288	88	93	*Podoviridae*	T7likevirus
26	42462	K1ind2	GU196280.1	42765	67	89	*Siphoviridae*	Unclassified
27	42462	K1ind2	GU196280.1	42765	68	91	*Siphoviridae*	Unclassified
28	42462	K1ind2	GU196280.1	42765	68	89	*Siphoviridae*	Unclassified
29	42462	K1ind2	GU196280.1	42765	68	89	*Siphoviridae*	Unclassified
30	42462	K1ind2	GU196280.1	42765	68	89	*Siphoviridae*	Unclassified
31	42462	K1ind2	GU196280.1	42765	68	90	*Siphoviridae*	Unclassified
32	42462	K1ind2	GU196280.1	42765	68	90	*Siphoviridae*	Unclassified

3.6 Comparative Genome Analysis of the Phages Active Against ESC Resistant *E. coli*

3.6.1 Annotated Phage Genomes

Analysis of phage genomes was conducted after phage genomes were annotated. Due to the high number of unknown proteins returned by the online phage annotation tool Phantome (www.phantome.org), all translated open reading frames of unrecognized proteins were manually searched against the NCBI database using the BLASTp tool and the region annotations updated if homologous proteins were found. The phage genome annotation was completed by grouping the proteins by an arbitrary colour scheme; tail proteins (green), lysin (blue), other known proteins (red) and hypothetical proteins (pink) (Figure 11). The large difference of the genomes representing each of the different families and cluster demonstrates the high rate of mutation of phages resulting in a highly divergent evolution between these groups, with the genes themselves, the number of genes and the size of genes differing between the groups.

Several alignments of whole genomes were constructed with phages within each cluster (excluding *Myoviridae* due to only one genome) using Mauve. These were used for visual comparison of the genomes between families. The alignment of the whole genome sequences of the three *Podoviridae* phages, all isolated from source sample S2 with isolate SA36 and SA72 demonstrated high similarity. In comparison the alignments of the whole genomes of clusters 1 and 2 demonstrated blocks of the similarity in different locations in relation to the start of the genome. These blocks were in the same arrangement and the nucleotides in these genomes were highly similar with the sequences being 99-100% identical. Phages within these clusters have different 5’ termini of each genome however the order of the genes and blocks are the same.

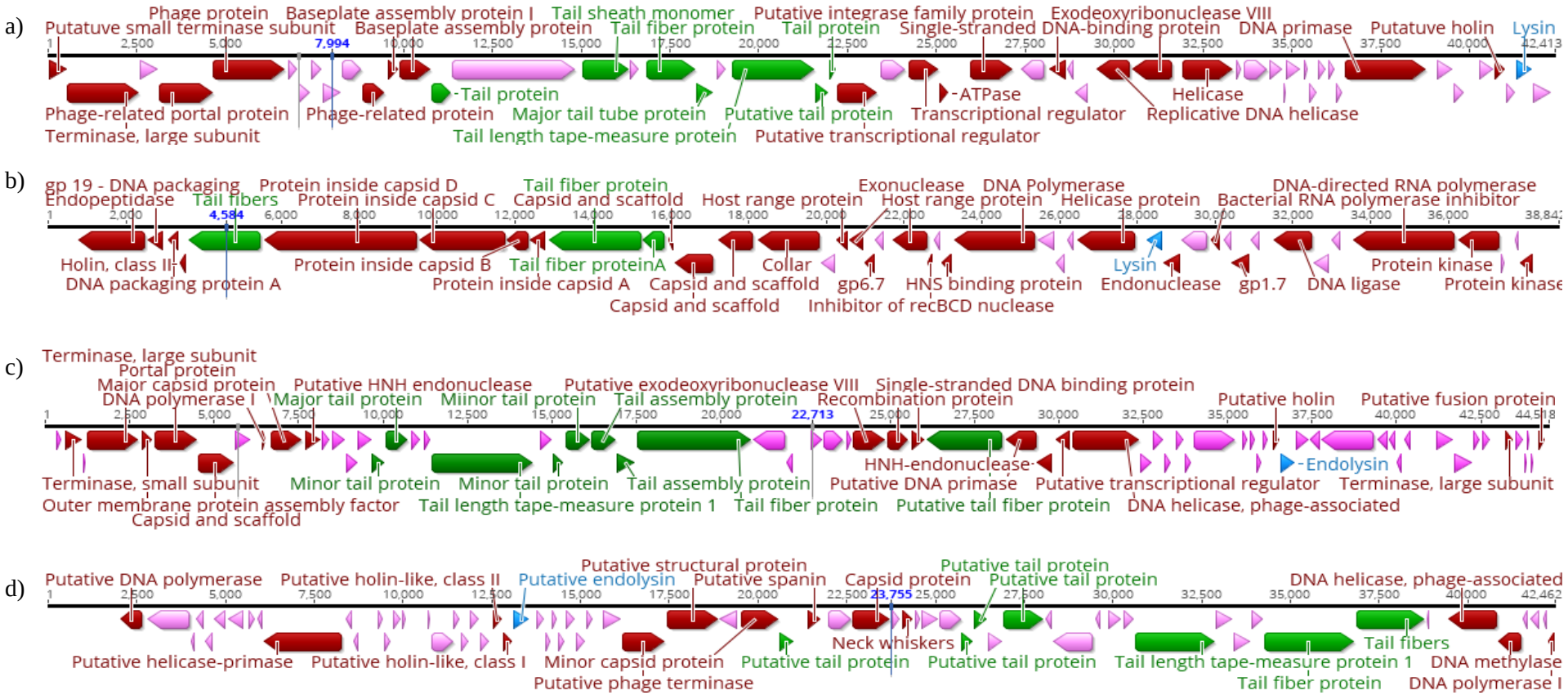

Figure 11. Annotated whole genomes of phages representing the four groups (*Myoviridae*, *Podoviridae*, *Siphoviridae* cluster 1 and *Siphoviridae* cluster 2) with lytic activity against ESC resistant *E. coli* isolates.

a) Phage 1 (*Myoviridae)*, b) Phage 13 (representative of phages 17 and 23 – *Podoviridae*), c) Phage 7 (representative of phages 2, 3.1, 4.2, 5, 6, 7, 8, 9, 10 and 11 – *Siphoviridae* cluster 1), d) Phage 26 (representative of phages 3.2, 4.1, 27, 28, 29, 30, 31 and 32 – *Siphoviridae* cluster 2). Tail proteins are coded green, other known proteins are coded red, hypothetical proteins (HP) are coded pink.

3.6.2 Phylogenetic Analysis of Phages

Two genes, lysin and DNA polymerase, were chosen for phylogenetic studies in order to determine relationships between the three bacteriophage families (Figure 12, Figure 13). Two clusters within the *Siphoviridae* family were identified using the lysin gene for analysis with further analysis using the DNA polymerase gene demonstrating a higher rate of differentiation between phages. These two genes were chosen as they were present across majority of phages. Phage 1, the only phage belonging to *Myoviridae*, did not have a DNA polymerase identified. The lysin and DNA polymerase genes varied in both length across the families.

The phylogenetic tree assembled using the lysin gene (Figure 12) shows three families with 2 clusters within the family *Siphoviridae*; Cluster 1 includes phages 2, 3.1, 4.2, 6, 7, 8, 9, 10 and 11 with cluster 2 including phages 3.2, 4.1, 26, 27, 28, 29, 30, 31 and 32.

The phylogenetic tree of DNA polymerase (Figure 13) indicates that this gene differs within the family *Siphoviridae*, with four different branches corresponding to this family. Cluster 2 showed three different branches despite 99 or 100% identity of the genome, suggesting that the mutations in DNA polymerase may be used to differentiate phages beyond the family *Siphoviridae* into genus or species.

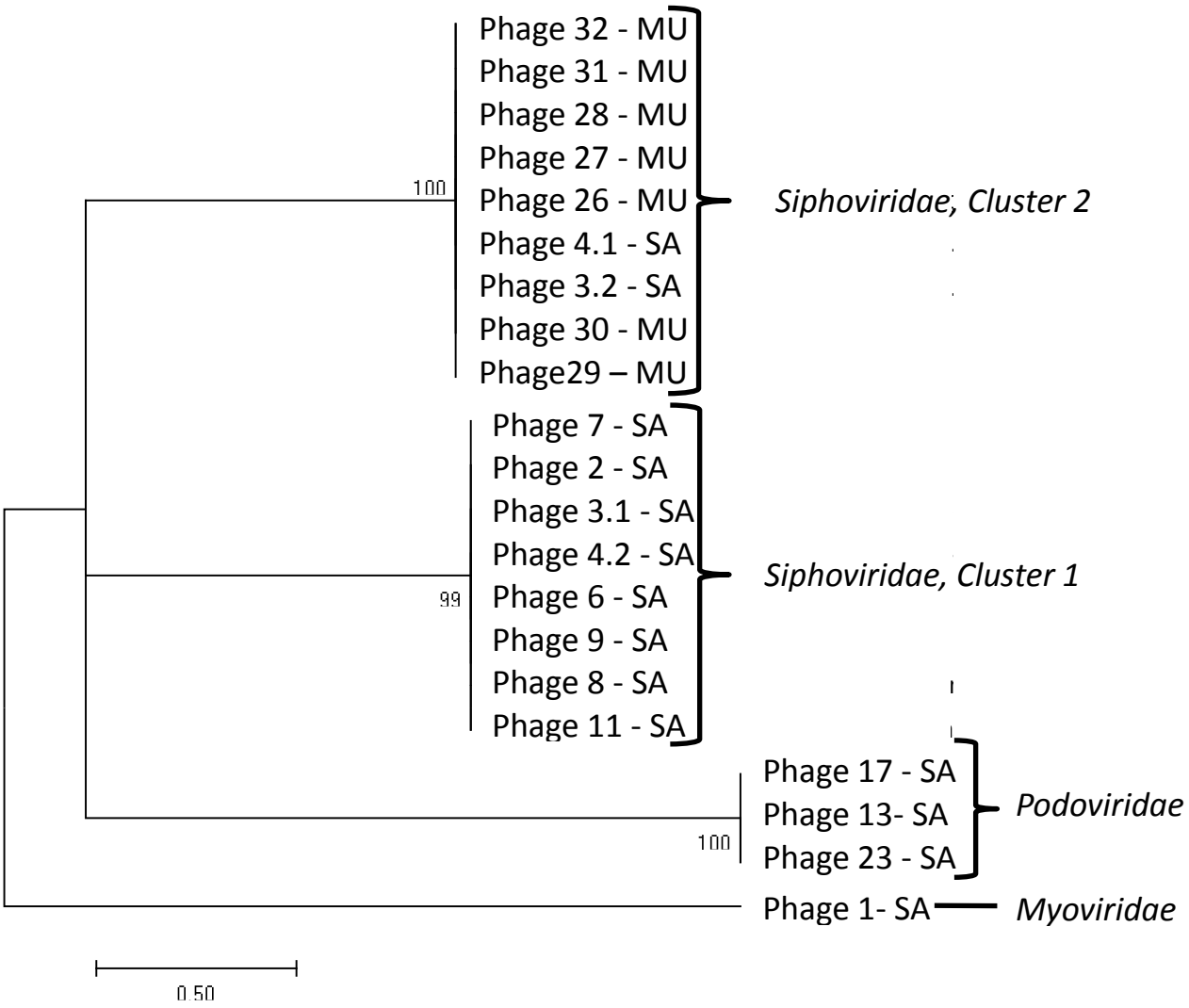

Figure 12. Maximum likelihood phylogenetic tree based on the lysin gene of phages from the three tailed phage families (*Myoviridae, Siphoviridae and Podoviridae).*

Scale bar = number of nucleotide substitutions per site. MU – Phages isolated from Murdoch University faecal material and water samples, SA – Phages isolated from South Australia faecal material. Tree created using WAG model.

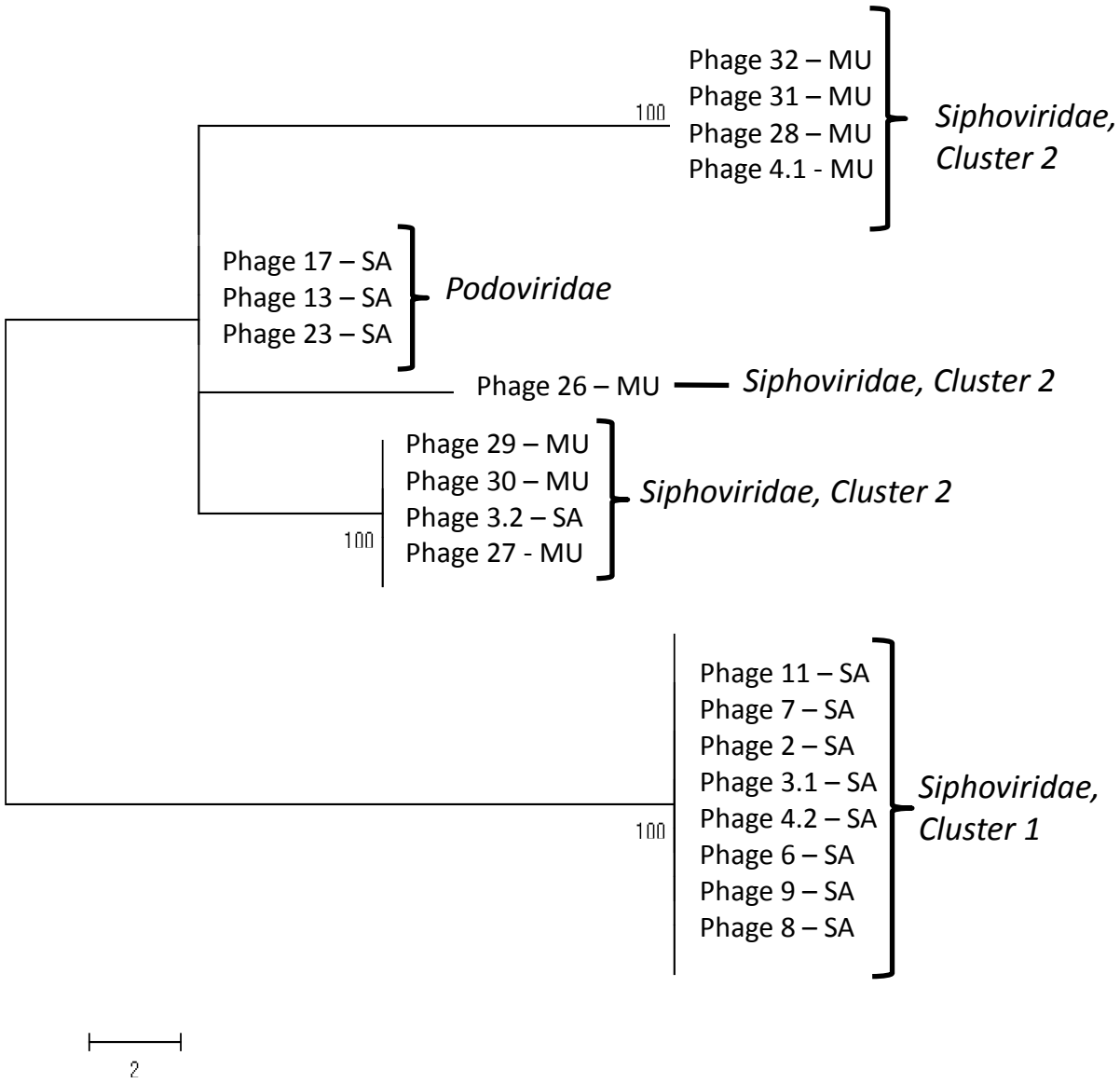

Figure 13. Maximum likelihood phylogeny tree based on the DNA polymerase gene of phages from the three tailed phage families (*Myoviridae*, *Siphoviridae and Podoviridae).*

Scale bar = number of nucleotide substitutions per site. MU – Phages isolated from Murdoch University faecal material, SA- Phages isolated from South Australia faecal material. Tree created using JTT model.

3.6.3 Molecular Comparison of Phage Tail Proteins

Analysis of tail proteins were performed to determine any differences between phages that may account for change in host range between phages, however no results of significance were found.

Each tail protein common within each cluster was aligned and checked for single nucleotide polymorphisms (SNPs). A SNP was found in the tail fiber of Phage 26 leading to a change in amino acid sequence. The adenosine to guanine change resulted in the change of the neutral amino acid histidine to a positively charged arginine. Further investigation using an online interactive protein model portal using RaptorX showed this amino acid change altered the exposure level of the amino acid at the position from 21% to 22.5% exposed [79]. Phyre2, a protein homology/analogy recognition engine, predicted the binding site of the protein was altered from phage 27 to include the arginine [80, 81]. These predictions suggest that the SNP may be involved with the protein binding site and could potentially alter the host range. Despite this Phage 26 showed the same host range of the isolates tested in this project when compared to other phages in this cluster.

Further analysis of the tail proteins was performed by building a concatenated tail protein segment for each phage (Figure 14). These were aligned for visual comparison using Geneious [73]. The concatenated tail segments of each phage cluster highlights the variation of the tail protein genes across the different phage families with the length of these sections being comparable at 11,000bp for *Myoviridae*, 12000bp for *Podoviridae* and 18000bp for *Siphoviridae*. There is variation within the genes present in these regions with *Myoviridae* having no identified tail fiber gene compared to *Podoviridae* and *Siphoviridae*. Each gene also varies in length between the clusters with the length of the tail fiber protein gene being 1,338bp, 2,388bp, 2,277bp and 2,550bp in *Myoviridae*, *Podoviridae*, *Siphoviridae* cluster 1 and *Siphoviridae* cluster 2 respectively. Despite these variations phages from each cluster have shown the same host range, only showing lytic activity of isolate SA35. This suggests that the

different genes may still recognize the same receptor or that a different receptor on this strain is being used for adsorption.

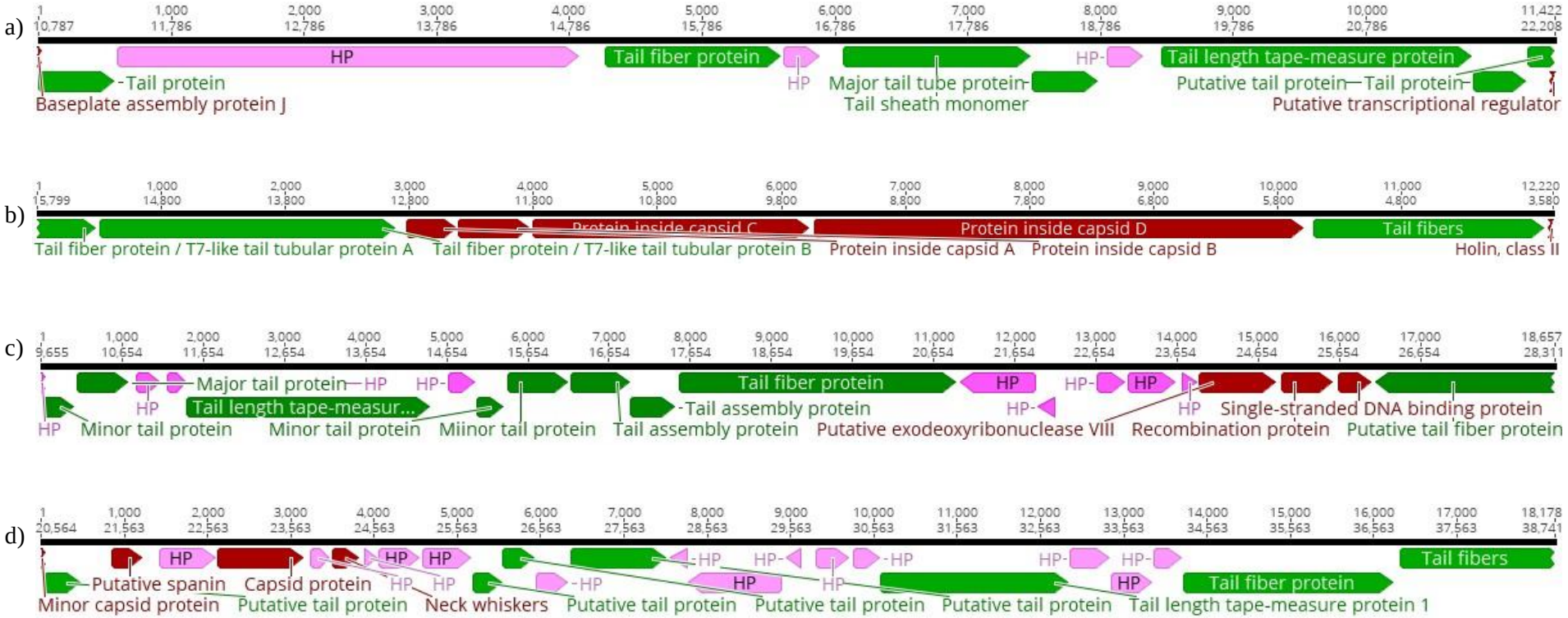

Figure 14. Concatenated tail protein segment of phages that target ESC resistant *E. coli* isolates, the phages represent each family and cluster identified.

a) Phage 1 (*Myoviridae)*, b) Phage 13 (representative of phages 17 and 23 – *Podoviridae*), c) Phage 7 (representative of phages 2, 3.1, 4.2, 5, 6, 7, 8, 9, 10 and 11 – *Siphoviridae* cluster 1), d) Phage 26 (representative of phages 3.2, 4.1, 27, 28, 29, 30, 31 and 32 – *Siphoviridae* cluster 2). Tail proteins are coded green, other known proteins are coded red, hypothetical proteins (HP) are coded pink.

4. Discussion

Antibiotic and multidrug resistant bacteria have emerged and rapidly spread, with antibiotics no longer able to be depended upon as the definitive treatment and prevention tool for diarrhoea induced bacterial infections in the swine industry [2, 12, 16]. Contamination of slaughter meat with bacteria resistant to critically important antimicrobials such as extended-spectrum cephalosporins is also a major concern due to its potential for the transfer of antimicrobial resistant bacteria from animals to humans via the food chain [13, 14]. The use of lytic, target-specific bacteriophages provides a strategy that can be implemented on a large scale to reduce the carriage of antimicrobial resistant bacteria in food-producing animals in order to limit the spread of critically important antimicrobial resistant bacteria [2, 32, 33]. This project aimed to isolate and characterise bacteriophages that target ESC resistant *E. coli* from pigs. The major findings arising from this study are as follows: Firstly, this study has successfully isolated and characterized 21 bacteriophages that specifically target ESC resistant *E. coli* from faecal material of pigs from different sources. Secondly, the morphological and genomic characterization revealed that all bacteriophages belong to the order *Caudovirales* and represent the tailed phage families *Myoviridae, Podoviridae and Siphoviridae.* Molecular analysis identified the DNA polymerase gene as a potential marker for the differentiation of bacteriophages within family groupings. Finally, *Siphoviridae* phages have the same order of genes however different 5' and 3' termini, suggesting these phages undergo headful DNA packaging.

Phages with lytic activity against different ESC resistant *E. coli* isolates were successfully isolated from faecal material sourced from South Australia (ESC resistant *E. coli* isolates present) and Murdoch University. Host specificity testing was conducted on all phages to determine phages with a broad target host range that have minimal lytic activity against other bacterial isolates, for identification of phages for future development into a therapeutic agent. Of the 12 ESC resistant *E. coli* isolates, 11 were lysed by the phages isolated in this study. Phages 26, 27 and 30 lysed a wide range of these isolates with phages 26 and 27 lysing nine

isolates and phage 30 lysing five isolates. These phages show potential for development into a phage therapy for decolonizing pigs from ESC resistant *E. coli*.

Further target specificity of the phages was determined through tests on a number of commensal and pathogenic *E. coli* and other bacterial genera and revealed that the majority of the phages were target specific. The host range testing also revealed that the phages from sources with no use of ceftiofur (pigs and environment) were able to specifically target ESC resistant *E. coli.* This indicates that naturally occurring phages could have specific lytic activity against antimicrobial resistant *E. coli* disproving one of the hypothesis of this project, that phages isolated from faecal material obtained from pigs that were treated with ceftiofur, are more specific than phages from faecal material from non-treated pigs for lysis of ESC resistant *E. coli*. The phages from both samples were highly specific to the ESC resistant *E. coli* isolates with only three other *E. coli* isolates (not resistant to ESCs) also lysed by phages from South Australia (Phage 1-13 and 24) and Murdoch University (26, 27 and 31) and no lysis of bacteria from other genera (Table 6). This demonstrates that the phages isolated from faecal material without the presence of ESC resistant *E. coli* are of similar specificity and faecal material can easily be sourced from outside samples for the isolation of phages. In addition to this, the phages isolated from the South Australian pig farm on average lysed 1.25 (range of 1-3) of the ESC resistant *E. coli* isolates compared to the faeces collected from Murdoch University causing lysis of 3.63 (range of 1-9) isolates (Table 5). This data set not only disproves this hypothesis, it shows that phages isolated from faecal material without ESC resistant *E. coli* isolates has an increased host range of the ESC resistant *E. coli* isolates. Comparison of the specificity between phages isolated from different locations evaluated that phages don't need to be sourced from locations in conjunction with target bacterial isolates.

Phages were characterized using electron microscopy and next generation sequencing due to characterization of phages used in phage therapy being a mandatory requirement [50]. All phages belonged to the order *Caudovirales* with the EM images capturing the presence of tails in all samples tested (Phages 1-4, 23 and 26) (Figure 10). Whole genome sequencing confirmed these classifications into families whilst determining the taxonomy of all other phages. 82.6%,

4.3% and 13% of phages isolated belonged to the family *Siphoviridae, Myoviridae* and *Podoviridae* respectively. Characterisation of isolated phages demonstrated an expected range and proportion of families similar to previous studies with 96% of all bacteriophages belonging to the order *Caudovirales* and 61%, 24.5% and 14% being *Siphoviridae, Myoviridae* and *Podoviridae* respectively [17, 39]. These three phage families have also been previously isolated from swine faecal samples [82-84].

Faecal sample 2 was the only sample containing phages belonging to the *Podoviridae* family. These phages were isolated using ESC resistant *E. coli* isolates (SA36 and SA72). This suggests that faecal sample 2 may have a different phage population and possibly collected from a different pig pen. All phages isolated from the Murdoch University faecal samples belonged to the second cluster of *Siphoviridae* (tree). This second group was also present in the South Australian samples however was the minority with a prevalence of 17% of all *Siphoviridae* isolated. These demonstrate an unexpected difference in phage population between host populations and locations.

In this study the DNA Polymerase gene was identified as a potential marker for phylogenetic analysis. Firstly, molecular analysis of the whole genome sequence of phages that target ESC resistant *E. coli* resulted in the recognition of four groups of phages with one group belonging to each family *Myoviridae* and *Podoviridae* and the family *Siphoviridae* divided into two clusters. This grouping was also supported by phylogenetic analysis using the lysin gene (Figure 12). Previous studies have performed phylogenetic analysis of phages using the major capsid gene and the large terminase gene. This study could not use these genes for phylogenetic analysis due to a major capsid protein not identified in the *Myoviridae* phage with three identified in the *Podoviridae* phages, and a large terminase gene not identified in *Podoviridae* with two identified in phages belonging to *Siphoviridae* cluster 1. Therefore, phylogenetic analysis was conducted using DNA polymerase as one copy of this gene was present in all phages (excluding the one *Myoviridae* phage), and viral polymerase genes are relatively conserved within families (Figure 13). This analysis resulted in the differentiation of *Siphoviridae* cluster 2 into three

separate groups. These groups potentially align with phage genus and species and therefore can be used for classification, with analysis of more phages needing to be conducted to confirm this.

The whole genome alignments of cluster 1 and 2 demonstrated the genes of each phage arranged in the same order, however in different locations in relation to the start of the genome. This change in the start of the genome is due to the type of DNA packaging of the phage. Headful DNA packaging results in genomes of differing lengths and different starting and ending locations. When phage DNA is inserted into the host cell, it can circularize. The terminase protein recognizes a specific site on the genome referred to as the *pac* sequence and starts synthesis of genetic material from this site. This newly synthesised genome is inserted into the capsid of a length of between 102 and 110% of the actual genome length. The synthesis of genetic material continues from the region past the 'end' of the genome, with the next genome packaged now starting at a different base. This cutting and inserting continues until the capsid is full, resulting in genomes of varying lengths and starting points as seen in these two clusters. The whole genome alignment of *Podoviridae* suggests a different DNA packaging method is used such as exact DTR's or cohesive ends, due to the identical alignments [85].

Two issues arose when determining phage lysis of ESC resistant *E. coli* isolates; bacterial contamination and the determination of lysis. To overcome the bacterial contamination 1 mL of phage suspension was filtered using a 0.20 µm filter syringe. This process resulted in the majority of the 1 mL suspension lost due to absorption in the filter and syringe. The next method applied to overcome the contamination was the addition of chloroform to the phage preparation. The chloroform resulted in elimination of bacterial contamination without altering phage lysis. The spot tests with contamination were then repeated. The addition of small amounts of chloroform should be used for future bacterial contamination.

The second issue was the determination of lysis and no lysis of phages on bacterial lawn plates. Some spots showed partial lysis with others showing complete lysis of all bacteria. To differentiate between the levels of lytic activity a scale similar to the study conducted by Kutter (2009) [86], could be implemented. This scale uses a number system from 0 to +4 with 0

showing no lysis and +4 showing complete lysis of bacteria [86, 87]. Future application of the scale method when recording lysis of phage will differentiate the degree of lysis of each phage, helping to identify which phage would have higher therapeutic potential against certain bacterial isolates.

Another issue of the project relates to the concentration of phages for host range testing. When comparing the host range of different phages, there is an expected difference between the genomic sequences. However, phages with different host ranges showed identical genome sequences. This could be due to the concentrations of the phages and the bacteria when conducting spot tests. The ratio of phage and bacteria (MOI) affect the lytic capability of the phage suspension [86, 87]. A previous study comparing the percentage reduction of bacteria against the phage and bacteria concentration demonstrated the importance of the correct MOI for phage lysis of target bacteria. Initially $4.6x10^2$ PFU/mL was incubated for 2 hours with differing concentrations of phages. Only 0.1% of the bacteria survived when incubated with $1.1x10^7$ PFU/mL of phage compared to 98.9% surviving when incubated with $1.8x10^4$ PFU/mL [87]. The significance of the phage concentration was further studied with a log reduction in phage concentration, from $1.5x10^6$ PFU/mL to $1.5x10^5$ PFU/mL, resulting in a 50% reduction in the percentage of bacteria lysed. In theory, phages 26, 27 and 30 may have a higher phage titer than other stocks, resulting in the ability to show lysis of various isolates of bacteria. This change in concentration may also account for the varying amounts of lysis in Figure 3 where phages 1-15 were spotted onto 35 and the variation in plaque sizes of the different phage lysates. MOI calculations were not performed in this study, and this may have had some affect on the results. Future characterization and development of the phages isolated in this study will require titrations and MOI to be performed.

The immediate future direction is the *in-vivo* testing of a successful phage cocktail in pigs to evaluate the efficacy of the phage cocktails in eliminating ESC resistant *E. coli.* Before use, the phage preparation would need to have all endotoxins removed using a commercial kit or an organic solvent method [88]. Endotoxins are the main contaminants of phage preparations from the bacterial host and if not removed, the *in-vivo* testing can cause cell injury and toxic shock to

the animal [89]. Another factor for preparation of a phage therapy for *in-vivo* testing is the survival of the therapy in the acidic levels of the gastrointestinal tract. The stability of phages differs between each phage with majority denaturing at pH 3 [56, 68]. The acidity of the GI tract of swine ranges from pH 1-2 before a meal up to pH 4-5 after a meal, with phage therapy combined into animal feed reducing the effect of the pH on the phages [56]. Another method to further protect phages against the GI tract is the encapsulation of the phages in liposomes [69]. This would further protect and improve the stability of the phage therapy in *in-vivo* trials.

Further study of ESC resistant *E. coli* targeting phages include the combination of phages into a cocktail, efficacy tests of *in vivo* animal trials and molecular analysis to identify the phage recognition binding protein for improvement of phage therapy. Single phages with high therapeutic potential are often combined into phage cocktails to increase the benefit of the phage therapy via an increased host range and decreased rate of bacterial resistance [58, 60, 61]. To create a cocktail with 100% coverage of the ESC resistant *E. coli* isolates in this study, further isolation of phages from faecal matter using isolate 37 as the host isolate for phage enrichment needs to be conducted in order to isolate a phage with lytic activity against isolate SA37. Combination of this phage with the broad host range phages (Phage 26, 27 and 30) isolated will create a cocktail that can theoretically lyse all of the ESC resistant *E. coli* isolates, limiting development of bacterial resistance against the phages [61]. Interaction between the phages may decrease the practical host range of such a cocktail with studies needing to be conducted to determine this [63].

Identification of the recognition binding protein of phages used in phage therapy is highly desirable in optimization of phage therapy as therapies can be targeted to bacteria with the corresponding binding site, or phages with strong lytic activity can be genetically modified to change or extend their host range [90]. This study identified a single nucleotide polymorphism (SNP) in the tail fiber of phage 26, changing the positively charged lysine to a neutral asparagine [90]. The SNP identified in this project didn't result in an altered host range. However studies have successfully identified a recognition binding protein by recognition of a SNP in mutagenesis studies. Le *et al.* (2013) identified ORF84 (putative tail fiber gene) as the

recognition binding protein of phage JG004. This was achieved through genetic analysis of phage JG004 and its mutants with lytic activity of a different range of hosts. Further extensive molecular analysis focusing on the tail fibers and baseplates may results in the identification of the recognition binding protein of phages that target ESC resistant *E. coli*. A potential therapeutic option following this is to have a library of lytic phages which can be mutated on demand using CRISPR-Cas9 to bind to newly isolated resistant bacteria.

Recent development of a novel method for identification of phage recognition binding proteins has shown promise recognizing the proteins by their attachment to a host cell and could also be conducted to identify the recognition binding protein of ESC resistant *E. coli* targeting phages. The method utilizes expression vectors containing phage DNA, transferring them into *E. coli* and probing with fluorescent bacterial hosts. The proteins that attached the host cell are then sequenced. This method allows for the identification of novel recognition binding proteins. Limitations are still present with only bacteria capable of being cultured available to use as the host [91], however it is foreseen that this method could be applied to ESC resistant *E. coli* which are easily cultured.

5. Conclusion

This project has resulted in the isolation of 21 phages that specifically target ESC resistant *E. coli* isolates, and is a significant first step in the process of developing an alternative antimicrobial therapeutic. Phages isolated from sources with and without the presence of ESC resistant *E. coli* isolates show similar specificity towards the ESC resistant *E. coli* strains, demonstrating that phages can be isolated from sources not infected by the target bacterial isolate, can be easily isolated from sources that are easy to sample, and providing promise for use against other antibiotic class resistant *E. coli*. These phages have been morphologically and genetically characterized by EM and NGS, a mandatory requirement for classification of phages by the ICTV and for use in phage therapy. In addition, this study has demonstrated the potential for the DNA polymerase gene to be used for phylogenetic analysis to differentiate phages within family groups, particularly within the *Siphoviridae*.

Future directions for this study include:

1. Retesting of host specificity of phages with controlled phage titre and bacterial concentration (MOI testing).
2. Molecular and mutagenesis studies directed towards identifying the phage recognition binding protein and the protein binding site on the ESC resistant *E. coli* isolates.
3. Phage cocktail preparation and *in vitro* and *in vivo* testing to demonstrate combinatorial efficacy of phages.
4. *In vivo* animal testing to determine the efficacy of the treatment in controlling ESC resistant *E. coli*.

In conclusion, the study has isolated and characterized highly specific bacteriophages that target ESC resistant *E. coli* from sources with and without the presence of these target bacterial isolates, identifying a novel method for the control of ESC resistant *E. coli* isolates within pigs.

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Appendix I

Reagents

5% sheep's blood agar plate	Micromedia, Australia
Agar bacteriological	Thermo Fisher Scientific, Australia
BBL ™ Muller Hinton II broth (cation adjusted)	BD Worldwide, USA
Brain heart infusion broth	Thermo Fisher Scientific, Australia
Ceftriaxone	Sigma Aldrich, Australia
Chloroform	Sigma-Aldrich, Australia
Ethanol	Sigma-Aldrich, Australia
Distilled water	
Gelatin	Ajax Finechem, Australia
Glycerol	Ajax Finechem, Australia
LB broth	Thermo Fisher Scientific, Australia
Magnesium sulphate	UNIVAR, Australia
Molecular grade water	
Potassium chloride	Ajax Finechem, Australia
Potassium phosphate	Ajax Finechem, Australia
Sodium chloride	VWR Chemicals, Belgium
Sodium hydroxide	Ajax Finechem, Australia
Sodium phosphate	Ajax Finechem, Australia
Tris hydrochloride	Amresco, Australia

Equipment and materials

Generic laboratory equipment is listed without manufacturers.

0.45 µm filter	Pall Life Sciences, Australia
0.5 mL microcentrifuge tubes	Sarstedt, Germany
1.5 mL microcentrifuge tube	Sarstedt, Germany
2 mL screw cap micro tube	Sarstedt, Germany
5 mL sterile tubes	Sarstedt, Germany
10 µL loop	Copen Labs, United Kingdom
15 mL FALCON tubes	Sarstedt, Germany
50mm Rapid-Flow 500 mL filter unit	Thermo Fisher Scientific, Australia

50mL falcon tubes	Sarstedt, Germany
96-well polystyrene flat bottom plate w/ lid	Thermo Fisher, United States
96-well polystyrene round bottom plate w/ lid	Thermo Fisher, United States
DNeasy blood and tissue kit	Qiagen, Australia
Electronic pipette, multichannel pipette and tips	Thermo Fisher, United States
LabChip GXII	Perkin Elmer, Australia
MagMAX viral RNA isolation kit	Thermo Fisher Scientific, Australia
Magnetic stand	Ambion, Australia
McFarland 0.5 standard	Thermo Fisher, United States
Nextera XT DNA library preparation kit	Illumina, Singapore
Petri dish	Thermo Fisher Scientific, Australia
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific, Australia
Qubit 2.0 Fluorometer	Thermo Fisher Scientific, Australia
Syringe	Terumo, Australia
Tecnai G^2 D1237	FEI, United States
Thermal cycler	BIO-RAD, Australia
Vivaspin protein concentrator spin column	GE Healthcare Life Sciences, Australia

Appendix II

1. **Preparation of Luria-Bertani broth**

Luria-Bertani (Thermo Fisher Scientific, Australia) broth was prepared by weighing 10 grams of LB broth powder and adding it to 400 mL of distilled water. After mixing the solution was autoclaved at 121 °C for 15 mins. The broth was allowed to cool and stored at 4 °C.

2. **Preparation of 2 x Luria-Bertani broth**

The 2 x Luria-Bertani broth was prepared by weighing 7.5 grams of LB broth powder and adding it into a bottle with 300 mL of distilled water. The broth was autoclaved at 121 °C for 15 mins, then stored at 4 °C until use.

3. **Preparation of Luria-Bertani agar**

Luria-Bertani agar was prepared by weighing 10 grams of LB broth powder and 6 grams of bacteriological agar powder and adding it to 400 mL of distilled water. The agar was autoclaved at 121 °C for 15 mins. Molten agar was poured into sterile petri dishes and the plates stored at 4 °C until use.

4. **Preparation of soft Luria-Bertani agar**

Soft Luria-Bertani agar was prepared by weighing 5 grams of LB broth powder and 6 grams of bacteriological agar powder (Thermo Fisher Scientific, Australia). These were added together into a bottle along with 200 mL of distilled water. The solution was autoclaved at 121 °C for 15 mins then stored at 4 °C. Prior to use, agar was heated in a water bath at 47 °C until it formed a liquid suspension.

5. **Preparation of SM buffer**

SM buffer was prepared by mixing 2.91 grams NaCl (VWR Chemicals, Belgium), 0.61 grams $MgSO_4$ (UNIVAR, Australia) and 0.05 grams of gelatin (Ajax Finechem, Australia). In a separate bottle 3.94 grams of tris-HCl (Amresco, Australia) was added into 25 mL of distilled water and mixed to dissolve. All 25 mL was added to the bottle with the other powders, made

up to 500 mL with distilled water, and the pH adjusted to 7.5 before the solution was sterile filtered through a 50mm Rapid-Flow 500 mL 0.2µm filter unit using a vacuum system.

6. **Preparation of Mueller Hinton broth**

Mueller Hinton broth (Thermo Fisher Scientific, Australia) was prepared by addition of 6.6 grams of MH broth powder to 300 mL of distilled water. To ensure all powder was completely dissolved, the bottle was heated with frequent agitation and boiled for 1 min. The broth was then autoclaved at 116-121 °C for 10 mins, before being stored at 4 °C.

7. **Preparation of PBS buffer**

PBS buffer was prepared by combining 8 g of NaCl (VWR Chemicals, Belgium), 0.2 g of KCl (Ajax Finechem, Australia), 1.44 g Na_2HPO_4 (Ajax Finechem, Australia) and 0.24 g of KH_2PO_4 (Ajax Finechem, Australia) to 1000 mL distilled water and the pH adjusted to 7.4, before autoclaving at 121°C for 15 mins and storage at 4 °C.

8. **Preparation of Brain Heart Infusion broth with 20% glycerol**

Brain heart infusion (BHI) (Thermo Fisher Scientific, Australia) broth was prepared by adding 3.70 grams of BHI broth, and 20 mL of glycerol to 80 mL of distilled water. This was autoclaved at 121 °C for 15 mins then stored at 4 °C.

Appendix III

Figure 15. Annotated genome of phage 1 (Myo*viridae*) with lytic activity against ESC resistant *E. coli*.

Figure 16. Annotated genome of phage 13 (*Podoviridae*) with lytic activity against ESC resistant *E. coli.*

Representative of phages 17 and 23.

Figure 17. Annotated genome of phage 7 (*Siphoviridae* cluster 1) with lytic activity against ESC resistant *E. coli*.

Representative of phages 2, 3.1, 4.2, 6, 8, 9, 10 and 11.

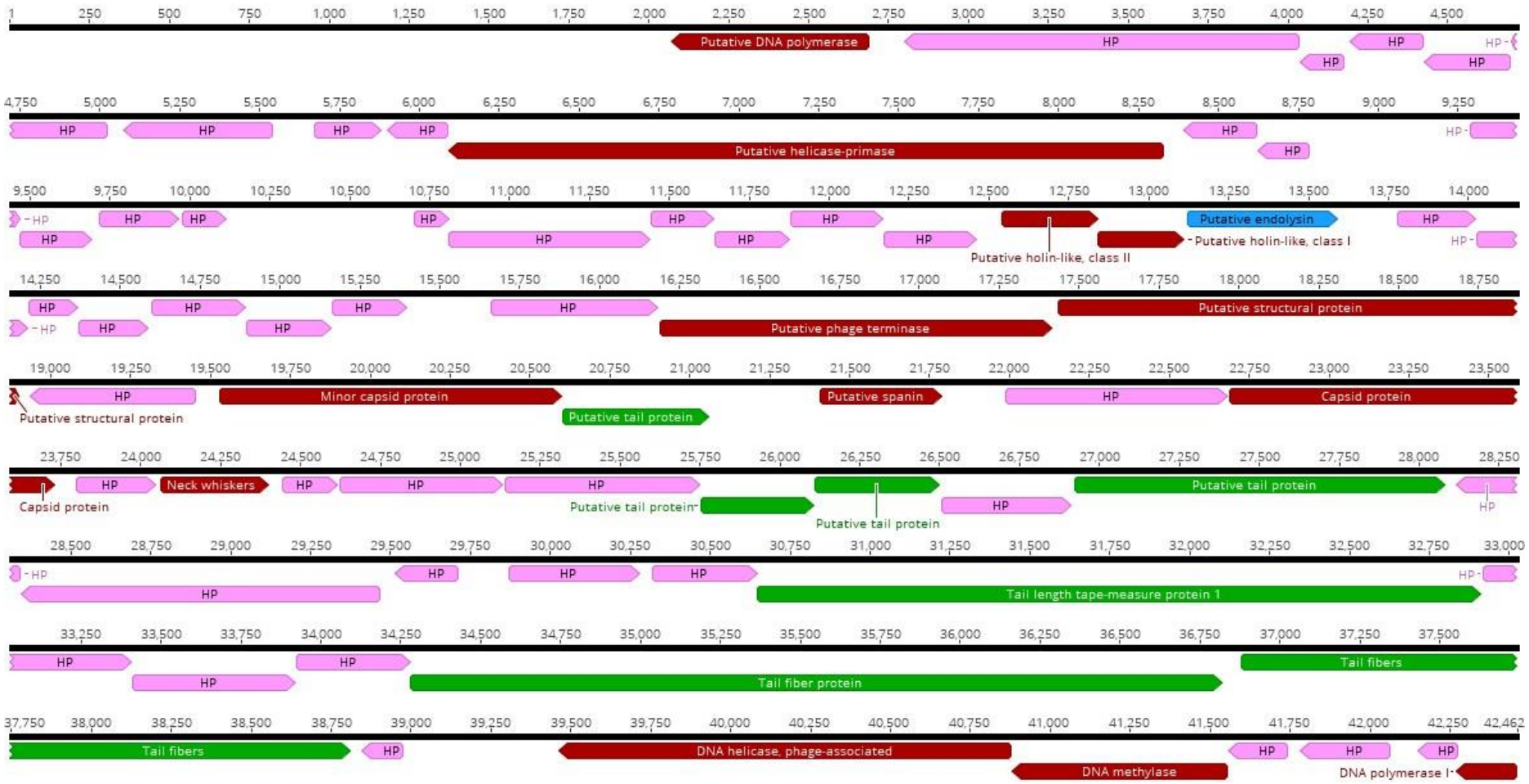

Figure 18. Annotated genome of phage 26 (*Siphoviridae* cluster 2) with lytic activity against ESC resistant *E. coli.*

Representative of phages 3.2, 4.1, 26, 27, 28, 29, 30, 31 and 32.