Isolation and Characterization of bacteriophages targeting uropathogenic *E.coli*

By

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Abbreviations

BV	Bacterial Vaginosis
$CaCl_2$	Calcium chloride
DNA	Deoxynucleic acid
ECOR	*E. coli* reference collection
LB	Luria Bertani
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
ml	Mililiter
NaCl	Sodium Chloride
OD	Optical density
PFU	Plaque forming units
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPM	Revolutions per minute
Spp.	Species
TEM	Transmission Electron Microscopy
Tris-HCl	Tris (Hydroxymethyl) Hydrochloride
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infections
UV	Ultraviolet
λ	Lambda
µl	Microliter
%	Percentage

ABSTRACT

Urinary tract infections (UTI) are one of the most common human bacterial infections, caused by uropathogenic *E. coli* (UPEC). Conventionally, UTI's are treated with antibiotics, such as sulpha-mathoxazole/trimethoprim and ampicillin. However, a significant proportion of UPEC strains have become resistant to these antibiotics, resulting in a significant burden on the health care system worldwide. Bacteriophages (phages) that target *E. coli* strains could provide an alternative treatment for UTIs, particularly those resistant to antibiotics. This study aimed to screen a variety of environmental samples for phages that target a UPEC strains and characterize these phenotypically and genotypically, towards the development of a candidate phage preparation for treatment for antibiotic-resistant UPEC.

Environmental samples (including faeces cattle, sheep, horse, goat faeces, river water, raw cow milk and mud) were collected from various sources around the City of Cape Town and screened for phages using an *E. coli* laboratory strain; K-12 MG1655. Lytic phages against *E. coli* MG1655 were purified and host-range testing was conducted against a panel of 11 UPEC strains form the *E. coli* reference collection (ECOR). These lytic phages were characterized phenotypically using transmission electron microscopy (TEM) using negative staining and genotypically using restriction enzyme HaeIII.

In total, 41 phages that were lytic against MG1655 were isolated from six different environmental samples. Of these, 31/41 (76%) were lytic against 7/11 (64%) UPEC strains tested. Four phages (16-3, 16-4, 16-5 and 16-7; all isolated from raw milk) had the broadest host range of all the phages screened, being lytic against 4/11 (36%) UPEC strains. UPEC strain ECOR-40 was the most susceptible of the *E. coli* strains tested, being susceptible to 18/31 (58%) of the lytic phages isolated. In contrast, four UPEC strains (including ECOR-14, -60, -62 and -64) were not susceptible to any of the phages isolated. Ninety percent (28/31) of the isolated phages were structurally similar to four known phage families; including *Myoviridae*, *Siphoviridae*, *Inoviridae* and *Rudiviridae*. The remaining 10% (3/31) had structures that did not resemble any of the known phage families. DNA was

isolated from all 31 phage isolates to screen for similarities in restriction patterns of isolates. Of these, RFLP banding was clear for 5/31 samples; which showed that 5/5 phages were unique based on their banding patterns. In conclusion, this study demonstrated the existence of several unique *E. coli* phages in nature and their ability to target several of the UPEC strains known to cause UTI. Although time did not allow for sequencing of the full genomes of those isolates with unique characteristics in this study, this should be a priority for this research going forward.

KEYWORDS: Urinary tract infections, uropathogenic *E. coli*, bacteriophages, antibiotic resistance

1 INTRODUCTION

The emergence of antibiotic resistance in South Africa and worldwide has made it challenging to treat bacterial infections. Over-prescription of antibiotics to treat human diseases, their casual use in treating plant/animal diseases, in agriculture to accelerate crop and animal growth, and the decline in development of new antibiotics over the past decades have contributed significantly to the increase in antibiotic resistance [1]. Combining multiple antibiotics together in treatment has been one approach implemented to control development of antibiotic resistance in bacterial pathogens. However, the dissemination of multi-drug resistant bacteria such as *Mycobacterium tuberculosis*, *Enterococcus* species, and *Pseudomonas aeruginosa*[2] indicates that this multi-antibiotic strategy may not be as effective as needed or too little too late. New and advanced approaches to microbial chemotherapy need to be explored urgently to avoid the occurrence of a catastrophic post-antibiotic hazard. As an attempt to address this, bacteriophages are being explored as biocontrol agents in food additives and in both veterinary and clinical settings, where they have been used to treat human bacterial infections in Eastern Europe for over 100 years.

1.1 Polymicrobial nature of urinary tract infections (UTIs)

One of the most common infections in humans are urinary tract infections (UTIs)[3–6], affecting the urethra, bladder, ureter and kidney[7]. Although commonly caused by uropathogenic *E. coli* (UPEC), several other bacterial species have been associated with UTI, including *Escherichia coli*, *Staphylococcus. aureus*, *Klebsiella pneumonia*, *Proteus mirabilis* and *P. aeruginosa*[8]. Of these, UPEC strains account for by far the majority (>75%) of UTIs[6]. In addition, *Enterococcus* and *Candida* spp. have also been associated with UTIs, although less commonly[9,10]. Although UTIs are more common in the lower urinary tract (urethra and the bladder), some UTIs ascend and reach organs in the upper urinary tract (ureters and kidneys) which results in more severe symptoms such splenomegaly (spleen enlargement as a complication of an abscessed kidney infection) and pyelonephritis (kidney inflammation)[7]. Uncomplicated UTIs are generally acute and community acquired, with less diverse etiology comprising almost exclusively of infections with UPEC.[6] Complicated UTIs,

on the other hand, are sometimes poly-microbial (involving a diversity of microbes including *E. coli*, *S. aureus*, *K. pneumonia*, *P. mirabilis* and *P. aeruginosa*[8]), and more likely to be influenced by underlying risk factors, such as catheterization, diabetes, old age or having a spinal cord injury[4]. Although the etiology of complicated UTIs is more diverse, uncomplicated UTIs are much more common[4]. In general, women are more prone to UTIs than men, with >70% of UTI cases being reported in women and these are further complicated by the high recurrence rate of UTI in most women.[6,11].

E. coli is a rod-shaped gram-negative bacterium, that is widely distributed in nature, inhabiting water and soil environmental niches, as well as the gastrointestinal tract (GIT) of most mammals[12–14]. *E. coli* has been associated with a number of clinical diseases in humans, mainly involving the intestines and urinary tract. *E. coli* K-12 MG16555 is a non-pathogenic laboratory strain of *E. coli*, originally derived from a commensal of the human gut, called K-12, and hence designated K-12 MG1655. The MG1655 *E. coli* strain is the first *E. coli* strain with a large genome (>4 megabases) to be sequenced in 1997 by F. Blattner and has been used as a model organism for wild-type *E. coli* over the years because it is uncomplicated to culture[14,15].

There has been ten *E. coli* pathotypes described to date, based on the clinical syndromes they exert and their place of action in the human body; including (1) uropathogenic *E. coli* (UPEC), (2) diffusely adhering *E. coli* (DAEC), (3) enterotoxigenic *E. coli* (ETEC), (4) enteropathogenic *E. coli* (EPEC), (5) enterohemorrhagic *E. coli* (EHEC), (6) enteroaggregative *E. coli* (EAEC), (7) shiga-toxin-producing enteroaggregative *E. coli* (STEAEC), (8) enteroinvasive *E. coli* (EIEC), (9) adherent invasive *E. coli* (AIEC), and (10) meningitis-associated *E. coli* (MAEC)[15,16]. Except for AIEC, which has been associated with Crohn's disease, these cause diarrhea (DAEC, ETEC, EPEC, EHEC, EAEC, STEAEC, and EIEC). Furthermore, both UPEC and MAEC are extra-intestinal pathogens causing infections such as UTIs, septicemia, osteomyelitis and neonatal meningitis[15–17] .

1.2 The virulence of UPEC strains

Women with shortened and/or wider urethras are at a higher risk of suffering from retrograde

ascent of microbes, in which microbes of fecal origin populate the bladder and sometimes the kidney[18]. Child birth and sexual intercourse also contribute to this, as they allow for the fecal microbes to be massaged up the urethra into the bladder[18,19]. Vaginal colonization with UPEC strains generally precede bladder epithelial colonization, and this is worsened in women who experience recurrent UTIs, whereby vaginal UPEC colonization is extended even during asymptomatic infections[20,21]. Recurrence of UTIs make them more challenging to treat, and more than 25% of women with UTIs experience recurrent infections within 3 to 6 months[22]. Of these recurrent UTI cases, ~60% are caused by the same bacterial strain that caused the initial infection[4,7]. Gilbert *et al*.[7] showed that vaginal microbiota play an important role in UTI recurrence using mice as a model, and that transient exposure to *Gardnerella Vaginalis*, a facultative anaerobe commonly associated with bacterial vaginosis (BV), triggers the movement of *E. coli* from latent reservoirs in the lower reproductive tract to the bladder lining[7]. Consistent with this, *Gardnerella* and *Lactobacillus* species have been isolated from urine, both of which are commonly found as part of the vaginal microbiota supporting the hypothesis that the urinary tract is exposed to vaginal bacteria, and vice versa [23–25]. Interestingly, there is a 3-fold increase in *E. coli* adherence to buccal, vaginal and voided bladder epithelial cells in women with recurrent UTIs[4], a phenomenon previously thought to be connected with diminishing antibacterial adherence host defense mechanisms[26].

Several specialized adhesive structures on the outer surface of uropathogens have been identified to play a central role in colonization, including the outer protein Lam B, the outer membrane protein C (OmpC), terminal glucose moieties with a βl,3 glycosidic linkage and filamentous cell surface structures (flagella[27] and pili[28])[29–32]. In the case of UPEC, these filamentous structures are called type 1 fimbrae and P pili, and have generally been found on the surface of most *E. coli* strains[4]. Unlike the *E. coli* P pili, type 1 fimbrae are thought to play a larger role since they are one of only two factors thought to contribute significantly to urovirulence, the second factor being the production of α-hemolysin[8,33]. Each *E. coli* fimbrae has an adhesion molecule at the tip, called FimH, which allows *E. coli* to interact with host cell receptors on the bladder epithelium, thereby enabling bacterial attachment[4]. Fimbriation by *E. coli* isolates has been associated with urovirulence[4,34], with pathogenic *E. coli* strains found to have higher expression of type 1 fimbrae compared to commensal *E. coli* strains[35–38]. High expression levels of fimbrae by *E. coli* is thought to enhance their recognition by receptors on

bladder epithelial and immune cells, which increase the likelihood of *E. coli* surviving antibiotic treatment[39]. In addition to mediating host cell entry or adhesion, *E. coli* type 1 fimbrae also allow UPEC strains to translocate out of the host cell or be internalized into the late endosomal compartment, where they establish a quiescent intracellular reservoir that contributes to UTI recurrence and other serious complications of recurrent UTIs (such as kidney damage and splenomegaly) [7]. In such a quiescent state, UPEC strains are better able to evade immune surveillance and resist antibiotic treatment which primarily targets actively replicating pathogens [40]. UPEC strains also avoid immune detection by suppressing host immunity [41,42]. UPEC strain CFT073 was able to block activation of host NF-κB, thereby decreasing secretion of inflammatory cytokines which was associated with an increase in type 1 pilus-mediated uroepithelial apoptosis[43]. The ability of UPEC strains to evade and/or suppress host immunity indicates the extent at which these organisms have evolved and will have to be studied and understood further in order to effectively treat or eradicate them.

Unlike the type-1 pili, an *E. coli* surface virulence factor, α-hemolysin is a pore forming intracellular virulence factor with a wide spectrum activity against leukocytes, erythrocytes, endothelial and renal epithelial cells[44,45]. At low concentrations α-hemolysin, it induces apoptosis of its target cells and stimulates bladder epithelial cell exfoliation[46,47]. Whereas, at high concentrations, it results in host cell lysis, which in turn facilitate UPEC dissemination across mucosal barriers to gain access to host nutrients, iron supplies and impair immune effector cell function[8,14,44,45,48,49].

Although type 1 fimbrae and hemolysin are the dominant mechanisms facilitating UPEC pathogenesis, there are other less common factors that can be of importance in facilitating urovirulence, such as afimbrial adhesins (filamentous structures without fimbriation; which have the same mode of action as the fimbriated structures i.e. type 1 fimbrae), iron-acquisition systems required to steal the host's iron stores; and other secreted toxins such as cytotoxic necrotizing factor 1 (which cripples the host's immune effector cells hence facilitating bacterial dissemination) [33].

Four phylogenetically distinct groups of *E. coli* have been described, using multilocus enzyme electrophoreses (MLEE) to compare allelic variation of 12 enzyme encoding loci [50]. These

include *E. coli* groups A, B1, B2 and D. Pathogenic *E. coli* strains primarily belong to group B2 and group D to a lesser degree, whereas commensal strains predominantly belong to either group A or group B1 [51–53]. Strikingly, some pathogenic strains of UPEC, specifically those in the ECOR collection belong to phylogenetic groups known to harbor commensal strains, such as group D[54,55]. A study characterizing UPEC isolates from South Korean patients showed that almost 80% (46/58) belonged to group B2, only 16% (9/58) belonged to group D, and less that 5% (1/58) belonged to group A and B1 (1/58)[56]. This indicates that it is not uncommon for some pathogenic strains of *E. coli* to be categorized under a phylogenetic group considered to contain commensal strains. This further suggests that there might be an overlap of alleles used to group the strains into phylogenetic groups amongst commensal and pathogenic strains or that the criteria used to group these strains may not be stringent.

1.3 Burden of antibiotic resistance in treating UTIs

International recommendations for the treatment of UTI involves one of the following first line antibiotics; trimethropim-sulphamethoxazole (TMP-SXT), amoxicillin combined with clavulanic acid, ampicillin, nitrofurantoin or fluoriquinolones for five days[57,58]. However, the frequency of treatment failure for conventional antibiotics to *E. coli* has increased over the last decade [19,59–64]. Amongst the 58 UPEC isolates tested from the South Korean cohort described previously, 13 of the 14 *E. coli* virulence factors (*fimH, sfa, papA, hylA, cnfl, aer, afac, feoB, Irp2, iroN, iha, eae, stb and It-1*) that were screened for antibiotic resistance against Aminoglycosides (gentamicin, tobramycin and amikacin), Penicillin (ampicillin), Carbapenems (imipenem and meropenem), Cephalosporins (cefotaxime, ceftazidime, cefepime and cefoxitin), Monobactam (aztreonam), Chloramphenicol, Sulfonamide (trimethoprim), Fluoriquinolones (ciprofloxacin) and Tetracycline were noted. *E. coli* groups B2 and D exhibited the highest number of the virulence genes and were the most resistant to the antibiotics tested for compared to the other phylogenetic groups.[56] However, since none of the UPEC isolates appeared to be resistant to cefoxitin, cefepime, ceftazidime, and chloramphenicol, the Korean study confirmed that these could still be used in therapy in that region[56].

Some of the strategies employed by *E. coli* to resist antibiotics include: (i) expression of antibiotic resistance genes[65–68]; (ii) biofilm formation (which renders them resistant to the immune system and antibiotics)[3]; and (iii) establishing a protected intracellular niche within bladder epithelial or immune cells[7]. In the face of antibiotic pressure, UPEC strains undergo horizontal gene transfer, involving the acquisition of mobile genetic material/cassettes (which could include plasmids, transposons or bacteriophages) that often result in antibiotic resistance[57,69]. Horizontal gene transfer is one of the mechanisms often used by bacteria to adapt to the new environment, by which they are able to transfer resistance from one bacterium to the next for survival [70–72]. Over time, the antibiotic sensitive UPEC strains will be targeted and destroyed while the strains that have acquired antibiotic resistance will survive through natural selection, and pass it on to their offspring[1,57,69]. A study investigating the antibiotic susceptibility profile of 227 UPEC isolates from Jordan University of Science and Technology found that 82% of the isolates that were resistant to at least three antibiotic classes (including amoxicillin, nalidixic acid, and cefoxitin) produced extended- spectrum beta-lactamase (ESBL) which is an enzyme encoded by antibiotic resistance genes SHV,TEM and CTX_M[73] and can confer antibiotic resistance to β-lactam antibiotics through the hydrolysis of the β-lactam ring (a four atom ring structure found in β-lactam antibiotics) which then deactivates the antibacterial properties of β-lactam antibiotics [74]. In addition, recombination within *E. coli* with foreign genetic material and spontaneous mutations are other mechanisms that enable emergence of drug resistance mutations[75] .A study conducted by Manikandan *et al*.[8] screened several different bacterial strains that have been associated with UTIs, including *E. coli*, but also *S. aureus*, *Klebsiela pneumonia*, *P. mirabilis* and *P. aeruginosa*, for susceptibility to various antibiotics (including amoxicillin, cephalexin, gentamycin, nalidixic acid, ciproloxacin, cotrimaxozole and TMP-SXT). They found that most strains were highly resistant to nalidixic acid and TMP-SXT (the drug of choice in treating UTIs), although *E. coli* were more resistant to most conventional UTI antibiotics than the other strains, followed by *L. pneumonia* and *P. aeruginosa*. The higher prevalence of antibiotic drug resistance in *E. coli* compared to the other UTI causing bacterial species was attributed to the presence of the type 1 dihydrofolate reductase (Dhfr) gene, which has been shown to confer resistance to TMP-SXT in particular and is widespread across gram-negative bacteria as a result of selective pressure resulting from the extensive use of TMP-SXT [8,77,78].

Trimethoprim is a folate analog which binds to the catalytic site of the Dhfr enzyme, however over- transcription of this enzyme can result in mutations which can reduce the affinity of folate analogs for this Dhfr enzyme and thus result in a bacterial phenotype that's resistant to drugs containing trimothoprim[78].

Alternatively, biofilm formation by *E. coli* is another potential mechanism for the decreased susceptibility of UPEC isolates to commonly prescribed antibiotics[3]. A study by Chibeu *et al*.[3] characterized 250 UPEC isolates for their biofilm forming competences and anti-microbial resistance, and reported that almost a fifth produced biofilms and more than 75% were resistant to at least one of the antibiotics tested. Another study examining the association between biofilm formation and antibiotic susceptibility in 100 UPEC isolates from 166 urine specimens of UTI patients demonstrated that of the 72 strains producing biofilm, 100% were resistant to chloramphenicol and amoxiclav (amoxicillin combined with clavulanic acid), 86% were resistant to gentamicin and cefotaxime, 84% were resistant to cotrimaxozole and piperacillin/tazbactam, 75% were resistant to tetracycline and 70% were resistant to amikacin[73].

Hiding within host cells is another mechanism used by *E. coli* pathogens to survive antibiotics and evade immune surveillance[40]. Internalization of *E. coli* into host cells is mediated by type 1 fimbrae which also enhance their intracellular survival within phagocytes such as macrophages and neutrophils[35,79,80] . However, the exact mechanism through which this process takes place is not known, as internalization of pathogens by phagocytes commonly leads to degradation through destructive enzymatic action and toxic host chemicals[42]. The ability of *E. coli* to escape from intracellular acidified phagosomes within phagocytes, either (1) by lysing the phagosome to escape into the cytosol where they will gain access to nutrients; or (2) by altering their physiology and characteristics of the phagosome compartment, has been proposed as a mechanism whereby immune evasion occurs[42,81,82]. While not all intracellular pathogens can escape these membrane-bound compartments within phagocytes (like *Chlamydia* and *Salmonella typhimurium*), *E. coli* shares this property with *Mycobacterium tuberculosis* that are able to propagate intracellularly by inhibiting phagosome acidification and maturation into a phagolysosome[42]. Inhibiting phagosomal acidification through the use of drugs like concanamycin and bafilomycin (which are potent inhibitors of a proton motive force V-ATPase shown to mediate this process), resulted in the

death of intracellular *E. coli* strains[42,83]. This implies that this may be one of the key intracellular strategies employed by *E. coli* to remain viable. The continued increase in cases of antibiotic resistant UPEC strains should serve as a warning of the potential dangers of antibiotic resistance among these common pathogens. In order to prevent the emergence and spread of antibiotic resistant UTI strains, empiric treatment directed at UTIs will have to change.

1.4 Bacteriophages as alternative treatment to antibiotics

As antibiotic resistance (particularly multi-drug resistance) increases, there has been renewed interest internationally in alternative microbicidal treatments, including the use of bacteriophages to kill specific bacterial pathogens. First tested in the early 1900's by Felix d'Hérelle and collaborators[1] at the Pasteur Institute in France, phage therapy uses specific viruses that target bacteria to kill disease-causing bacterial pathogens. Although this method of phage therapy was overtaken by the antibiotic revolution in the America's and most other parts of the occidental world, it has successfully been used for the treatment of bacterial infections in a number of eastern European countries such as Russia, Armenia, Ukraine, Georgia and Poland[84]. Phage treatment was most often used as last resort to treat chronic bacterial infections that did not respond to antibiotic treatment[85]. And although used in previously mentioned European countries, the republic of Georgia is the only place that has officially incorporated phage therapy in their standard of care for both prophylactic and treatment purposes[86]. For instance, Intestiphage and Pyophage are the two primary phage cocktails primarily used in Georgia [86]. Both these phage cocktails target different bacterial species, Pyophage targets pus-causing infections caused by *E. coli*, *P. aeruginosa, S. aureus, Streptococcus* and 2 *Proteus* species[86].In contrast, Intestiphage cocktail targets enteric bacteria, particularly gut-acquired strains of *P. aeruginosa* and *S. aureus*[86].

One of the major concerns of burn wounds in the past was the development of bacterial infections which usually impeded skin grafting[85]. To overcome this concern, phage cocktails were applied topically to the wound with high success rates. Thirty patients with burn wounds from Egypt that were experiencing sepsis caused by antibiotic-resistant *P. aeruginosa*, bandages immersed in 10^{10} pfu/ml of phages were applied three times daily, of which 60% of patients (18/30) reported good skin grafting, 40% (12/30) experienced outstanding skin

grafting, and 26.7% (8/30) were negative for bacterial infections[87]. More evidence on the successful use of phages to treat burn wounds came from Georgia during the Soviet era. For examples, three laborers with methicillin-resistant *S. aureus* (MRSA)-infected burn wounds were treated with phage preparations made up of a polymer saturated with ciprofloxacin and lytic phages, following antibiotic failure, and all subsequently were shown to have cleared MRSA within a week of treatment. Since MRSA had already been shown to be resistant to ciprofloxacin, treatment success was attributed to the presence of phages in the mixture[88]. In Poland, *E. coli* and *S. aureus* were the two main bacterial species that were targeted with phages as they were the main cause for most bacterial infections with antibiotic resistance[85], and a panel of phages against these two bacterial species are typically kept in a central repository to be accessed for treatment.

1.5 General characteristics of phages

Phages have been classified into 14 subfamilies, including 204 genera and 873 species [89–91], with four distinct morphotypes having been described (Figure 1.1): tailed, polyhedral, filamentous, or pleomorphic (meaning they can modify their structure in response to environmental conditions)[92]. Some morphotypes are more prevalent than others, with tailed phages being the most ubiquitous in any geographical niche[92,93]. Phages can either be double or single stranded DNA or RNA viruses, that either have linear or circular genomes,[92,94] often influenced by the phage morphotype[94]. DNA phages have more commonly been described in the literature than RNA phages. Phage genome size tends to correlate with the size of the capsid, and tailed phages tend to have larger genomes than non-tailed phages[92].

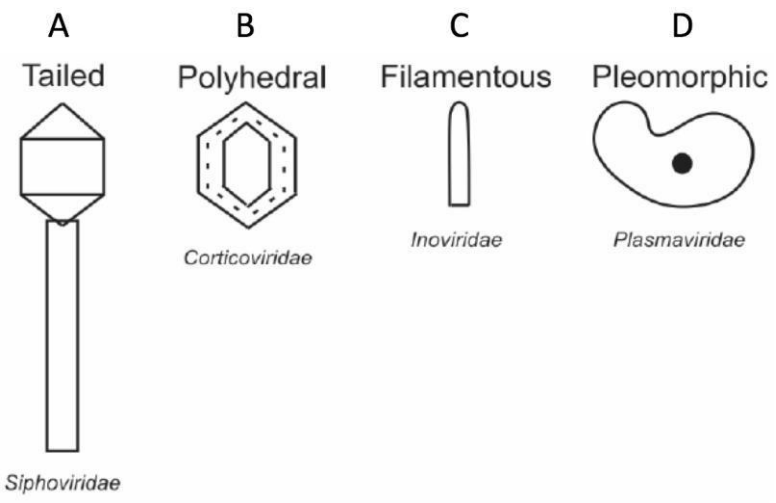


Figure 1.1: A schematic representation of the four phage morphotypes. (A) Tailed structure seen for phages such as T4 and Lambda belonging to the families *Myoviridae* and *Siphoviridae*, respectively. (B) Polyhedral structure seen in phages such as φX174 belonging to the family *Microviridae*. (C) Filamentous structure seen in phages such as fd and MVL1 belonging to the family *Inoviridae*. (D) Pleomorphic structure seen in phages such as L2 and PSV belonging to the families *Plasmaviridae* and *Globuloviridae*, respectively. Figure adapted from Ackerman *et al* [92]

Tailed phages either have contractile or non-contractile tails (Figure 1.1A). Phages of this morphotype are the most prevalent described in nature, including three phage families: *Myoviridae*, *Siphoviridae* and *Podoviridae* and the order Caudovirales[92]. Tailed phages have regularly been identified across a wide range of host organisms, making up ~96% of bacterial viruses from 14 subfamilies, with *Siphoviridae* making up about 61% of those identified, *Myoviridae* about 25%, and *Podoviridae* about 14%[92].*Myoviridae*, *Siphoviridae* and *Podoviridae* all have hexagonal heads, with *Myoviridae* family generally having bigger heads than the other types to accommodate their larger genome size[94]. Although the tail length of *Myoviridae* and *Siphoviridae* are similar, the rigid contractile tails of *Myoviridae* phages discriminates them from flexible and usually spiral non-contractile tails found in *Siphoviridae* phages [92]. Phages in the *Podoviridae* family have a non-contractile tail which is shorter than their heads[94].

Polyhedral phages are distinguished by their polygonal nature, which is often the shape of

most phage capsid (Figure 1.1B). This morphotype is demonstrated in phages belonging to the families *Corticoviridae*, *Tectiviridae*, *Leviviridae*, *Microviridae* and *Cystoviridae*[92]. The most studied phage with this morphotype are those belonging to the family *Microviridae* and are often in association with tailed phages in nature[92,93].

Filamentous phages (Figure 1.1C) are long and flexible in structure but lack the typical polygonal capsid of tailed phages. The phage families *Inoviridae*, *Rudiviridae* and *Lipothrixiviriadae* exemplify this morphotype[92], with *Inoviridae* being the most common and best characterized. Some of the best characterized *Inoviridae* phages include M13, f1 and fd and have been isolated from *E. coli*[95]. Phages of this morphotype strictly infect gram negative bacteria, and their length is determined by the genome size they encapsulate[92,94]. Filamentous phages are released from their bacterial host by secretion, requiring a proton pump and ATP, rather than by causing bacterial lysis [95–97]. As a result, filamentous phages frequently cause chronic infection of their bacterial hosts.

Pleomorphic phages (Figure 1.1D) tend to acquire unique shapes which has been attributed to their ability to survive under more diverse environmental conditions [92,93]. Phages families with this type of morphotype include *Plasmaviridae*, *Globuloviridae*, *Bicaudiviridae* and *Apullaviridae* – all of which are not well characterized due to their how scarce they are in nature [92,93]. Phages of this morphotype are known to have a very limited host range[92,93].

1.6 The two distinct phage lifecycles

Given the vast abundance and diversity of phages in various ecosystems that exist in nature, it is important to understand how they interact with other organisms in each niche, as this interaction is likely to play an important role in shaping other biological systems [98]. The interaction of phages with other organisms is best understood through their lifecycles. Traditionally, phages have two distinct types of lifecycles: the lytic and lysogenic lifecycle (Figure 1.2).

The lytic lifecycle involves the phage infecting its host and, within the cytoplasm, using the host's resources to assemble its particles and consequently resulting in host lysis (without

integrating is genetic material into the DNA of the host) [48,70,99]. Phages that undergo the lytic lifecycle are termed virulent phages.

The lysogenic lifecycle occurs when the phage does not lyse the host following infection, instead it integrates its genome into the circular DNA chromosome of the host and continues to replicate with host's genomic material [48,70,99]. These phages are alternatively known as temperate phages Although less common, temperate phages can also exist as an extrachromosomal plasmid within the cytoplasm of the host (Figure 1.2F).[12,98] During their lysogenic cycle, they are called prophages [103,104]. Once a bacterial host acquires a prophage, the host is then designated a lysogen[69]. In some instances, bacteria that harbor more than one prophage are said to be poly-lysogenic [69,103,105]. An interesting example of a poly-lysogenic bacteria is an enterohemorrhagic *E. coli* strain O157 which was found to harbor eight prophages (sp1 to sp8) and six prophage-like elements (SpLE1 to SpLE6) which are basically sequences indicative of genetic mobility, also called the mobilome [103,106]. Some of these prophages have been linked to virulence of the bacterial host because they encode genes for some of the bacterial hosts most vigorous virulence factors, such as shiga toxins 1 and 2 [103]. Furthermore, the poly-lysogenic nature of some bacteria has been associated with phenotypic changes [69,103,105]. For instance, a prophage can interfere with an expression of a specific gene by inserting itself in the middle of the bacterial gene locus. The host gene can later be reestablished as a consequence of prophage excision, which leads to the manifestation of multiple phenotypic traits in the bacterial hosts [69,103,105].

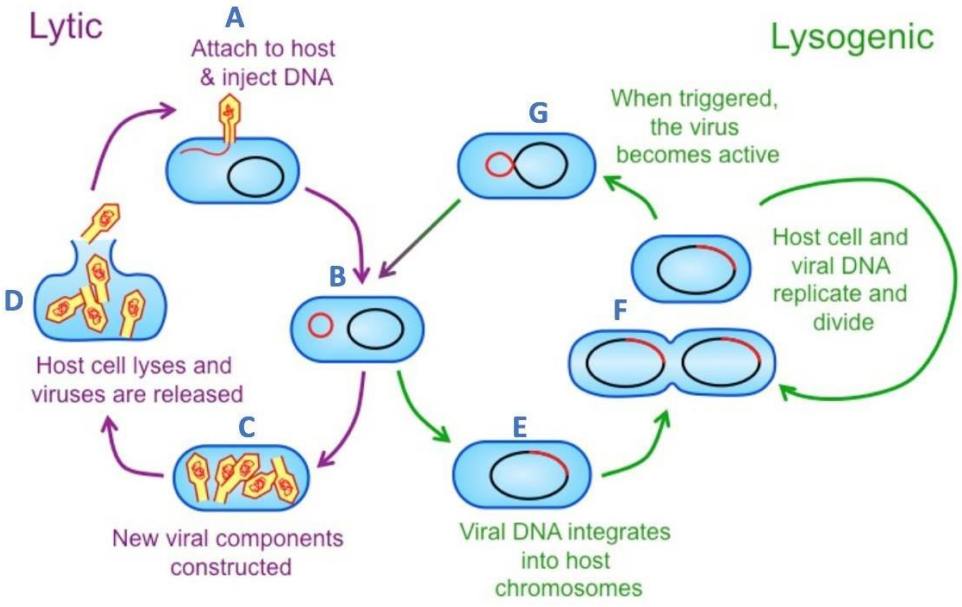


Figure 1.2: Schematic diagram of lytic and lysogenic lifecycles of phages. Initially a phage adsorbs (A) and inserts its genetic material into the host cell (B), through a series of factors, the genetic material will either be incorporated into the bacterial chromosome where it is now a prophage (E), or the phage will use the host replication machinery to produce new viral components and assemble (C), then lyse the host in a lytic infection (D). If the prophage is established, it will replicate and divide with the host (F) and if its triggered through prophage induction it will then enter the lytic lifecycle and result in host lysis (G). [69,70]

1.7 Factors that induce expression of prophages

Although lysogeny is a common amongst bacterial populations [101,103,104,107], environmental stressors can trigger the release of the prophage from the genome of the bacterial host. This process is known as prophage induction [100,101,103]. Among the many factors that result in induction of prophages from their bacterial hosts, treatment with Mitomycin C (an antitumor agent known to bind to the CpG island for DNA crosslinking[108]) or exposure to UV C radiation are among the best described[100–102]. Prophage induction results in the activation of the SOS response in lysogens[109]. The SOS response in bacterial hosts is a high-fidelity DNA repair mechanism that is induced in response to DNA damage and involves the use of two bacterial proteins: LexA and RecA. LexA acts by repressing DNA transcription hence known as negative regulator, whereas RecA activates transcription and is termed a positive regulator of this repair system [109]. For instance, exposing lambda (λ) phage lysogens to UV radiation or

Mitomycin C was shown to result in RecA-dependent cleavage of the λ prophage repressor λcl, which represses the phage lytic genes. Studies using RecA and/or λcl deficient bacteria have shown that λcl is not cleaved and the lytic cycle is no longer inhibited [110]. Following prophage induction, viral replication occurs through the lytic lifecycle (Figure 1.2D).

1.8 Alternate phage life cycles: pseudo-lysogenic or the carrier state

Although lytic and lysogenic phage lifecycles have been well-described in literature [67,98,104], there is evidence to suggest that there might be other less studied and understood lifecycles [67,98,104]. An example of a less understood phage lifecycle is pseudolysogeny and the carrier state [98,100,101]. Pseudo-lysogeny does not involve phage genome integration or bacterial host cell lysis and has caused some debate about whether this is an actual phage lifecycle or a suspended state of phages after infection. It has been suggested that this might be a protective state, by serving as (1) an intracellular retreat for phages in an environment with low host cell concentrations, or (2) a state in which phages avoid poor replication or degradation of their DNA in a nutrient starved host cell that is not conducive to neither the lytic or lysogenic lifecycle [98,111,112]. An example of a pseudo-lysogenic phage is P22 that was isolated from *Salmonella typhimurium*. P22 was only expressed when its genetic material was not integrated into the host genome and it was found to be asymmetrically separated following cell division [113]. This state has also been observed in phages against *P. aeruginosa.* [103] Although considered to be a recent discovery, it was originally witnessed by Frederick Twort [114] in the early 1900s.

With the carrier state, the phage neither enters the lysogenic nor the lytic lifecycle. Instead, the phage particles persist within the host cell and their progeny is released through budding or asymmetrically passed on to daughter cells following cell division [116]. Unlike pseudolysogeny, the carrier state is thought to be established and maintained in a nutrient-rich environment, and results in either a chronic or a productive infection [116,117]. For instance, it is suggested that virions of the filamentous phage M13 known for strictly infecting gram negative bacteria[92,94], undergoes this state since they are not be released by host cell lysis but by leaking through holes created by extruding virions that form mesosomes on the bacterial cell wall (which are folded invaginations on the surface of bacterial membrane meant to

increase the surface area of the membrane) [118,94]. It has also been suggested that some *E. coli* filamentous phages undergo the carrier state, resulting in high concentrations of phages while concomitantly slowing down the growth rate of the host replication. [95]

Pseudolysogeny, lysogeny and the carrier state may all represent states of coexistence between the phage and their hosts. In some cases, it has been suggested that these two states allow for the preservation of the phage nucleic acid material for an extended period of time and could partly explain the large environmental reservoirs of phages in nature[112].

1.9 Phages against *E. coli* and other UTI causing pathogens

There is very little published data on the use of phages as treatment for UTIs. Most of the literature that has focused on demonstrating the existence of lytic phages against uropathogens is derived from *in vitro* studies [119–124]. *E. coli*-specific phages, termed coliphages, have commonly been described in the literature, which may reflect the vast abundance and diversity of *E. coli* in nature[14,90,99,120,125–132]. Several well-characterized coliphages that have been described to date, including tailed (belonging to the families *Myoviridae*, *Siphoviridae* and *Podoviridae*), filamentous (belonging to the *Inoviridae* family), and polyhedral phages (belonging to the *Microviridae* family)[92]. Microscopic analysis of

coliphages isolated in nature revealed that they are mostly tailed and predominantly belong to the *Siphoviridae* family [92,93]. Demerec *et al*.[133] first isolated coliphages from an *E. coli* strain at the Cold Springs Harbor laboratories, isolated from sewage or fecal matter around the mid-1940s, that are available to the public today through the ATCC [134]. The Cold Springs Harbor repository was later accessed by Delbrück *et al*.[135] who propagated seven coliphages using *E. coli* strain B, which he later designated type 1 (T1), T2, T3, T4, T5, T6 and T7. Of these, T3 and T7 are thought to be related to each other whereas T1 and T5 are different to the rest of the T phages and to each other. [136] T2, T4 and T6 are the commonly described coliphages and have been shown to be genetically related and are now termed T-even enterobacterial phages. [137,138] Morphological analysis of the T-even phages revealed a contractile tail and elongated head, suggesting that they belong to the *Myoviridae* family. The T-even phages continue to be explored for their utility in treating *E. coli* infections [120,137].

Lambda (λ) is another well described coliphage, discovered in 1950 by an American microbiologist Esther Lederberg, [139] while she was streaking mixtures of *E. coli* strains. The mixture of a UV-light treated mutant strain of *E. coli* K-12 crossed with its wildtype parental strain yielded plaques on agar plates. She later realized that the plaques were caused by bacterial viruses, which were later named phage Lambda (λ) [139]. Lambda phage is one of the notable phages that can undergo both the lytic and lysogenic lifecycle[139,140]. It belongs to the family *Siphoviridae*, being tailed with a capsid encapsulating its dsDNA genome [140]. Phage Lambda continues to be used as a model organism in microbial and genetic settings.

KEP10 is one of the seven coliphages isolated from environmental water in Kochi, Japan [120]. Its morphology and genotype resemble that of T4 (classified as a *Myoviridae* phage) and was shown to have a broad host range (able to lyse 67% of 42 UPEC strains tested). The ability of KEP10 and T4 phages to treat UTIs were tested *in vivo* using BALB/c mice who had been given UTI through injecting bacterial cells of UPEC ECU5 suspended in saline transurethrally. In this UTI model, mice were injected with 1.3X10 KEP10 or 1X10 T4 viral particles peritoneally, which resulted in a significant decrease in mortality compared to the control mice [120]. KEP10 continues to be explored further due to its broad host range.

Three more tailed phages ϕAPCEc01, ϕAPCEc02, and ϕAPCEc03, isolated from three adult patients, were also found to infect UPEC strains [125]. Morphological analyses revealed that the first two phages belonged to the family *Myoviridae* and resembled the T4 and rV5 phages. The last phage resembled the T5 phage, belonging to the family *Siphoviridae*. These three phages were tested for their ability to clear 16 *E. coli* strains in culture. Each one of the three cleared at least two of the 16 *E. coli* strains to the multiplicity of infection (MOI) between 10^{-3} and 10^{5}. One strain in particular, called *E. coli* DPC6051, was susceptible to all phages individually and in the cocktail [125]. Combining the phages with ciprofloxacin, individually or as a cocktail, led to the inhibition of *E. coli* growth and establishment of resistant strains[125].

It is clear that *E. coli* is predominantly sensitive to tailed phages, this susceptibility suggests that tailed phages may have adapted to infecting *E. coli* over time, possibly increasing the

sensitivity of *E. coli* strains to these phages and hence increasing the robustness of these coliphages to *E. coli* strains. The abundance and diversity of coliphages further increases their chances of discovery which perhaps explains why most studies focus on isolating them from environmental sources such as raw sewage, rivers, dams and fecal matter [120,125,134].

1.10 Advantages and disadvantages of phages versus antibiotics

Although the use of phages as therapy was eclipsed by antibiotics in the twentieth century in most developed countries (including the United States of America, the United Kingdom and Western and Central Europe), this approach has some advantages over antibiotics [141]. Certain antibiotics, like tetracycline, are bacteriostatic rather than bactericidal, and therefore do not kill the bacteria but limit their growth by interfering with DNA replication, protein production and overall metabolism [142], which can facilitate development of antibiotic-resistance [143,144]. In contrast, lytic phages tend to be bactericidal, resulting in the bacterial cell death [142]. Owing to their narrow host range, phages tend to minimally disrupt commensal bacterial communities [4,70,145]. In most instances, phages are capable of infecting only a few strains even within one bacterial species. Phages are rarely able to infect two closely related bacterial geneses[145]. Resistance strategies developed by bacteria against antibiotics cannot be adopted by phages since they kill bacteria differently from antibiotics [1,4,70,71,84,141]. As a result, phages can be applied to treat antibiotic-resistant bacteria [1,4,70,71,84,141].

Despite some of the advantages that phages offer over antibiotics, they do pose a certain level of risk. Phages being administered as replication-competent entities could potentially aid in evolution of commensal bacterial strains into pathogenic ones [121]. By lysing their hosts, phages can result in the release of bacterial components (including toxins like lipopolysaccharides [LPS]) that may induce immunity in the human or animal host [146] and lead to septicemia. While cases of septicemia have been observed, this occurs much less frequently than observed with the use of antibiotics[147]. Some clinical trials using phages have shown that immune responses are elicited by phages [148]. Most studies have shown that the innate immune system is primarily responsible for systemic clearance of phages [68,149,150]. For example, in experiments using mice injected intravenously with phages showed that Kupffer cells (which are specialized phagocytes in the liver) and spleen macrophages phagocytosed

T4 phages rapidly after the mice were exposed to phages, leading to rapid clearance of the therapeutic agents [149]. It was argued that some of these side effects can be mitigated by carefully removing endotoxins from phage preparations, which can be removed using organic solvents [151].

Only obligate lytic phages are used for phage therapy, since they have to be able to target and kill bacteria. The use of lysogenic phages in therapy carries a higher risk of introducing foreign genetic material such as antibiotic resistance genes, and pathogenicity islands into the bacterial host genome by transduction. Transduction, which can either be generalized or specialized, involves transfer of genetic material among bacterial strains or genera using the phages as the transducing vehicle [70,71,152]. Generalized transduction is often the result of a lytic phage infection whereby the phage encapsulates host nucleic acids, burst the bacterial host cell, and moves to infect surrounding susceptible bacteria. Specialized transduction, on the other hand, involves the incorporation of targeted bacterial genetic segments into the phage genome, which often confers a beneficial trait to the phage [98]. In both cases, the newly introduced bacterial genetic material could result in the existing prophage blocking another temperate phage from infecting that same bacterial host. This phenomenon is known as superinfection immunity [153,154], and is facilitated by a prophage-encoded repressor, which inhibits the expression of the newly introduced phage nucleic acid material [70–72,86,155]. In rare cases, this can convert a phage sensitive bacterium into an insensitive one and might reduce the efficacy of phages as therapeutics. As new phages are being discovered and characterized, more research should focus on ensuring that phages undergoing superinfection immunity are avoided at all cost and should not be used in clinical settings for therapeutic purposes.

1.11 Hypothesis

While UTIs continue to be such a common and recurrent infections in humans, the increase in multi-drug resistant UPEC strains is alarming as this poses a major threat to public health [4,156]. Use of phages, with or without antibiotics, to treat drug resistant bacterial infections offers a promising alternative or adjunctive treatment to antibiotics for UTIs. The success of using phages in certain eastern European countries for clinical treatment of antibiotic resistant infections and their current use as a food additive in bio-control suggests this avenue

of microbial control should be researched more aggressively

This study hypothesized that lytic phages will be present in environmental samples that are likely to contain *E. coli* (such as cow, goat, sheep faeces, river water, sewage), and these will then be used to target UPEC strains and may later have the potential to be developed for phage therapy.

1.12 Rationale

Phages require bacterial hosts for survival hence they are more abundant where their hosts are highly concentrated [157]. *E. coli* is a common commensal of the human and animal gastrointestinal tract (GIT). As a result, *E. coli* will commonly be excreted into the environment, including soil, sewage, rivers, dams and oceans. Lytic phages against UPEC strains should be found in abundance in environments where their host prevails.

1.13 Aim of this study

To isolate and characterize virulent phages from the environment that target uropathogenic *E. coli* strains.

1.14 Objectives of the study

Specific objective 1: To screen a panel of environmental samples for the presence of lytic phages against *E. coli* laboratory strain K-12 MG1655.

Specific objective 2: To determine the host range of the purified *E. coli* K-12 MG1655-specific phages against a panel of clinical strains of UTI *E. coli* from the *E. coli* reference collection (ECOR).

Specific objective 3: To phenotypically and genotypically characterize pure phage isolates that are lytic against UPEC strains using electron microscopy, restriction mapping and sequencing.

2 MATERIALS AND METHODS

2.1 Culture media

The reagents and culture media constituents used in the study were purchased from Sigma Aldrich (St. Louis Missouri, USA) unless otherwise stated. Luria Bertani (LB) media (containing 10g/L Tryptone, 10g/L NaCl and 5g/L yeast extract, pH 7.2), supplemented with 10mM $CaCl_2$ and 10mM Maltose/$MgSO_4$ was used to culture *E. coli* strains and for phage plaque/spot assay.

2.2 Bacterial strains and culture conditions

Eleven UPEC strains from ECOR, the *E. coli* reference collection (Table 2.1; kindly provided by Dr. Rémy Froissart, from the French National Research for Scientific Research [CNRS], Montpellier, France), were selected from 72 reference strains which have been well described

and used in previous published studies, by Ochman H. and Selander [54] and Herzer P.J.[55]. All selected UPEC strains were isolated from urine samples of patients with clinical UTIs from varying geographical locations in Sweden. The ECOR strains represent a spectrum of genotypic variants of *E. coli*. In addition, the laboratory strain *E coli* K-12 MG1655 which is known to be susceptible to a number of different of phages,[158] was included as a propagating strain and a positive control. The *E. coli* strains were cultured aerobically [constant agitation at 120 rpm using a shaking incubator, (model FSM-SPO, Labcon)] at 37^0C overnight. Following an overnight culture, the cultured strains were frozen in 20% glycerol (v/v) at -80^0C until further use.

Table 2.1: UPEC standard reference strains included from the ECOR collection

Strain No.	Previous Designation	Phylogenetic group
ECOR-11	C97	A
ECOR-14	P62	A
ECOR-40	P60	D
ECOR-48	C90	D
ECOR-50	P97	D
ECOR-56	P106	B2
ECOR-60	C89	B2
ECOR-62	P69	B2
ECOR-64	C70	B2
ECOR-71	ABU84	B1
ECOR-72	P68	B1

Table adapted from Ochman H. [54] and Herzer P.J. [55]. The phylogenetic allocation of the ECOR strains into their respective groups was derived using the NJ algorithmic method which compares the genetic distances of the 38 enzyme loci of these strains, based on polymorphisms.

2.3 Collection and processing of environmental samples

Environmental samples were collected from different locations in Cape Town, South Africa into sterile 50ml Falcon tubes using a sterile spatula (Sterile Cellstar, Sigma Aldrich, USA) for solid samples and Pasteur pipette for liquid samples (Sterile Cellstar, Sigma Aldrich, USA). Each tube was labeled with the description of content and the location at which the sample was collected. Samples were then transported at room temperature to the laboratory and stored at 4^0C until they were processed. SM buffer [100mM NaCl, 10mM $MgSO_4 \bullet 7H_2O$, 50mM Tris-HCl (pH 7.4)] was added to the solid and semi-solid samples in a 1:1 ratio and mixed thoroughly, while liquid samples were processed without SM buffer. Samples were centrifuged at 2465 g (Eppendorf 5810 centrifuge) for 30 minutes to remove any particulate

matter. The supernatant was added to a new 50ml tube (Sterile Cellstar, Sigma Aldrich, USA) and 10% v/v chloroform (Sigma Aldrich, USA) was added to remove residual bacterial debris and inverted 10 times. Finally, the samples were centrifuged at 1258 g for 10 min followed by harvesting the chloroform-free supernatant and transferred to a new 50ml tube and stored at 4^0C until further use.

2.4 Bacteriophage enrichment and isolation

To amplify phages from the environmental samples, 100 µl of the supernatant was added to 4.9 ml *E. coli* K-12 MG1655 overnight culture at an OD_{600nm} of 0.3-0.4. The mixture of *E. coli* and environmental samples were incubated for 15 min at 37^0C without shaking and then overnight at 37^0C under aerobic conditions, shaking at 120 rpm. The enriched phage-*E. coli* cultures were stored at 4^0C for an hour and then centrifuged at 2465 g for 30 min. Chloroform (10%v/v) was added to the supernatant and centrifugation progressed at 1258 g for 10min. The chloroform-free supernatant was transferred to two Eppendorf tubes and stored at 4^0C until needed.

E. coli K-12 MG1655 was used to purify single plaques from serially diluted phage concentrates using the double layer plaque assay as follows; 20ml of LB agar (1.2%) was poured onto petri dishes and allowed to set as bottom agar at room temperature for 15 minutes. Soft overlay LB agar (0.6% agar; 4ml) was added to 15ml tubes and maintained at 45^0C in a heating block (Accublock™ Digital dry bath, Labnet). The phage concentrates and the overnight *E. coli* K-12 MG1655 culture were mixed in a 1:1 ratio (200µL each) and added to the soft overlay agar, then mixed gently using a vortex mixer. This mixture was poured over the bottom agar and allowed to set. Plates were incubated at 37^0C overnight. Plaques were identified by the appearance of clear zones on the bacterial lawn, which indicated bacterial lysis by phages. The plaques were isolated based on their morphologies, which includes different sizes (small, medium or large), characteristics of the zone clearance (turbid or clear), and shape of the plaque (circular or irregular). Phages were selected from plaques with varying size and shape although all picked plaques were clear. To increase the likelihood that one type of phage was picked per plaque and to decrease the likelihood of identical phages

being propagated from environmental sources, the plaque assay was repeated at least three times.

2.5 Characterization of phage host range against UPEC isolates

To determine whether the isolated phages will be lytic to more than one UPEC strain, an agar assay was carried out in which the susceptibility of different UPEC strains to the isolated phages was tested. Twenty milliliters of LB agar (1.2%) was poured onto petri dishes and allowed to set as before. Ten microliters of the bacteria-free phage supernatant were aliquoted to the LB agar plates inoculated with different UPEC isolates. The plates were incubated overnight at 37^0C and inspected for the presence of plaques.

2.6 Purification of phages for transmission electron microscopy (TEM)

Two methods were employed for the purification of phages: (1) the first method used PEG 8000 (10% wt/vol) and NaCl (1 M final). The tubes were inverted 3 times and kept at 4^0C overnight 100K and centrifuged for 2 h at 15 000g, 4^0C (Sorval RC 5C plus, Labnet). The supernatant fraction was discarded, and the pellet was resuspended in 1ml of SM buffer. The phage suspensions were aliquoted into Eppendorf tubes a day later and stored at 4^0C for further analysis. (2) The second method used Chloroform (10% v/v), which was added to the phage suspension and vortexed vigorously followed by 5 minutes of room temperature incubation. This procedure was repeated twice. The phage suspension with the chloroform were centrifuged for 10 min at 16 000g (Eppendorf 5810R centrifuge) at 4^0C. The upper layer containing the phages was recovered without disturbing the interface and transferred to a new 50ml tube. The phage suspension was filtered through 0.2 μm filter and stored at 4^0C until further use. To increase the phage titer, a large volume of phage sample was used (≥4 X 1.5ml Eppendorf tubes per sample). The phages were pelleted for 1 h at 20 800g at 4^0C. Without disturbing the pellet, the supernatant was discarded, and the phages were resuspended in 100 μl of TEM buffer [0.1 M NH4-acetate; pH 7.0]. All the content of the Eppendorf tubes was pooled together in a single 2 ml Eppendorf tube for each of the phage isolates and TEM buffer was added to fill the Eppendorf tube. This washing step was repeated twice. The final resuspension volume was 100 μl of TEM buffer and the phages were stored at 4^0C until

visualized by TEM.

Prior to doing TEM, phage concentrations (plaque forming units [PFU]/ml) were determined for each isolate (Table 2.2). This was done to ensure that sufficient quantities of each phage are utilized to optimize their recovery under the microscope. To carry this out, a spot assay was conducted with 10μl of each phage suspension at the following dilutions; 10^{0}, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10} on a lawn of *E. coli* MG1655. If a phage could not form a plaque at a dilution of $<10^{-6}$ then ≈8 X1.5 ml tubes of each phage sample were purified using chloroform and centrifuged, then

then the recovered phage lysate was viewed on TEM. If a phage could form plaques at a dilution of $\geq 10^{-6}$ then 4 x 1.5 ml Eppendorf tubes were purified for that sample and the recovered phage lysate was viewed on TEM. The rationale was that, phages not being able to form plaques at $<10^{-6}$ had a very low titer which would not allow for visualization under the microscope hence the titer had to be increased by increasing the number of tubes which consequently led to an increase in phage volume.

Table 2.2: Titration of phages in each sample for TEM analysis

	Plaque formation per dilution					
	10^{-0}	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}
Phage ID						
16-1	–	–	–	–	–	–
16-2	–	–	–	–	–	–
16-3	–	–	–	–	–	–
16-4	–	–	–	–	–	–
16-5	–	–	–	–	–	–
16-6	–	–	–	–	–	–
16-7	–	–	–	–	–	–
16-8	+	+	+	+	–	–
16-9	+	+	+	+	–	–
16-10	+	+	+	+	–	–
16-11	+	+	+	+	–	–
16-12	+	+	+	+	–	–
16-13	+	+	+	+	–	–
16-14	+	+	+	+	–	–
16-15	+	+	+	+	–	–
16-16	+	+	+	+	+	–
16-17	+	+	+	+	–	–
16-18	+	+	+	+	–	–
17-1	+	+	+	+	+	–
17-2	+	+	+	+	+	–
17-3	+	+	+	+	+	–
17-4	+	+	+	+	+	+
17-5	+	+	+	+	+	–
17-6	+	+	+	+	+	–
17-7	+	+	+	+	+	–
17-8	+	+	+	+	+	–
17-9	+	+	+	+	+	–
17-10	+	+	+	+	+	–
17-11	+	+	+	+	+	–
17-12	+	+	+	+	+	–
17-13	+	+	+	+	+	–

NOTE: Phages are indicated on the y-axis from 16-1 to 16-18 for those isolated in 2016 and 17-1 to 17-13 for those isolated in 2017. Plaque formation

is indicated "+" and no plaque formation is indicated with "–".

2.7 Characterizing phage morphology by TEM

The purified phage particles were transported in TEM buffer at 4^0C to the Center for Imaging and Analysis, housing the Electron Microscope Unit, University of Cape Town. To visualize phages by TEM, 10 μl of each phage suspension was pipetted on parafilm and carbon coated copper grids (Agar Scientific, UK), which were glow discharged using an EMS100 Glow

Discharge Unit (Electron Microscopy Sciences, USA) in order to create a hydrophilic effect on the coppers surface. The phage samples were coated onto the grids for 10 min. Grids were washed twice with distilled sterile water and were negatively stained by 2% uranyl acetate (SPI Supplies, USA). The phage preparations were visualized under the supervision of Dr Mohammed Jaffer (UCT Electron Microscope Unit), using a FEI Tecnai 20 transmission electron microscope (FEI, Eindhoven, Netherlands), operating at 200 kV (Lab6 emitter) and fitted with a Tridiem energy filter and Gatan CCD camera (Gatan, UK). Phages were classified to recognized phage families according to their respective tail length, tail width and capsid diameter, as described in Table 2.3.

Table 2.3: Summary of characteristics by which phages will be grouped

Phage Families	Phage Morphologies	Nucleic Acid Type	Size	Lifecycle	Shape	Genome Sizes
Myoviridae		DS linear DNA	Capsid 50-110nm Tail <114 nm	Virulent	Hexagonal head rigid tail with a contractile sheath and tail fibers	33-244kB
Siphoviridae		DS linear DNA	Capsid 60nm Tail 150x10nm	Temperate or Virulent	Hexagonal head, with a flexible/contractile tail and may/may not contain tail fibers	35 - 70kb
Podoviridae		DS linear DNA	Capsid <60nm Tail <17nm	Virulent	Hexagonal head and a non-contractile tail shorter than their head	40-42kb
Inoviridae		SS circular DNA	Length 700nm-2000nm Diameter 6-10nm	Temperate	Long and filamentous rod like shape	4.5-8kb
Rudiviridae		DS linear DNA	Length 600-900nm 23nm in width	Neither temperate nor virulent; Chronic	Rigid, thick rods	24 – 35kb

Table was adapted from Ackerman (2007) with minor modifications.

2.8 Bacteriophage DNA extraction

A high titer lysate ($\geq 10^{-6}$ PFU/ml) was generated for all the phage isolates using the *E. coli* K-12 MG1655 strain to ensure optimal DNA yield. To extract phage DNA, two methods were employed:

1. The first method used phenol-chloroform-isoamyl alcohol (25:24:1). To maximize the DNA yield, the volume of each sample was increased by using 12-30 Eppendorf tubes depending on the phage titer of each sample, with more tubes being required if the number of phages were low (determined by calculating the PFU/ml). The phages were washed by pelleting them for 1 h at 20 800g at 4^0C. The supernatant was discarded, and pellets were resuspended in 100 μl of buffer containing 100mM Tris-HCl, 25 mM $MgCl_2$ (pH 7.5), and 1M NaCl (buffer A). All the contents of the Eppendorf tubes were pooled into a single 2 ml Eppendorf tube for the respective phages and filled with buffer A. This washing step was repeated once. To avoid host genomic DNA contamination, 10 μl of DNAse I (11U/μl), 5 μl of RNAse I (4 μg/μl) and 1 μl of DpnI (1X) SuRE/Cut buffer M was added to the respective phage suspensions and incubated at 37^0C for 1 h without agitation. Following digestion, each tube was filled with buffer containing 100mM Tris-HCl, 25 mM $MgCl_2$ (pH 7.5) (buffer B). To wash the phages, suspensions were pelleted for 1 h at 20 800g at 4^0C. The pellets were washed with buffer B as before. This washing step was repeated once more, and the pellets were resuspended in 800 μl of buffer B and transferred to 2ml Eppendorf tubes. DNA was purified by adding the Phenol/Chloroform/Isoamyl alcohol mixture to the phage suspensions, vortexing, and then centrifuging for 10 min at 16 000g at 4^0C. Without disturbing the interface, the upper aqueous layer was transferred to a clean 2 ml Eppendorf tube and this process was repeated twice. The final elution was centrifuged for 10 min at 16 000g at 4^0C. The supernatant containing DNA was harvested and divided equally into two new 1.5ml Eppendorf tubes. Next, 2.5 x the DNA eluent volume of absolute ethanol (99% final) (Sigma Aldrich, USA) was added, in addition to 0.1 x volume of 3M Sodium-acetate. This suspension was vortexed and stored at -20^0C overnight. The sample was then centrifuged for 30 min at 20 800g at 4^0C. The supernatant was discarded and 1ml of 70% ethanol was added, without disturbing the

pellet and centrifuged. The supernatant fraction was then carefully removed without disturbing the pellet and the tubes were left to dry on the bench to allow the ethanol to evaporate. The DNA pellet was resuspended in 50 μl of nuclease free water and stored at 20^0C for further use.

2. The second method used a Phage DNA Isolation Kit (Norgen Biotek Corp, Canada). Host genomic DNA was degraded using DNAse I (2U/μl), followed by DNAse I inactivation at 75^0C for 5 minutes. Five-hundred microliters of lysis buffer was added to the phage DNA elution and vortexed vigorously for 10 seconds. To increase the DNA yield, 4μl of 20mg/ml proteinase K was added and incubated at 55^0C for 30 min. The lysate was incubated at 65^0C for 15 min, inverting the tube continuously during the incubation period to ensure that the solution is homogenous before applying it to the spin column. Isopropanol (320μl) was then added and mixed briefly. Then, 650 μl of the lysate was applied to the spin column and centrifuged at 6 000g for 1 min. The flow-through was discarded. To ensure that the entire lysate passed through the column, the binding step was repeated until the entire sample had passed through. The column was washed with 400 μl of wash solution A (provided with the kit) and centrifugation proceeded as before. The flow-through was discarded. The washing step was repeated twice. The column was centrifuged at 14 000g for 2 min to dry the column and ensure that all wash buffers are removed. To elute the DNA, the column was placed into a fresh 1.7ml elution tube and 700 μl of elution buffer (provided with the kit) was added. The column was centrifuged as before. The elution step was repeated once using a separate elution tube.

2.9 Genome fingerprinting using restriction fragment length polymorphism

To determine the genetic diversity of the individual phages, digestion was conducted on phage DNA purified with DNA isolation kit as the DNA concentrations isolated using the phenol-chloroform method was either too low or had poor quality to be visualized on a gel hence it was excluded from further analysis (data not shown). Their DNA was digested with restriction enzyme HaeIII (Roche Diagnostics GmbH, Germany) according to the

manufacturer's recommendations. HaeIII recognizes 5'-GG/CC-3', and cleaves between G and C [159,160]. Briefly, one unit of HaeIII that can cleave 1 μg of DNA at +37^{0}C for 1hr 1 h in a final volume of 25 μl (1X) SuRE/Cut buffer M was added to the DNA. Following incubation, the digestion mixture was immediately run for 1 hour at 100V on a 0.8-1% agarose gel in TAE buffer at pH 8.0 (Thermo Fisher Scientific company, USA). Undigested genomic DNA was used as a negative control whereas Lambda phage DNA was used as a positive control. On each gel, a GENERULER 1kb DNA ladder (Thermo Fisher Scientific Company, USA) was included. The gel was stained with Gel Red (Roche Diagnostics GmbH, Germany).

3 RESULTS

3.1 Isolation of phages from environmental samples

Between 2016-2017, environmental samples were collected from 31 different sources (animal faeces raw milk, mud, river, pond and dam water, organic fertilizer, compost heap) at seven different locations around Cape Town, South Africa (Table 3.1). Sampling locations were selected because *E. coli* is from faecal samples and sewage waste are occasionally deposited into soil and water associated areas [14]. Twelve of these 31 samples were in solid form (including faeces and fertilizer), 8 were in a semi-solid (including mud and sludge), and 11 were liquid (including effluent and water from various nearby rivers and dams). Although all samples were screened for phages, a total of 41 phages were isolated from only six of these samples, including mud from the Liesbeeck River collected in Rondebosch; goat faeces and raw milk collected from Skaapkraal farm, Ottery; dam and pond water from Dreyersdal farm, Bergvliet). This suggested that *E. coli* phages were more likely to be found in samples with high water content, although not all semi-solid or liquid samples contained phages.

Each of these environmental samples were tested against *E. coli* K-12 MG1655 [15,161]. Since different phages form plaques of different morphotypes, individual plaques with diverse morphotypes were selected from each sample. Only clear plaques were collected for further evaluation since those that were turbid could indicate the presence of temperate phages [123]. Plaques with an irregular shape, even if clear, were excluded. Figure 3.1 shows a series of representative phage plaques on a lawn of *E. coli* MG1655.

Table 3.1: Screening of environmental samples for lytic phages against *E. coli* MG1655

Sample location (GPS coordinates)	Sample type	Nature of sample	Number of phages isolated
Liesbeeck River, Rondebosch (33.9658° S, 18.4810° E)	Bird faeces	Solid	0
	Mud by Liesbeeck Road	Semi-Solid	0
	Liesbeeck River Mud	Semi-Solid	5
	Water from Liesbeeck river	Liquid	0
Observatory (33.9376° S, 18.4721° E)	Organic Fertilizer	Solid	0
Skaapkraal Farm, Ottery (34.0112° S, 18.5092° E)	Cow faeces	Semi-Solid	0
	Horse faeces	Semi-Solid	0
	Sheep faeces	Solid	0
	Goat faeces	Solid	6
	Duck faeces	Solid	0
	Dam Water	liquid	0
	Mud (by the dam)	Semi-Solid	0
	Sheep faeces	Solid	0
	Raw Milk	Liquid	7
Qurbaan Farm, Ottery (34.0112° S, 18.5092° E)	Horse faeces	Semi-Solid	0
	Sheep faeces	Solid	0
	Mud (from the sheep pen)	Semi-Solid	0
Master Organics Compost Supplier; (34°02 51.8''S18°31'42.4''E)	Cow faeces 1 (deep layer)	Solid	0
	Cow faeces 2 (superficial layer)	Solid	0
	Compost Heap	Solid	0
	Cow faeces 3 (superficial layer)	Solid	0
	Cow faeces 4 (superficial layer)	Solid	0
	Water from stagnant Pond	Liquid	0
Dreyersdal Farm, Bergvliet (34°03'21" S18°27'22" E)	Water from stagnant Pond (site a)	Liquid	4
	Water from stagnant Pond (site b)	Liquid	4
	Dam Water	Liquid	15
Athlone Water Treatment Works (33.9653° S, 18.5018° E)	Primary sedimentation tank overflow[a]	Liquid	0
	Primary sludge[b]	Semi-solid	0
	Mixed liquor[c]	Liquid	0
	Final effluent [d]	Liquid	0
	Vygieskraal river[e]	Liquid	0

[a]Primary sedimentation tank liquid overflow was pre-treated overflowing wastewater without biological solids; [b]Primary Sludge was settled sludge, following primary treatment of sewage; [c]Mixed Liquor was wastewater and raw biological mass; [d]Final effluent was treated wastewater flowing out of treatment plant into the Vygieskraal River in Athlone; [e]Vygieskraal Riverwater, Athlone was river water upstream of the Water Treament works that neighbours the plant, into which processed effluent is drained

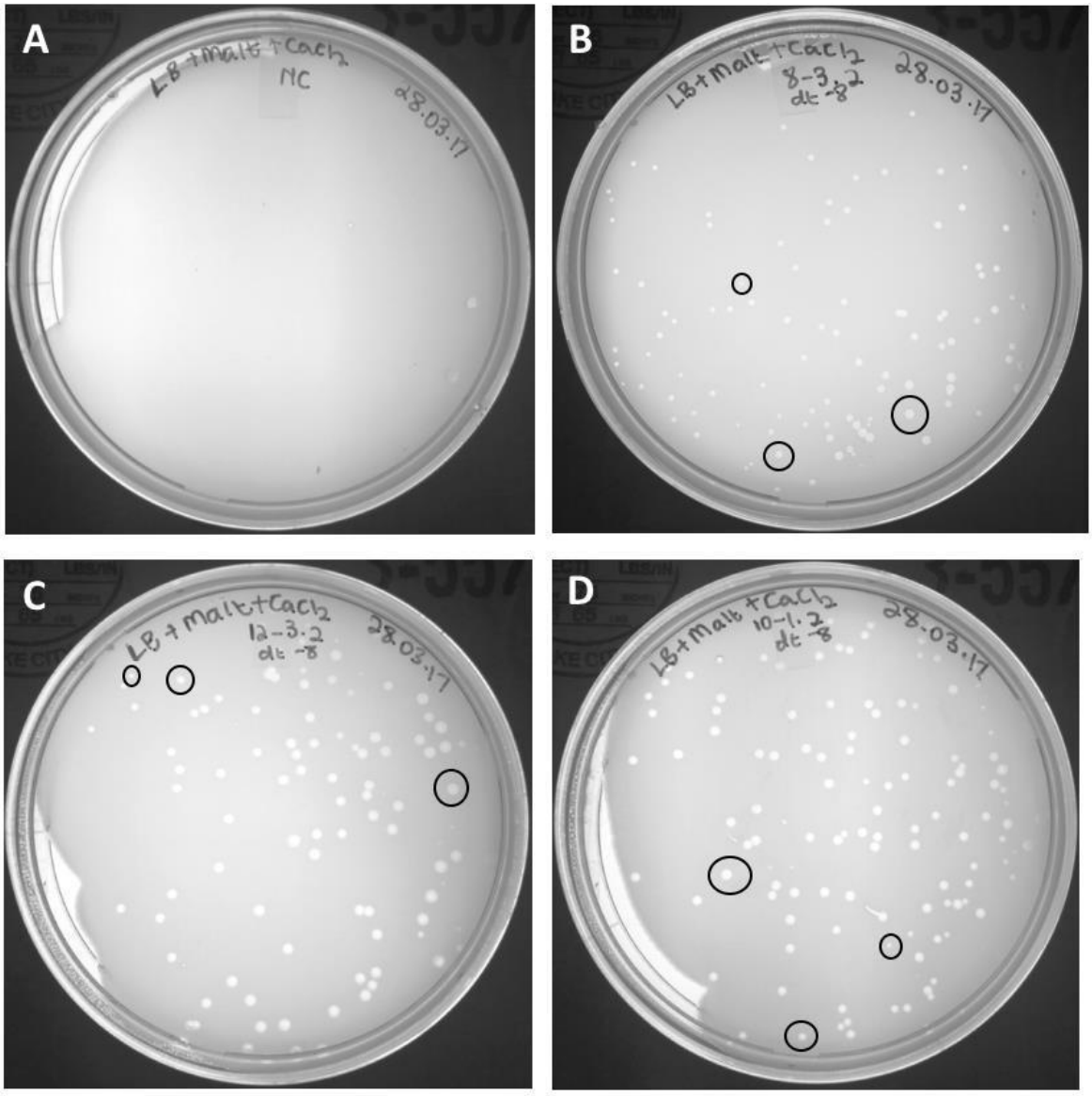


Figure 3.1: Plaque morphology of coliphages on an *E. coli* MG1655 lawn, from (B) Raw milk from Skaapkraal Farm, (C) goat faeces from Skaapkraal Farm, and (D) river mud from Liesbeeck River and a negative control (A). Shown in black circles are their different sizes (small, medium or large), circular shape and clear plaques that were chosen. Images were taken using Panasonic DMC-TZ8 camera.

3.2 Identification of phages lytic to UPEC strains

The isolated 41 phages were tested for their host range against the 11 UPEC strains from the ECOR collection (listed on Table 2.1). Figure 3.2 shows representative plaques formed by phages 16-1 to 16-5 that were lytic on ECOR 40 and phages 16-10 to 16-13 on ECOR-50. Only clear and round plaques were acknowledged for further evaluation. Plaques formed against *E. coli* MG1655 were generally clearer than plaques against the UPEC strains. Furthermore, the sizes of the plaques varied against each *E. coli* strain, with plaques formed against *E. coli* MG1655 tending to be larger than those against UPEC strains. Phages 16-1 to 16-7 isolated from raw milk from Skaapkraal Farm, 16-8 to 16-13 isolated from goat faeces collected at Skaapkraal Farm, and 16-14 to 16-18 isolated from the Liesbeeck River mud yielded larger plaques against *E. coli* MG1655 than any of the UPEC strains, and all these phages could infect at least one UPEC strain.

Not all of the 41 isolated phages could lyse the UPEC strains. Only 31/41 (76%) of the phages that were isolated could infect UPEC strains, and only 7/11 (64%) of the UPEC strains were susceptible to phage lysis (Table 3.2). It is important to note that since a 1/3 of these phage samples were contaminated, their host range could have been altered and thus Table 3.2 might not be a true reflection of the activity of theses phages. The, four phages isolated from Skaapkraal raw milk (16-3, 16-4, 16-5 and 16-7) had the broadest host range, being lytic against 4/11 (36%) UPEC strains. Five phages isolated from dam water or a stagnant pond at Dreyersdal Farm infected only one UPEC strain: ECOR-72. Of the 11 UPEC strains, ECOR-72 was determined to be the third most susceptible to phage lysis in this study, following ECOR-50 and ECOR-40 (Table 3.2). Four of the UPEC strains, including ECOR-14, ECOR-60, ECOR-62 and ECOR-64 were not susceptible to any of the phages tested (Table 3.2). The phages isolated in this study had a relatively narrow host range, suggesting that they would make good candidates for use in phage therapy.

The similarity in host range lysis profiles seen for some phages suggests that they could possibly be either identical phages or closely related (Table 3.2). Phages 16-1 and 16-2 were both isolated from raw milk from the same farm, and both infected UPEC strains ECOR-11, - 40 and -50. Similarly, phages 16-3, 16-4, and 16-5 (also all isolated from raw milk) all

infected UPEC strains ECOR-11, -40, -48 and -50. Phages 16-8 and 16-9, both isolated from goat faeces collected at Skaapkraal Farm, infected UPEC strains ECOR-40, -50 and -71. Phages 16- 10, -11, -12, and -13, also isolated from goat faeces from the same farm, all infected UPEC strains ECOR-40 and 50. Phages 16-14, -15, -16, -17 and -18, all isolated from mud collected form the Liesbeeck River, also had a similar infectivity pattern that was seen for phages 16- 10 to 16-13. Similarly, phages 17-1, -2, -3, -4, -5, -8, -9 and -11, all isolated from Dreyersdal Dam water, all caused lysis of UPEC strains ECOR-56 and ECOR-72. Phages 17-6, -7, -11, -12 and -13, which were all isolated from Dreyersdal dam water or pond water at the same location, all infected only ECOR-72. Two phages (16-6 ad 16-7) isolated from raw milk, had infectivity pattern that was not similar to any of the other host range patterns generated by these phage isolates, which implies that they are not identical nor closely related to any of the other phages isolated from Skaapkraal raw milk.

Since only 31 of the 41 phages isolated in this study could infect at least one of the UPEC strains, subsequent characterization of the morphology of only these 31 strains was performed. Overall, phages from this study generally had narrow host ranges (infecting a median of 4 of the UPEC strains screened). This finding is advantageous in phage therapy because only specific bacterial strains could be targeted and in the case of attempting to widen phage host range, different phages could be combined to increase their bacterial targets[116]. Furthermore, phages isolated from the same samples had a tendency of having similar UPEC host ranges, suggesting that they were related genetically. In the event that they were genetically different, it's possible that they had common receptors hence making them display similar host ranges. This data also suggests that UPEC strains may also exist in nature since phages targeting UPEC strains were isolated.

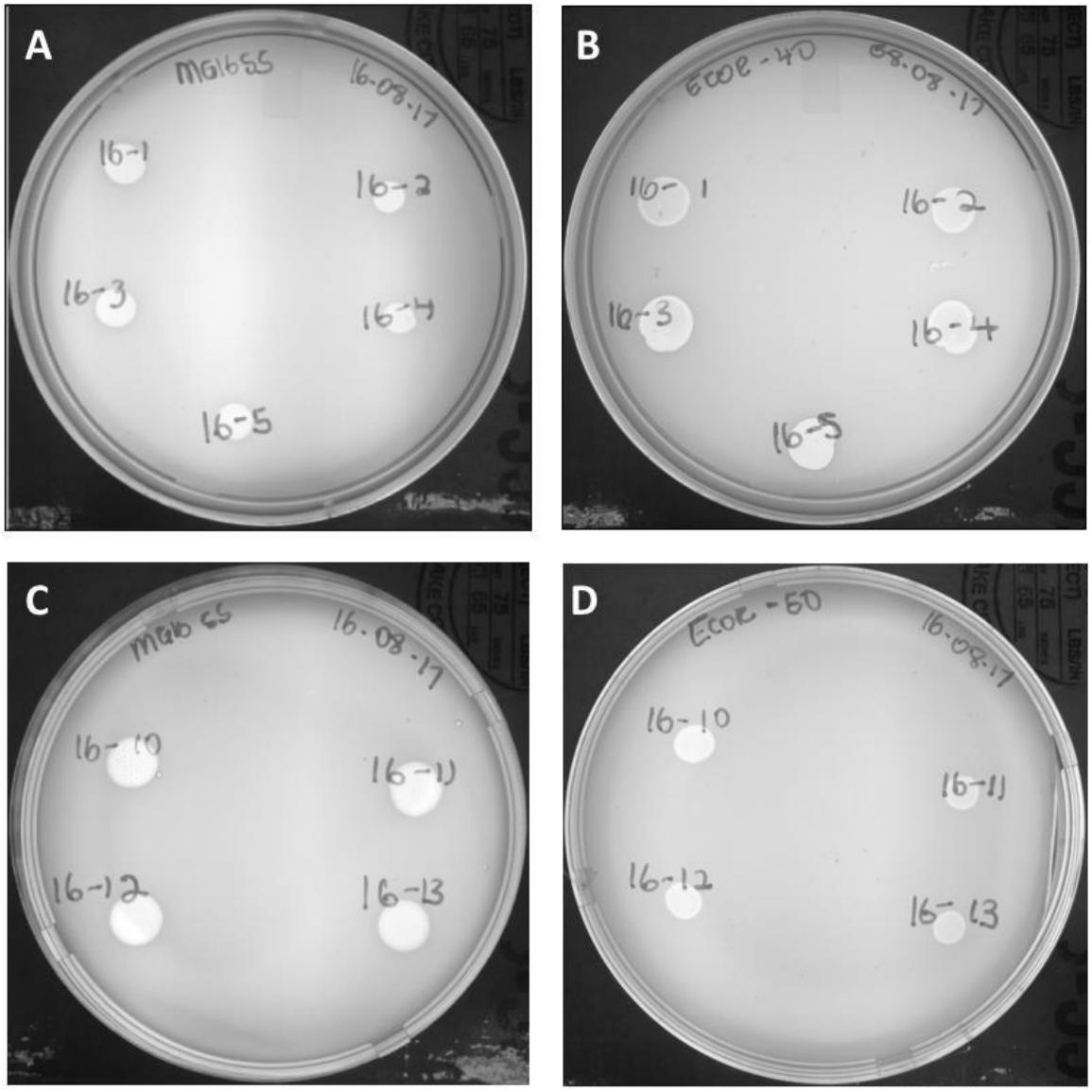


Figure 3.2: Comparison of plaque morphology against *E. coli* MG1655. (A and C) or UPEC strains ECOR-40 and ECOR-50 (B and D) for phages 16-1 to 16-5 (isolated from raw milk) and phages 16-10 to 16-13 (isolated from goat faeces). Images were taken using Panasonic DMC- TZ8 camera.

Table 3.2: Host range testing of isolated coliphages against 11 UPEC strains

Sample location	Phage isolates	UPEC Strains 40	50	72	56	11	71	48	14	60	62	64	Lytic activity (%)
Raw milk	16-3	+	+			+		+					36
	16-4	+	+			+		+					36
	16-5	+	+			+		+					36
	16-7	+	+			+	+						36
	16-1	+	+			+							27
	16-2	+	+			+							27
	16-6	+				+	+						27
Goat manure	16-8	+	+				+						27
	16-9	+	+				+						27
	16-10	+	+										18
	16-11	+	+										18
	16-12	+	+										18
	16-13	+	+										18
River mud	16-14	+	+										18
	16-15	+	+										18
	16-16	+	+										18
	16-17	+	+										18
	16-18	+	+										18
Dam water	17-1			+	+								18
	17-2			+	+								18
	17-3			+	+								18
	17-4			+	+								18
	17-5			+	+								18
	17-8			+	+								18
	17-9			+	+								18
	17-11			+	+								18
	17-6			+									9
	17-7			+									9
	17-10			+									9
Pond water (site a &b)	17-12			+									9
	17-13			+									9
Susceptibility of UPEC strains to phages (%)		58	55	42	26	23	13	10	0	0	0	0	

The different colors indicate coliphages that infected similar UPEC strain ranges. Plaque formation is indicated with "+".

3.3 Coliphage phenotype by TEM

To further characterize the 31 phages that were lytic to UPEC strains, their morphologies were determined using electron microscopy. From these 31 phages that were isolated from local environmental samples, 28/31 appeared to belong to one of the four major phage families, including *Myoviridae* (representative images shown in Figure 3.3), *Siphoviridae* (representative images shown in Figure 3.4), *Inoviridae* (representative images shown in Figure 3.5) and *Rudiviridae* (representative images shown in Figure 3.6). Three phages that were isolated (including 16-14, 17-12a and 17-13) could not be categorized, as their dimensions did not match any of the phage families previously described (representative images shown in Figure 3.7). In addition, 12/31 samples appeared to contain mixtures of different phage populations, despite several rounds of serial purification being carried out for each of the phage preparations (including phage samples 16-12, -15, -16, -17, 18 and 17-5, -6, -3, -7, -9, -11 and -12, Table 3.3) (representative images shown in Figures 6, 7, 8 and 9). Phage samples with mixed populations are designated with either letter a or b next to the phage ID (Table 3.3).

The majority of the phages that were isolated were tailed, with either icosahedral or round heads, most probably belonging to the family *Myoviridae* or *Siphoviridae*. A total of 16/31 (52%) samples contained phages belonging to the *Myoviridae* family. These phages had rigid, contractile tails with fibers (usually 6 tangled fibers) at the one end spread apart (sample 16-1; Figure 3.3). The fibers were seen on phages isolated from raw milk, goat faeces and river mud. Samples 17-5a and 17-6a (Figure 3.3), isolated from Dreyersdal dam water did not contain fibers. Unlike *Siphoviridae* phages, the presence of fibers is common amongst *Myoviridae* phages. However, the absence of fibers in these two phages could have been a result of them being lost through mechanical damage during purification. Although not seen

in any of the phages isolated in this study, *Myoviridae* phages commonly have a collar with short spikes emerging from the base plates around collar. The length of *Myoviridae* phages isolated in this study ranged between 103nm to 127nm, with a mean tail width of 13 nm (standard deviation [SD] ± 0nm) and a mean capsid diameter of 72nm (±4) (Table 3.2).

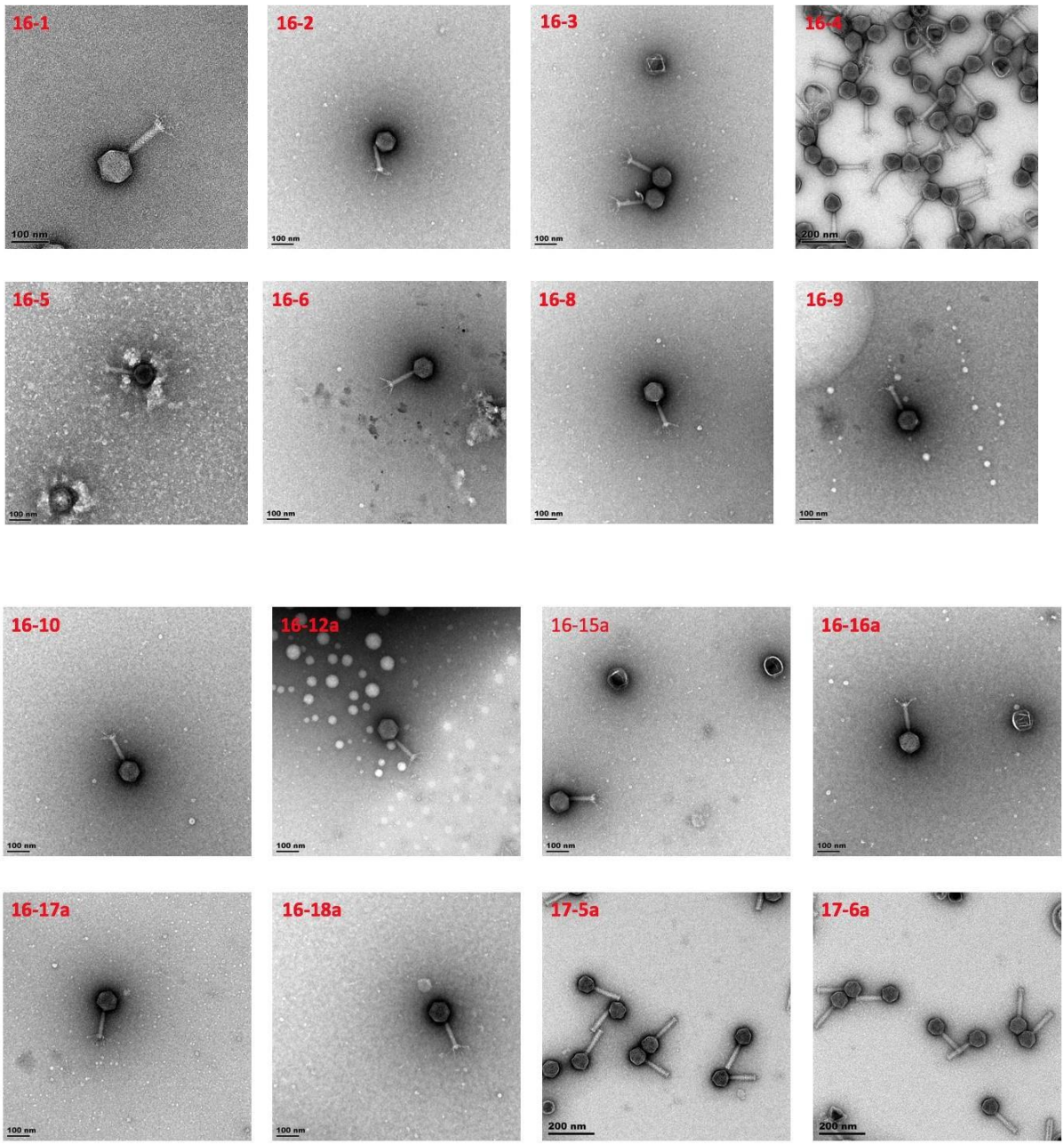
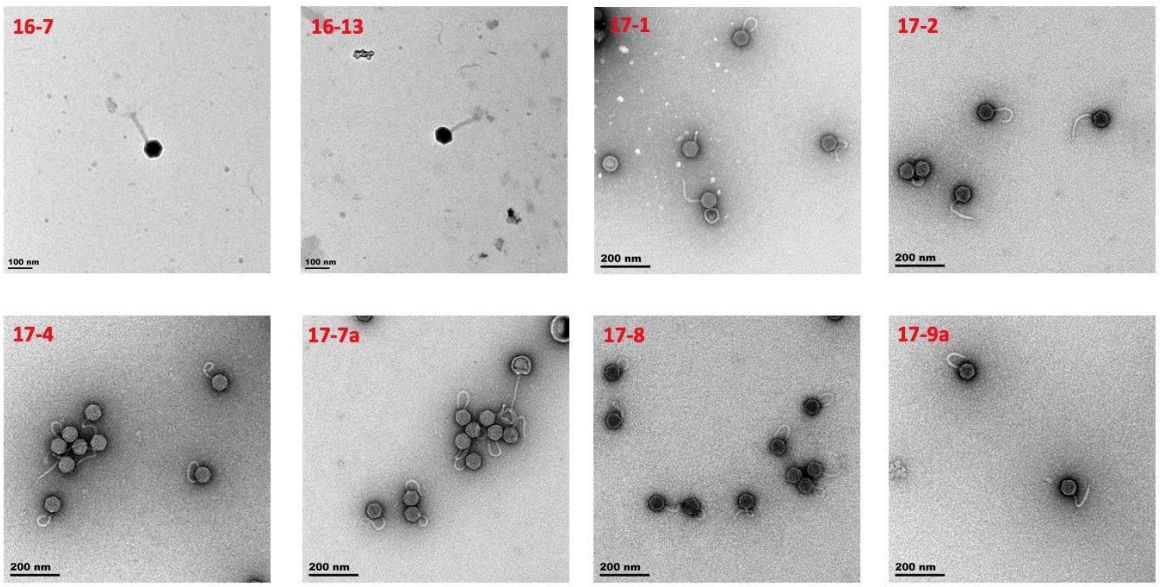


Figure 3.3: Electron micrographs of coliphages with a structural resemblance of phages belonging to the *Myoviridae* family. Phage images of contaminated samples are indicated with "a". Images were taken using a FEI Tecnai 20 TEM operating at 200K at a magnification of 100K.

The second largest group of phages isolated was assigned to the *Siphoviridae* family as a result of their long thin, non-contractile tails ranging from 116nm to 170nm in length and 11 (±0.5) to 22±27 nm in width. In general, *Siphoviridae* phages tend to acquire an S-spiral morphology which has been suggested to be a result of an artefact resulting from EM preparation[162]. Samples 17-1, 17-2, 17-3, 17-7a, 17-8, 17-9a, 17-10, 17-11a and 17-13a (Figure 3.4) all had phages with a spiral morphology and that clumped together possibly as a result of a high phage titer. Samples 16-7 and 16-13 have a similar structure with tails of almost size (1nm difference between the tail length and width) suggesting that they could be the same kind of phage although they were isolated from different sources, raw milk and goat faeces. Although there is an overlap between *Myoviridae* and *Siphoviridae* phages, what distinguishes the two families is the presence of a neck found in *Myoviridae* phages connecting the tail to the capsid, the rigidity of tails found in *Myoviridae* phages compared to the flexible thin tails of *Siphoviridae* phages. Moreover, *Siphoviridae* phages tend to have round and smaller capsids (>60nm), compared to *Myoviridae* phages which have been shown to encapsulate large DNA material due to their enormous capsids that can go up to 110nm in diameter and the phage particles are generally more heavier[163]. *Myoviridae* and *Siphoviridae* may appear similar due to some structural similarities hence careful analysis is required to distinguish them.



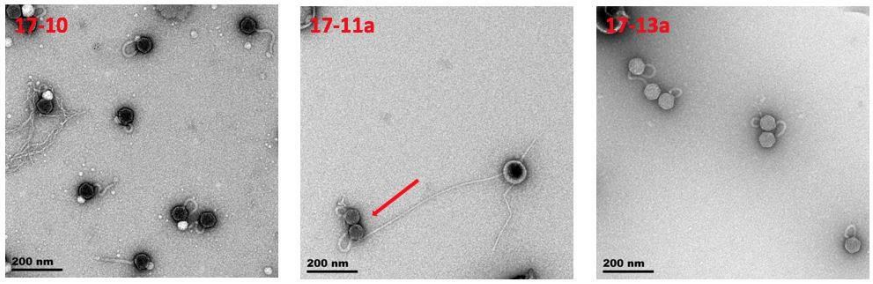


Figure 3.4: Electron micrographs of coliphages with a structural resemblance of phages belonging to the *Siphoviridae* family. Phage images of contaminated samples are indicated with "a". Red arrows indicated possible mixed phage populations and the phage(s) that were measured Images were taken using a FEI Tecnai 20 TEM operating at 200K at a magnification of 100K.

Phages belonging to the families *Inoviridae* and *Rudiviridae* were the two main culprits in co-existing with phage of other families in samples of "mixed" populations. *Inoviridae* phages, known for their long, flexible and filamentous rod-like structures were found to co-exist with four *Siphoviridae* phages (17-3b,17-7b,17-9b and 17-11b; Figure 3.5) and thee *Myoviridae* phages (including samples 16-16b,16-17b and 16-18b; Figure 3.5).Three samples (16-11,16- 12b and 16-15b; Figure 3.5), isolated from goat faeces and Liesbeeck River water, only contained filamentous phages that belonged to the family *Inoviridae*. All the phages found to belong to the family *Inoviridae* were fd type phages which are filamentous and long (up to 2000nm in length) as opposed to MV-L1 type phages which are known for their short rod nature that has one oval end and another end which can acquire different shapes[92,94].

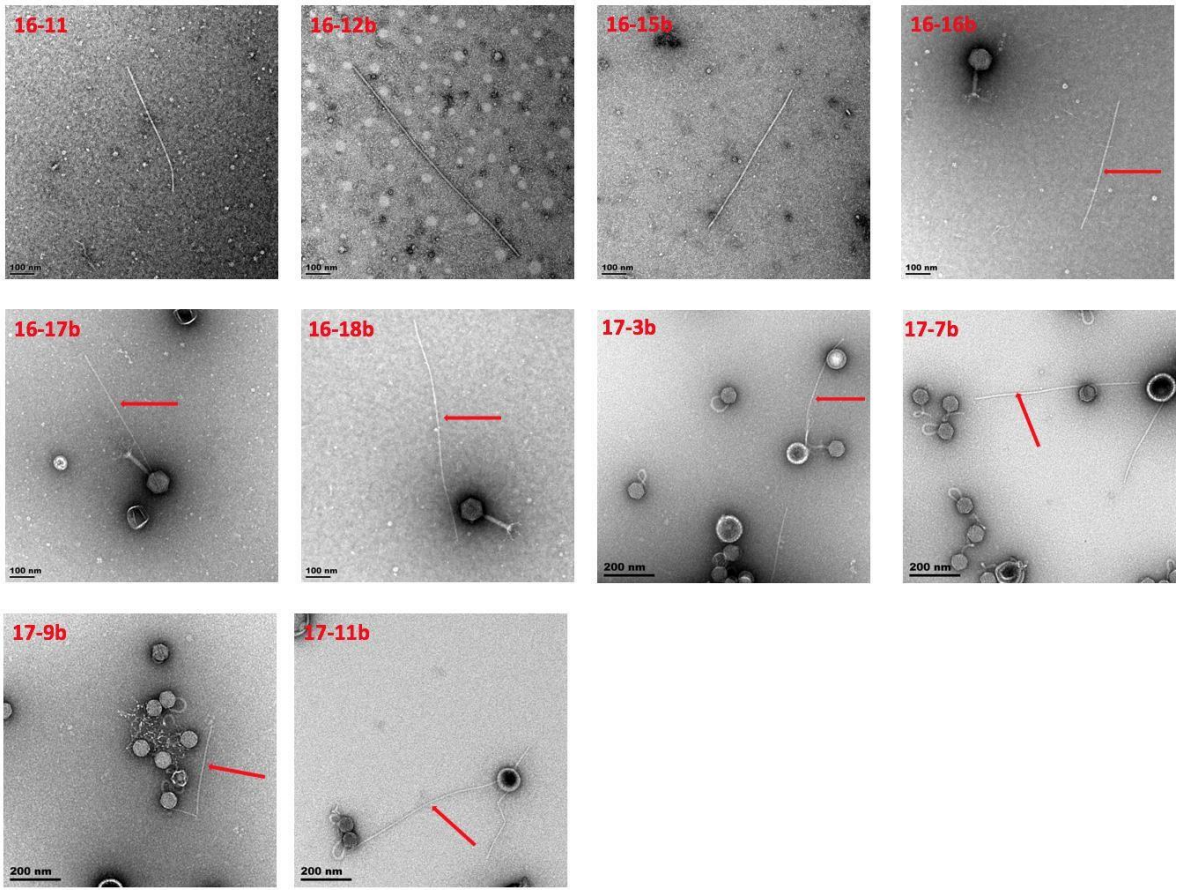


Figure 3.5: Electron micrographs of coliphages with a structural resemblance of phages belonging to the *Inoviridae* family. Phage images of contaminated samples are indicated with "a" and "b". Red arrows indicated possible mixed phage populations and the phage(s) that were measured Images were taken using a FEI Tecnai 20 TEM operating at 200K at a magnification of 100K.

Three samples contained phages belonging to the family *Rudiviridae* (Figure 3.6). These phages were found to be associated with two samples containing *Myoviridae* phages (including samples 17-5b and 17-6b; Figure 3.6) and one sample containing phages that could not be identified (including samples 17-12b; Figure 3.6). Although they both consist of rod-shaped phages, *Inoviridae* and *Rudiviridae* phages are different in that *Rudiviridae* phages are not flexible and are often shorter with a length ranging between 600nm-900nm and are at least 23nm wide.

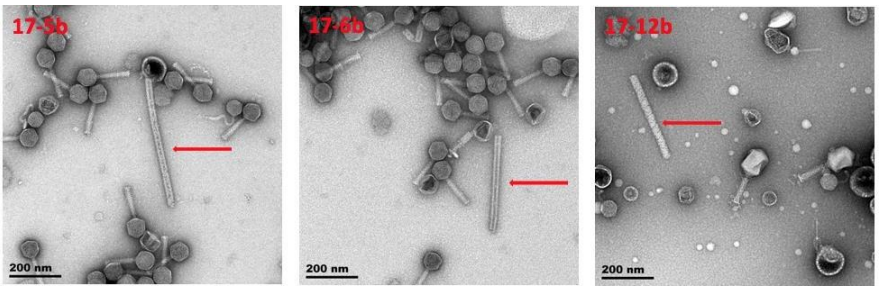


Figure 3.6: Electron micrographs of coliphages with a structural resemblance of phages belonging to the *Rudiviridae* family. Phage images of contaminated samples are indicated with "b". Red arrows indicated possible mixed phage populations and the phage(s) that were measured. Images were taken using a FEI Tecnai 20 TEM operating at 200K at a magnification of 100K.

The next group of phages had dimensions that did not fit with any of the known phage families that have been described in the literature (Figure 3.3 to 3.6 and Table 2.3; Methods Section). Sample 16-14 (Figure 3.7) isolated from river mud harbored a phage with a round head and a diameter of 107nm resembling that of *Myoviridae* phages, however the length of the tail was too short belong to the family *Siphoviridae* nor *Myoviridae* but longer that the family *Podoviridae* hence indicating that the phage does not belong to any of the tailed phage families. The overall shape of the phage resembles that of the *Podoviridae* phages, with a large capsid and relatively short tail. Two samples (including 17-12a and 17-13; Figure 3.7) isolated from Pond Water (site b) each contained phages of similar structure, with capsids resembling those of *Myoviridae* phages except they are more elongated, however the diameter (79 ± 0.7 nm and 80 ± 3 nm; Table 3.3) mirrors that of *Myoviridae* phages which ranges between 50 nm to 110 nm. The tail size of phages from sample 16-13 (116 nm x 18 nm; Table 3.3) resembles that of *Myoviridae* phages whereas the tail size of phages from sample 17-1 (131 ± 59 nm x 11 ± 0.5 nm) is challenging to be categorized since the standard deviation is very high, however taking the length of 131 nm into consideration without the standard deviation and the width size of 11 ± 0.5 nm, these phages would be assigned to *Siphoviridae* family.

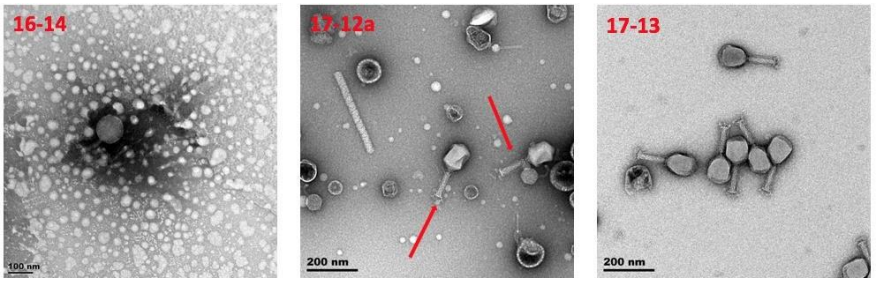


Figure 3.7: Electron micrographs of unidentified coliphages. Phage image of contaminated samples is indicated with "a". Red arrows indicated possible mixed phage populations and the phage(s) that were measured. Images were taken using a FEI Tecnai 20 TEM operating at 200K at a magnification of 100K.

The noise in the background of some micrographs could have been caused by residual bacterial debris resulting from lysate purification and could be reduced by increasing the purification steps of the phage lysates. Individual heads and tails were seen in some samples possible due to mechanical smearing that could have taken place during TEM preparation. Overall, phages isolated in this study were predominantly tailed with 39% of the samples consisting of mixed phage populations. As some most phages were observed to have similar structures, the possibility of genotypic variation exists thus this could be addressed through genotypic assays such as pulsed field gel electrophoresis, restriction fragment polymorphism or sequencing for accurate analysis.

Table 3.3: Morphological prediction of phage families

Phage ID	No. of phage particles measured	Tail Length (nm) Mean (± SD)	Tail Width (nm; mean ± SD)	Capsid meter (nm; mean ± SD)	Proposed Family
16-1	1	103	19	89	*Myoviridae*
16-2	1	118	20	86	
16-3	2	121 (± 9)	13 (± 0)	93 (± 0)	
16-4	12	114 (± 9)	17 (± 3)	83 (± 4)	
16-5	1	124	18	90	
16-6	1	127	19	96	
16-8	1	111	15	92	
16-9	1	117	20	89	
16-10	1	122	18	93	
16-12a	1	118	16	95	
16-15a	1	110	17	98	
16-16a	1	123	18	97	
16-17a	1	107	17	88	
16-18a	1	120	17	89	
17-5a	7	117 (± 5)	21 (± 3)	75 (± 5)	
17-6a	8	115 (± 6)	17 (± 3)	72 (± 4)	
16-7	1	117	16	73	*Siphoviridae*
16-13	1	116	18	80	
17-1	4	131 (± 59)	11 (± 0.5)	65 (± 5)	
17-2	3	153 (± 8)	12 (± 3)	65 (± 1)	
17-3a	5	120 (± 19)	22 (± 27)	66 (± 6)	
17-4	5	149 (± 17)	13 (± 2)	63 (± 4)	
17-7a	6	148 (± 21)	12 (± 2)	63 (± 3)	
17-8	9	131 (± 26)	12 (± 3)	63 (±4)	
17-9a	2	170	12	65	
17-10	8	135 (± 24)	12 (± 2)	60 (± 4)	
17-11a	2	138 (± 43)	13 (± 3)	55 (± 3)	
16-11	1	580	7	None	*Inoviridae*
16-12b	1	1100	8	None	
16-15b	1	671	12	None	
16-16b	1	545	9	None	
16-17b	1	633	8	None	
16-18b	1	923	9	None	
17-3b	1	430	11	None	
17-7b	1	673	9	None	
17-9b	1	380	10	None	
17-11b	1	888	9	Non-existent	
17-5b	1	503	23	None	*Rudiviridae*
17-6b	1	367	30	None	
17-12b	1	350	30	None	
16-14	1	91	16	107	Unidentifiable
17-12a	2	113 (± 2)	22 (± 1)	120 (± 1)	
17-13	6	112 (± 2)	20 (± 2)	121 (± 3)	

Italicized phage numbers are those with mixed populations evident; Alphabet "a" and "b" indicate phage micrographs taken from samples with mixed phage populations.

4 DISCUSSION

UPEC strains are responsible for over 70% of UTIs worldwide,[1] although, many of them have developed resistance to the antibiotics commonly used to treat them[1,2]. The emergence and spread of antibiotic resistance to this common human pathogen thus warrants urgent research into new treatments and effective ways to control it. Phage therapy, focusing on lytic phages against *E. coli*, is one such approach that could be useful in this setting. UPEC strains are a common resident of the human and animal GIT thus sewage water is often enriched with *E. coli* due to the presence of human and animal excreta[164]. In this greater context, this study aimed to isolate and characterize environmental virulent phages against UPEC strains. It was hypothesized that phages infecting UPEC strains could be found in environmental sources with high water content such as dams, rivers, river mud and/or animal faeces. Consequently, 31 environmental samples were screened for the presence of phages against UPEC strains and a total of 41 virulent phages that demonstrated the ability to lyse *E. coli* MG1655 were isolated. To determine their host range, these phages were then screened against a broader panel of UPEC strains of which 31/41 were lytic against these strains and as a result were further characterized morphologically using TEM. Based on morphology, 28/31 isolates were determined to belong to four phage families whilst 3/31 isolates did not fit morphologically into any of the characterized phage groupings. Many phages were tailed, representing the phage families *Myoviridae* and *Siphoviridae*, while a fraction belonged to non-capsid phage families namely *Inoviridae* and *Rudiviridae*.

4.1 Phage isolation and typing

4.1.1 Environmental screening for phages lytic against *E. coli* MG1655

A wide range of environmental samples, collected from seven different locations including Rondebosch, Observatory, Master Organics compost supplier, wastewater treatment plant, Skaapkraal, Qurbaan and Dreyersdal were screened for their presence of lytic phages against *E. coli*. The MG1655 strain of *E. coli* was used to propagate the phages in this first round of screening since it is considered highly susceptible to coliphages as a result of being "cured" of

integrated prophages and is uncomplicated to culture[15,33]. The MG1655 strain used in this study was specifically cured of the F-factor and lambda prophages using acridine orange and UV light respectively[15]. Of environmental samples that were screened, six out of 31 were found to contain phages. Of these samples, dam water from Dreyersdal farm yielded the highest number of phage isolates (n=15/41 phages isolated), followed by raw milk (n=7/41), goat faeces (n=6/41) and river mud (n=5/41). The dam water sample from this study was isolated from a site were most animals from this farm including (ducks, cattle and goats) drink water from and farmers deposit sewage matter. This could have resulted in that large number of phage isolates from this sample since it is a potential dump site for multiple types of animal excreta and an end point for sewage hence increasing the chances of *E. coli* retrieval and the subsequent recovery of coliphages. A number of previous studies have recorded the presence of coliphages targeting WT *E. coli* K-12 strains from environmental water, wastewater treatment plant and lake water[120,162,165]. For instance an *E. coli* K-12 derivative, strain K803, devoid of prophages like the MG1655 strain used in this study, was used to isolate 22 T4 like phages from environmental water in Bangladesh[128].T-4 like phages are notorious for their broad host range and are unequivocally abundant in nature[122,126,128,166].This highlights the abundance of *E. coli* in water-associated ecosystems, confirming one of the assumptions made in this study that natural water sources contain bio-matter that allows for the growth and survival of *E. coli* strains.

The second most abundant environmental sample screened for coliphages was raw cow milk, from Skaapkraal farm, Otterry, from which 7/41 phages were isolated. Although raw cow milk may contain *E. coli*[167,168], coliphages were not expected from raw milk, as there have been no published studies that could be found reporting on isolation of *E. coli*-targeting phages from this source. However numerous studies have reported on the abundance and diversity of phages against *Streptococcus* spp. and Lactic acid-producing bacteria in raw milk[167,169], commonly associated with manufacturing delays, production of a lower quality product, immature product spoilage or product loss altogether, leading to a significant economic loss in the dairy industry[170]. Since some phages have been reported to be polyvalent (defined as being able to infect more than one strain per bacterial species)[166], and in some rarer instances can cross the bacterial species barrier, [145]it is therefore possible for coliphages isolated from raw milk in this study to have a host range extending to lactic acid producing

bacteria and/or streptococcus that also infect *E. coli* MG1655 used to screen in this study. Since polyvalency is often witnessed in phages residing in marine-like environments and infecting the bacterial family *Enterobacteriaceae* of which *E. coli* is a member[145] , it is thus possible for coliphages isolated from raw milk to also have that ability. Additionally, two coliphages AR1 and LG1 have been isolated from raw milk and were to infect two serotypes of *E. coli* and many other enterobacteria strains of different genera including *Shigella dysenteriae*, *P. mirabilis* and two strains of *Salmonella*[171] . Another possibility would be that the raw milk from Schaapkraal farm could have contained *E. coli,* as *E. coli* is recognized as one of the most prevalent food-borne pathogens [172–174]. A study conducted in Pakistan isolated *E. coli* from 57% of raw milk samples tested and 67% of household milk products sampled, using gram staining and culture techniques[166]. Another study undertaken in Mid-western Brazil screened 50 raw milk cheese samples using PCR for toxigenic *E. coli* and 96% of the samples were found to contain 48 different types of *E. coli*. Although several studies have reported the presence of toxigenic *E. coli* strains in raw milk, there is currently no evidence that any of *E. coli* commensal strains, specifically *E. coli* K-12 derivatives have been isolated from raw milk. However, considering that different *E. coli* serogroups do share common phage receptors (of which lam B[175], OmpF, OmpC[137], protein Ia, protein Ib[176] have been best characterized), it is possible for phages infecting one serogroup to able to infect another serogroup.

The environmental sample that yielded the third most abundant coliphages was goat faeces from Schaapkraal farm, Ottery, yielding 6/41 phage isolates. Since other farm animals, including cows, have been shown to harbor *E. coli* and coliphages in their GIT[177–179], the presence of phages in any of the samples with animal faeces was not unexpected. Although there has been no published evidence that goat faeces harbors any of the *E. coli* K-12 derivative strains, it has been established that sheep and goats are reservoirs of shiga-toxin producing *E. coli* (STEC) strains[180,181] and more importantly STEC-specific phages[131,180,182]. As mentioned before that serogoups of gram-negative bacteria such as *E. coli* share phage receptors[166,175,176], it is therefore possible that phages isolated from fecal samples of goat meant to originally target STEC could have been able to recognize some receptors on the surface of the MG1655 strain. It was surprising that only one sample out of 12 containing animal excreta (goat faeces) contained coliphages, also suggesting that the

animals to which the samples belonged either did not harbor *E. coli* or coliphages, or that they were not viable

The absence of phages from the City of Cape Town wastewater treatment plant in Athlone was unexpected as these treatment plants receive households and industrial waste usually in the form of sewage and are designed to remove contaminants and monitor faecal microbial pollutants (including *E. coli* counts) from these sources and treat the water to be recycled. *E. coli* counts tend to be very high in sewage [124,183], so it was expected that these samples would be filled with coliphages[177–179]. This has also been corroborated by a study that isolated two phages (OP7061 and OP10081) that were lytic against *E. coli* MG1655 from an urban sewage water plant called Opfikin in Switzerland[183]. Additionally, 42 virulent phages isolated from sewage water in Australia were all lytic against an *E. coli* K-12 strain derivative of which the MG1655 strain is a member[124]. Since none of the sources from this waste treatment plant contained phages, this may suggest that the coliform count is undetectable or that they are free of *E. coli* and therefore coliphages and that the byproducts from the treated water indeed have minimal contamination as desired by the City of Cape Town municipality. This was however, unanticipated since the coliform count in the effluent of the Athlone wastewater treatment plant has been shown to remain at relatively the same levels (≈1000 counts/100ml), an arguably acceptable level for human contact[184].

The absence of phages in most faeces samples and sewage water was unexpected since literature suggests that coliphages are abundant in these samples however, since most of the phages in this study were isolated from sources with high water content (dam water, river mud, pond water), this supports the assumption made during this study that coliphages would be found in close proximity to their host, where we would also expect to find *E. coli* in abundance.

4.1.2 Phage isolation from environmental samples using *E. coli* MG1655 strain

Although it has previously been suggested that *E. coli* MG1655 is susceptible to a wide range of virulent phages[15], this was not observed in this study as only 19% of the samples that were screened using this strain were confirmed to contain coliphages. Although this was not

confirmed in the MG1655 strain used in this study, there have been reports of newly acquired phage resistance mechanism by the MG1655 strain since its isolation from the *E. coli* K-12 wildtype strain [122,185,186]. *E. coli* and other gram-negative bacteria have been shown to modify or lose their phage receptors altogether to evade phage attack[31]. A mutant *E. coli* K-12 strain derivative that had its FhuA receptor removed through mutations in the N-globular domain (close to the β-barrel channel of FhuA), became resistant to coliphage ϕ80[187]. Another study with *E. coli* K-12 mutant strain derivatives, that had undetectable levels of phage receptor protein 3A due to a defect in the outer membrane composition, were found to be resistant to coliphage K3 [185]. FhuA is not only a phage receptor but also involved in the transport of ferrichrome and antibiotics (including rifamycin and albomycin[187]), whereas protein 3A is involved in conjugation and colicin sensitivity[185]. Other functions of these receptors are compromised as a result of receptor loss[185,187], indicating that bacterial receptor loss can come with a fitness cost to the organism[145]. Regretfully, these mutations are then maintained in the population for generations, when natural selection is imposed by exposure to phages [1,188].

Spontaneous mutations are not the only mechanisms through which *E. coli* can develop resistance. Since most phage receptors are multifunctional, some bacterial hosts use structures such as extracellular polymers or proteins to block phage attachment [145]. Since the polymers or proteins involved in this process are often involved in virulence, they have not yet been well documented in commensal strains of *E. coli*. However, some F^+ strain derivatives of *E. coli* K-12 which uses the F factor for conjugation[189], secrete TraT, a lipoprotein that modifies and disguises the outer membrane protein A (ompA) of *E. coli* which are receptors for multiple T phages[185] hence blocking phage attachment. Additionally, phages also produce molecules that can block their own adsorption into bacterial hosts: T5, produces lipoprotein that binds to the outer membrane FhuA receptor found on *E. coli* K-12 strain derivatives including MG1655[187]. This lipoprotein is produced at the onset of phage infection and is thought to prevent superinfection[185]. As much as bacterial hosts have developed ways to protect themselves against phages, this has not stopped their phage opponents from evolving strategies that counteract these. Another approach that coliphages use to counteract bacterial phage-resistance mechanisms involve genetic recombination with another virulent phage, prophage or a non-phage entity (such as a bacteria). This then results

in the recognition of a different receptor on the same bacterial host[185]. Both these strategies result in an increase in phage infectivity thus expanding the phage host range [145]. These altered phage protein(s) that evolve to interact directly with phage receptors on the surface of bacterial host are termed "anti-receptors", and any changes to them are often confined to small sections of the protein that interact directly with the bacterial host[190–192].

Other less common mechanisms that bacterial hosts use to evade phage infection include the use of restriction enzyme modification and the CRISPR system, which is often observed following exposure to non-lytic phages[193]. Restriction modification systems function by cleaving foreign DNA (including phage DNA), resulting in non-viable phages [116]. The *E. coli* MG1655 strain has been shown to contain restriction modification systems, such as EcoKI type I restriction modification system[194]. Provided that the invading phage does not get methylated by methylase enzymes encoded by the host bacterium, which would render the phage insensitive to the host restriction enzyme, restriction modification systems are often rapid thus protecting the bacterial host against invading phage genetic material[193]. Since CRISPR loci have previously been identified in *E. coli* MG1655 strain [195], it is possible that this system might have played a role in any possible phage resistance in this study. It has been debated for decades that the CRISPR system may not confer such an immune function in *E. coli* because their CRISPR sequences have not changed over time, as would be expected if they were incorporating foreign genetic material from phages[196,197]. However, a pivotal study by Cornelissen A. *et al*.[198] suggested that CRISPR loci played a role in immunity to invading DNA in *E. coli*, using a laboratory strain of *E. coli*, called *E. coli* BL21AI that was protected against lysogenization and prophage induction by lambda phage when transformed with CRISPR encoding plasmids, despite not coding for any known CRISPR loci[195]. All of these phage resistance mechanisms may in part contribute to the rapid evolution of bacterial populations since they involve genetic variation as a means to ensure their survival, and any one of these mechanisms may have played a role in any possible evolution of the MG1655 strain used in this study that may have been accumulated since its derivation from its parental wildtype. Whole genome sequencing of the MG1655 strain used in this study would need to be performed to determine this conclusively. Although the removal of known prophages from the MG1655 *E. coli* strain in the 1990s should have made it highly permissive to phage attack, a previous study described four transducing coliphages that could not form plaques on the

MG1655 strain but could form plaques on the UPEC strains CFT073, UTI89, NU14,536 and J96[162] suggests that in future it may be useful to screen the same environmental samples for phages using classic UPEC organisms which could have increased the chances of isolating more diverse phages.

4.1.3 Phage isolation based on plaque morphology

A total of 41 phages were isolated from separate plaques based on them having different sized and shaped morphologies. Turbid and irregularly-shaped plaques were excluded from the study for the following reasons: (a) turbid plaques often indicate that the phages are temperate and not virulent,[123] and (b) plaques formed by virulent phages are generally round and clear in shape [94,123]. Since the study aimed to isolate virulent phages that could be used for phage therapy, temperate phages were excluded because (a) they could result in the bacterial population acquiring phage resistance through selective pressure from the high titers of phages administered during phage therapy (hence hindering clearance of the bacterial infection being treated in the first place); (b) they could result in the introduction of unfavorable genetic segments such as pathogenicity islands, virulence factors; and (c) in some cases result in super-infection immunity. [70,71,152]

The majority of the plaques selected in this study were clear and transparent. However, three samples (17-10,17-5 and 17-3) all isolated from dam water from Dreyersdal farm, which contained tailed phages) had plaques with a "bull's eye morphology" - with intense turbidity towards the periphery – which has previously been described for T-even phages (like T4)[199]. It has been suggested that this "bull's eye" plaque morphology is a consequence of reduced lytic activity due to the bacterial lawn being over grown, or through inhibition of lysis by the bacterial host[199,200]. The inhibition of phage lysis allows for more time for the phages to mature which resulted in phages being adsorbed to already infected bacterial hosts, especially in cases where the host is present at low concentrations, thereby increasing the phage burst size (which reflects the average number of virions released per infected cell) and lengthening the period of phage dormancy[117,199,200]. Depolymerase activity is another phenomenon characterized by the appearance of a "bull's eye" morphology for plaques[198,201].

Double stranded DNA phages, like those belonging to the tailed families *Myoviridae*, *Siphoviridae* and *Podoviridae*, also encode depolymerases (including dextranases, xylosidases, peptidases and hyaluronidases) which degrade cell surface polymers (such as membrane lipids, peptidoglycan and lipopolysaccharides) such as K30 antigens in the cases of pathogenic strains that make up the extracellular matrix of the biofilm on the surface of *E. coli* strains, which may facilitate diffusion of phages through bacterial biofilms [198,201–203]. Since *E. coli* MG1655 has also been shown to form type 1 fimbrae-mediated biofilms[183], it is thus possible that the *E. coli* MG1655 strain used in this study for phage screening could have produced a biofilm that resulted in the formation of the bull's eye plaque morphology observed for the three phage isolates. However, the formation of a biofilm by *E. coli* MG1655 has only been demonstrated in liquid cultures[183,204–206] and not in the type of double agar assay used in this study. It is possible that these strains behaved differently since the conditions of each assay system were distinct.

Another property of plaque morphology that was observed was varying plaque sizes during the initial phase of phage isolation from environmental samples (using *E. coli* MG1655) and when the host range of each phage was subsequently tested (against ECOR UPEC strains). In general, plaques formed against the MG1655 strain were larger in size than those formed against UPEC strains. Plaque size has been shown to be affected by several factors including virus morphology, whereby phages with larger capsid diameters (such as those belonging to the *Myoviridae* family) result in plaques with smaller diameters than those with smaller capsid diameters (like phages belonging to the *Siphoviridae* family) [117,123]. This is thought to be because larger viruses do not maneuver as easily through the top layer of agar as smaller viruses, which diffuse more freely and rapidly through sloppy agar to infect surrounding susceptible cells hence increasing the size of their plaques[123]. The length of the latent period is another factor thought to negatively affect plaque size. It is reasoned that the more viruses spend time within their bacterial host, the less they'll be able to diffuse and travel to other surrounding hosts [123,207]. Overall phages isolated in this study had varying plaque sizes in terms of size, turbidity and shape, phenotypes that are affected by the biology of the phage. To gain a better understanding of properties of plaque morphologies, understanding phage biology is paramount and critical parameters such latent period and adsorption rate of each phage isolate should be measured in future studies as they are also

implicated in phage therapy.

4.2 Phage host range testing against UPEC strains

One of the advantages of using phages as therapeutics is their typically narrow host range, with most phages infecting only a few bacterial strains generally within the same bacterial genera[116]. To characterize the host range of phages isolated in this study, 11 UPEC strains from the ECOR panel were tested for susceptibility to each of the phage isolates. In total, 31/41 of the isolated phages formed plaques against 7/11 of the UPEC strains. Phages 16-3, 16-4, 16-5 and 16-7 displayed the highest lytic activity against these UPEC isolates, infecting 4/11 UPEC strains. Interestingly, all four phages were isolated from the same raw milk source, which was harvested from cattle farmed at Schaapkraal Farm, Ottery, Cape Town. Although this sample yielded phages with the highest lytic activity against UPEC strains, only two families were represented in these four phages: *Myoviridae* (3/4 phages) and *Siphoviridae* (1/4 phages). It is not unusual for *Myoviridae* phages to have a broad host range, as a previous study investigating the host range of three phages (ACG-C91, ACG-C40 and ACG-M12) against biofilm-forming UPEC strains also found that the *Myoviridae* phages had the broadest host range[119]. Another study investigating the host range of T4 phages found that they lysed 41% of the UPEC isolates that were tested[59]. The high lytic activity of these four phages against UPEC strains possibly indicates how well adapted they are in infecting UPEC strains and that they might be identical especially since three of the (16-3, 16-4 and 16-5) could all infect similar strains. A property that has been previously favored in choosing phage agents in studies similar to this one[120,171,208].

In contrast to these highly lytic phages, ten phages could not infect any of the 11 UPEC strains and three of the UPEC strains were not susceptible to any of the phages tested. Of interest was the tendency of phages that could infect UPEC strains to have similarities in their infectivity pattern. It was rationalized that phages from the same environmental source with the ability to form plaques against similar strains are identical or closely related hence were grouped together as one phage since they appeared to be clones of other samples. Although 31 pure phages isolates were evaluated in this study, it is likely that there were only nine different phages (Table 3.2).

4.2.1 Characteristics of UPEC strains that could influence phage host range

Another factor that could have affected the host range spectrum of the panel of phages tested in this study was the UPEC strains that were used. The strains used in this study were all clinical isolates from the ECOR collection. UPEC strains are primarily found in the gut, another primary reservoir of coliphages[14,209,210], suggesting that some of them might have previously interacted with these phages in the gut. This interaction could have resulted in some of the UPEC strains acquiring prophages that would influence a number of phenotypic traits to the bacterial host. Although this study did not evaluate the presence of prophages in these UPEC isolates, their presence has been demonstrated previously in other UPEC isolates. Analysis of UPEC 26-1, a highly virulent strain of UPEC isolated from a patient with a UTI in Korea, was shown to contain as many as eight prophage sequences[211]. Similarly, genomic annotation of another UPEC strain - UTI89 - revealed the presence of the prophage P4 integrase a gene encoding the integrase protein only utilized by temperate phages when integrating their genomes into their bacterial hosts[212]. The major concern with acquiring prophages is that it has been linked to superinfection immunity in the bacterial hosts, which often inhibits infection by other phages[70,71].

Some phages, like those belonging to the *Siphoviridae* family, can undergo both a lytic and lysogenic lifecycle. It is therefore likely that provided these phages are undergoing the lysogenic lifecycle, they could have a smaller spectrum if some of these UPEC strains are harboring prophages. This could explain why three UPEC strains namely; ECOR-60, ECOR-62 and ECOR-64 used in this study were not susceptible to any of the isolated phage as they could be harboring prophages preventing superinfection. Additionally, two of these strains (ECOR-60 and ECOR-64) are cystitis causing UPEC strains, [213] which have been shown to encode a plasmid pEC14_114 [214] with high sequence similarity to plasmid pUTI89 which has been associated with intracellular invasion. Plasmid pUTI89 is encoded by UPEC UTI89 and is believed to have acquired this plasmid through horizontal gene transfer mediated by phages[215]. Phage transduction has been linked to superinfection immunity[153,154] possibly explaining the lack of susceptibility of these two strains to phages tested in this study. However, this does not account for the resistance of ECOR-62 strain to phage lysis since it is

not a cystitis causing strain. It is clear that genome plasticity is quite common amongst pathogenic strains of *E. coli* and often associated with pathogenesis[214–216].

The two pairs of the CRISPR loci were also found in all of the 72 strains of the ECOR collection[217,218], suggesting that this CRISPR mechanism could have played a role in phage resistance and the limited phage host ranges generally noted in this study. However, the majority of UPEC strains belonging to *E. coli* phylogenetic group B2 do not have the CRISPR system[197]. In the context of this study, it would suggest that strains not belonging to phylogenetic group B2 (including UPEC strain ECOR-11, -14, -40, -48, -50, -71 and -72) would have the CRISPR system that might contribute to phage resistance. However, this was not tested in the current study. Strikingly, strains ECOR-60, -62 and -64, belonging to phylogenetic group B2 were not susceptible to any of the phage isolates tested, even though strains belonging to this group were previously described as being devoid of the CRISPR system[197]. In addition to this UPEC strain produce the K1 capsule, a structure of polysaccharide often associated with septicemia and can block the adsorption of coliphage T7 by secreting this capsule[186].This data suggests that there are other mechanisms by which UPEC strains resist infection by the phages that were isolated or that these UPEC strains are just biologically not susceptible to the phages because they lack the correct entry receptors.

A number of other non-genetic factors could have affected the host range spectrum of phages. For instance, the propagating *E. coli* strain (MG1655 in this study) used to isolate phages could influence whether or not the isolated phages were able to infect other bacterial strains. A study that used the UPEC strain CFT073 used to isolate 49 unique phages, found that the same strain was resistant to an *E. coli* K-12 phage P1[162]. The implication of this being that had phage P1 been isolated using the K-12 strain and had the phage been used to infect strain CFT073, it might have not been susceptible to that phage. Therefore, it is possible that using the *E. coli* MG1655 strain to screen for phages that targeted UPEC strains was not ideal in this study. A UPEC strain should instead have been used to isolate phages from these environmental samples and even to enrich for the phages that were isolated.

Other non-genetic factors that may have influenced phage host range include the

concentration of bacteria that was used, properties of the bacterial culture medium (such as pH or temperature) which may have affected the viability of the phages[123]. Since this study focused on isolating phages from convenient environmental samples in Cape Town that were not yet identified, their ideal conditions of growth were not yet determined. This is something that should be considered once the panel of novel phages have been sequenced and annotated to gain a better understanding of their genetic background and biological properties. In general, phages isolated in this study had a relatively narrow host range. This suggests that they have good potential for use in phage therapeutics. However, their ability to infect an *E. coli* strain, such as MG1655, that was originally isolated from the human gut and therefore represent a human commensal[212] may pose a challenge.

4.3 Characterization of phage morphology by TEM

The majority of the phages screened in this study mapped to four known phage families, with the majority of the phages belonging to the order Caudavirales of tailed phages [92]. Since order Caudavirales makes up approximately 96% of phages in nature[93] it was not a surprise to have isolated many tailed phages. In addition, three samples appeared to contain phages that could not be identified since their morphologies did not match that of any of the previously characterized phages.

The majority of the phages isolated in this study appeared to belong to the phage family *Myoviridae*, previously shown to harbor some of the most well characterized coliphages, including T-even phages[92,93]. There have been several previous studies[59,119,121] that isolated coliphages belonging to this family from natural resources, suggesting their abundance in nature and their ability to survive under diverse environmental conditions, adapted to their target hosts[119]. Despite this study having isolated more phages belonging to the *Myoviridae* family than any of the other phage families, TEM analysis of tailed phages isolated in nature indicated that *Siphoviridae* phages are the most abundant of all tailed phages, making up 61% of the tailed phage group, while *Myoviridae* phages made up only 25% and *Podoviridae* only made up 14%[92]. In line with this, the second most abundant phages identified in this study belonged to the *Siphoviridae* family. Since *Siphoviridae* phages are temperate phages and can therefore undergo both the lytic and lysogenic lifecycles, it is possible that these phages were

underrepresented as they could have resulted in turbid plaque morphologies that were specifically excluded in this study.

The seven phages (16-1 to 16-7; all from raw milk) that could not reproduce plaques were visible by TEM. Of these seven, six were structurally similar and appeared to belong to the phage family *Myoviridae* known for their rigid, contractile tails with tail fibers which they use for adsorption[92] and one belonged to the family *Siphoviridae* known for their long, thin and non-contractile tails which often acquire an S-spiral morphology. Four of these phages (16-3, 16-4, 16-5 and 1-7) had the broadest host range against UPEC strains. The inability of these seven phages to reproducibly form plaques could be attributable to a known mechanism in phages whereby lysis is inhibited. Lysis inhibition has been most commonly described for T phages, belonging to the family *Myoviridae*.[20,63] It often occurs when the number of infected bacterial hosts exceeds that of uninfected hosts, and this state is thought to exist to prolong the latent period of phages to allow for a larger phage burst size[219]. Although this process usually ends up with lysis of the bacterial host, it can delay the process by several hours[219], which does not provide the phage with enough time to lyse the hosts. The loss of phage virulence could have also have resulted in the lack of ability to reproducibly form plaques [123]. For instance, injecting DNA material into the host is a temperature dependent process for Lambda phage and that this phage cannot inject its DNA at temperatures below 22^0C, which results in in a non-successful infection of the bacterial host [220]. Similarly, phage T4, a T-even *Myoviridae* phage that cannot be fully assembled to lyse its host at temperatures below 19^0C
[221]. Considering the vast number of coliphages that exist in a variety of ecosystems and niches, the anticipation was for them to be able to survive in diverse environmental conditions thus their inability to reproduce plaques was unexpected.

One of the requirements in using phages as therapeutics is that the phage lysate has to be thoroughly purified to avoid host protein contamination or phage mixtures. While apparently pure phage preparations were evident in the majority of the samples evaluated, some samples appeared to have mixed phages populations. As this study was focusing on lytic phages only, *Inoviridae* phages were intentionally disregarded since they are generally temperate[92,97] and hence should have turbid plaques. Unfortunately, of these 12 "mixed"

phage samples, 10 were found to contain phages belonging to the *Inoviridae* family, with four of these being a mixed population with *Siphoviridae* phages and three being mixed with *Myoviridae* phages. This "mixed" *Inoviridae* sample may explain why these plaques appeared clear, as turbid *Invoviridae* phage plaques could have been masked by clear plaques formed by *Siphoviridae* phages and *Myoviridae* phages. However, three samples (16-11, 16-12b and 16-15b) only contained *Inoviridae* phages, suggesting a problem with the isolation process. A major cause of mixed phage populations comes from phages that are acquired through contaminated surfaces, often observed in the dairy industry[222,223]. In addition to mixed phage populations, some samples appeared to have either individual phage heads and capsids observed by TEM, that may have resulted from mechanical shearing during the stringing process or from incomplete phage assembly[121].

Despite there are 13 families of phages that have been classified; only four families were identified in this study. This could have been affected by the strain of *E. coli* that was used to screen the environmental samples for the presence of phages. As previously mentioned, the strain of *E. coli* that is used to propagate phages can select for certain types of phage morphologies. Conversely, certain phage morphotypes may not be isolated hence resulting in underrepresentation of some phage families. This has been previously demonstrated whereby phages of several different morphotypes were isolated from the same human stool from an infant with diarrhea, depending on which different indicator bacterial strains were used: when *E. coli* strain C was used as a selecting host strain, phages belonging to the family *Siphoviridae* were isolated[224]; when *E. coli* K803 was used to select, phages with T4-like morphology belonging to the family *Myoviridae* were isolated[128].

4.4 Limitations and prospects

The use of a commensal *E. coli* strain such as MG1655 to isolate pathogenic strains (UPEC) was one of this study's main limitations, as this could have overestimated the number of potential phages that can be used for the treatment of bacterial pathogens that were less permissive to phages and could result in selection of phages that were infectious to human commensal *E. coli* strains. Since it is also known that bacterial strains used to propagate the

phages can affect the types of phages isolated, so using the "wrong" propagating strain may lead to underrepresentation of certain phages and limit potential phage agents that can be used as therapeutics. Future directions for this study will be to use a UPEC strain that has high sequence homology to strains against which the phages are to be isolated. Additionally, this UPEC strain must first be sequenced to ensure that it does not harbor any prophages that may be conducive to superinfection immunity, phage resistance or antibiotic resistance for situations were an antibiotic-phage cocktail is to be used. The use of a UPEC strain to isolate phages is thought to also increase the bacteriolytic spectrum of the phages being isolated [119,121]. In addition to this, performing phage adaptation experiments which aim to aggressively increase the virulence of the phage against the target strain can also increase the phage spectrum of bacteriolytic activity[162,119]. This study also isolated *Siphoviridae* phages, which are known to both virulent and temperate. To differentiate between virulent and temperate *Siphoviridae* phages, they could be sequenced in future to check for the presence of genes that may result in phage integration such integrase, cI and cII gene required for establishment and maintenance of lysogeny to confirm whether they are lytic or temperate[99]. Other critical parameters that may affect phage stability (such pH, temperature, detergents and organic solvents) should also be more carefully controlled for in future to ensure the stability and viability of phages.

Adsorption rate, burst size and latency period are some of the major parameters of phage therapy that need to be measured. Although some of the phage advantages of phages as therapeutics exceed those of antibiotics, it should be noted that phages can be directly immunogenic to humans[70,71]. Prospective studies should focus on identifying immunogenic phage components to alleviate the risk of inducing host immune responses. Phage lysates should be thoroughly purified to ensure that crude phage lysates do not contain endotoxins such LPS that could react with the immune system once administered. Although phages are still more desirable than antibiotics, unlike antibiotics which can be broadly bactericidal or bacteriostatic[142], phages tend to be highly bacterial host-specific and therefore the disease-causing microbes would first have to be isolated and identified from individual patients and then screened for phage sensitivity *in vitro* before they could be administered as treatment[85]. Finally, although the study aimed to conduct DNA sequence analysis of the isolated phages, this could not be achieved due to limited time.

4.5 Concluding remarks

Overall, the data obtained from this study supports the hypothesis that coliphages are abundant in nature and can be enriched using a commensal *E. coli* strain. However, not much could be concluded about their diversity since most of the phages that were isolated were structurally similar, representing only four phage families and one group of phages which could not be identified. An analysis of the phage genomes would have shed more light on the phage diversity and the presence of pathogenicity islands, antibiotic resistance cassettes, and virulence factors, all of which should be considered for phage therapy. The abundance and diversity of coliphages and the easy cultivation techniques for their host makes them ideal to work with as therapeutics since they are relatively easy to isolate from nature and can survive in a vast number of environmental conditions. In addition, there are historically good reasons that make coliphages ideal for phage therapy; Félix d'Hërelle and his colleagues[226] first described the use of coliphages to treat diarrhea caused by *E. coli* with good success rates. Moreover, there have been several reports on the successful use of coliphages for veterinary application in the United Kingdom[227,228]. None of the phages that have been described in the literature are infectious to all known pathogenic strains of *E. coli*, warranting the continued search for a novel highly lytic phage with a narrow host range. To date, most studies have described the lytic activity of coliphages *in vitro*, with only a few studies having done *in vivo* validation in animal models, such as mice and chickens.[227,229] This warrants more in vivo studies that are followed by well-designed clinical trials. The global spread of antibiotic resistance continues to have hard-hitting implications for future of bacterial control that will impact the quality of lives of most humans and cause massive financial loss to health care systems around the world. With the significant recent progress in our understanding of phage biology and our growing appreciation of the advantages of phages compared to antibiotics to control bacterial pathogens in light of growing antibiotic resistance, phage therapy clearly has merit to be explored as therapeutics in the Western world.

5 REFERENCES

1. Kutateladze M, Adamia R. Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends Biotechnol*. 2010;28(12):591-595. doi: 10.1016/j.tibtech.2010.08.001.
2. Magiorakos a, Srinivasan A, Carey RB, et al. Bacteria: An International Expert Proposal for Interim Standard Definitions for Acquired Resistance. *Microbiology*. 2011;18(3):268-281. doi:10.1111/j.1469-0691.2011.03570. x.
3. Chibeu A, Lingohr EJ, Masson L, et al. Bacteriophages with the ability to degrade uropathogenic Escherichia Coli biofilms. *Viruses*. 2012;4(4):471-487. doi:10.3390/v4040471.
4. Ronald A, Ronald A, Nicolle L, et al. The etiology of urinary tract infection: Traditional and emerging pathogens. *Disease-a-Month*. 2003;49(2):71-82. doi:10.1067/mda.2003.8.
5. Malki K, Shapiro JW, Price TK, et al. Genomes of Gardnerella strains reveal an abundance of prophages within the bladder microbiome. *PLoS One*. 2016;11(11):1- 16. doi: 10.1371/journal.pone.0166757.
6. Foxman B, Brown P. Epidemiology of urinary tract infections. *Infect Dis Clin North Am*. 2003;17(2):227-241. doi:10.1016/S0891-5520(03)00005-9.
7. Gilbert NM, O'Brien VP, Lewis AL. Transient microbiota exposures activate dormant Escherichia coli infection in the bladder and drive severe outcomes of recurrent disease. *PLOS Pathog*. 2017;13(3): e1006238. doi: 10.1371/journal.ppat.1006238.
8. Manikandan S, Ganesapandian S, Singh M, Kumaraguru AK. Antimicrobial Susceptibility Pattern of Urinary Tract Infection Causing Human Pathogenic Bacteria. *Asian J Med Sci*. 2011;3(2):56-60.
9. Khan SW, Ahmed A. Uropathogens and their Susceptibility Pattern-a Retrospective Analysis. *Journal-Pakistan Med Assoc*. 2001;51(2):98-99.
10. Sobel JD, Kauffman CA, McKinsey D, et al. Candiduria: A Randomized, Double-Blind Study of Treatment with Fluconazole and Placebo. *Clin Infect Dis*. 2000;30(1):19-24. doi:10.1086/313580.
11. Seminerio JL, Aggarwal G, Sweetser S. 26-Year-Old Man with Recurrent Urinary Tract Infections. *Mayo Clin Proc*. 2011;86(6):557-560. doi:10.4065/mcp.2010.0600.
12. Nicastro J, Wong S, Khazaei Z, Lam P, Blay J, Slavcev RA. *Bacteriophage Applications - Historical Perspective and Future Potential*.; 2016. doi:10.1007/978-3-319-45791-8.
13. Bien J, Sokolova O, Bozko P. Role of uropathogenic escherichia coli virulence factors in

development of urinary tract infection and kidney damage. *Int J Nephrol*. 2012;2012. doi:10.1155/2012/681473.

14. Liu D. *Escherichia Coli*☆. Elsevier Inc.; 2014. doi:10.1016/B978-0-12-801238-3.02291- 1.
15. Blattner FR, Plunkett G, Bloch CA, et al. The Complete Genome Sequence of Escherichia coli K-12. *Science (80-)*. 1997;277(5331):1453 LP- 1462. http://science.sciencemag.org/content/277/5331/1453.abstract.
16. Bekal S, Brousseau R, Masson L, Prefontaine G, Fairbrother J, Harel J. Rapid Identification of < em> Escherichia coli Pathotypes by Virulence Gene Detection with DNA Microarrays. *J Clin Microbiol*. 2003;41(5):2113 LP-2125. http://jcm.asm.org/content/41/5/2113.abstract.
17. Bingen E, Denamur E, Brahimi N, Elion J. Genotyping may provide rapid identification of Escherichia coli K1 organisms that cause neonatal meningitis. *Clin Infect Dis*. 1996;22(1):152-156. http://www.ncbi.nlm.nih.gov/pubmed/8824985.
18. Inabo H, Obanibi H. African journal of biotechnology. *African J Biotechnol*. 2002;5(5):487-489. https://www.ajol.info/index.php/ajb/article/view/137888.
19. Al Sweih N, Jamal W, Rotimi VO. Spectrum and antibiotic resistance of uropathogens isolated from hospital and community patients with urinary tract infections in two large hospitals in Kuwait. *Med Princ Pract*. 2005;14(6):401-407. doi:10.1159/000088113.
20. Amato R. Prevention of recurrent urinary-tract. 1999; 353:7-8.
21. Cardozo L, Lose G, McClish D, Versi E, de Koning Gans H. A systematic review of estrogens for recurrent urinary tract infections: Third report of the hormones and urogenital therapy (HUT) committee. *Int Urogynecol J Pelvic Floor Dysfunct*. 2001;12(1):15-20. doi:10.1007/s001920170088.
22. Mabeck CE. Treatment of uncomplicated urinary tract infection in non-pregnant women. *Postgrad Med J*. 1972;48(556):69-75. doi:10.1136/pgmj.48.556.69.
23. Hilt EE, McKinley K, Pearce MM, et al. Urine is not sterile: Use of enhanced urine culture techniques to detect resident bacterial flora in the adult female bladder. *J Clin Microbiol*. 2014;52(3):871-876. doi:10.1128/JCM.02876-13.
24. Pearce MM, Zilliox MJ, Thomaswhite KJ, et al. HHS Public Access. 2016;213(3):1-22. doi: 10.1016/j.ajog.2015.07. 009.
25. Pearce MM, Hilt EE, Rosenfeld AB. without Urgency Urinary Incontinence the Female Urinary Microbiome: A Comparison of Women with and. 2014;5(4):1-12. doi: 10.1128/mBio.01283-14.Editor.
26. Ruggieri MR, Levin RM, Hanno PM, Witkowski BA, Gill HS, Steinhardt GF. Defective

Antiadherence Activity of Bladder Extracts from Patients with Recurrent Urinary Tract Infection. *J Urol*. 1988;140(1):157-159. doi: https://doi.org/10.1016/S0022- 5347(17)41517-5.

27. Shin H, Lee JH, Kim H, Choi Y, Heu S, Ryu S. Receptor diversity and host interaction of bacteriophages infecting Salmonella enterica Serovar Typhimurium. *PLoS One*. 2012;7(8). doi: 10.1371/journal.pone.0043392.
28. Silverman SJ, Petti AA, Slavov N, et al. Metabolic cycling in single yeast cells from unsynchronized steady-state populations limited on glucose or phosphate. *Proc Natl Acad Sci*. 2010;107(15):6946-6951. doi:10.1073/pnas.1002422107.
29. Ronald A. The etiology of urinary tract infection: Traditional and emerging pathogens. *Disease-a-Month*. 2003;49(2):71-82. doi:10.1016/S0011-5029(03)90001-0.
30. Schaeffer AJ, Rajan N, Cao Q, et al. Host pathogenesis in urinary tract infections. *Int J Antimicrob Agents*. 2001;17(4):245-251. doi:10.1016/S0924-8579(01)00302-8.
31. Bertozzi Silva J, Storms Z, Sauvageau D. Host receptors for bacteriophage adsorption. *FEMS Microbiol Lett*. 2016;363(4):1-11. doi:10.1093/femsle/fnw002.
32. Alicja Niewiadomska JK, Niewiadomska A, Klama J. Polish Journal of Microbiology. *Polish J Microbiol*.2005;54(1):43-48.
33. Liu D. Escherichia coli☆. In: *Reference Module in Biomedical Sciences*. Elsevier; 2014. doi:10.1016/B978-0-12-801238-3.02291-1.
34. Lane MC, Simms AN, Mobley HLT. Complex interplay between type 1 fimbrial expression and flagellum-mediated motility of uropathogenic Escherichia coli. *J Bacteriol*. 2007;189(15):5523-5533. doi:10.1128/JB.00434-07.
35. Sokurenko EV., Hasty DL, Dykhuizen DE. Pathoadaptive mutations: Gene loss and variation in bacterial pathogens. *Trends Microbiol*. 1999;7(5):191-195. doi:10.1016/S0966-842X (99)01493-6.
36. Adiciptaningrum AM, Blomfield IC, Tans SJ. Direct observation of type 1 fimbrial switching. *EMBO Rep*. 2009;10(5):527-532. doi:10.1038/embor.2009.25.
37. Iida K, Mizunoe Y, Wai SN, Yoshida S. Type 1 fimbriation and its phase switching in diarrheagenic Escherichia coli strains. *Clin Diagn Lab Immunol*. 2001;8(3):489-495. doi:10.1128/CDLI.8.3.489-495.2001.
38. Hultgren SJ, Porter TN, Schaeffer AJ, Duncan JL. Role of type 1 pili and effects of phase variation on lower urinary tract infections produced by Escherichia coli. *Infect Immun*. 1985;50(2):370-

377.

39. Avalos Vizcarra I, Hosseini V, Kollmannsberger P, et al. How type 1 fimbriae help Escherichia coli to evade extracellular antibiotics. *Sci Rep*. 2016;6(July 2015):1-13. doi:10.1038/srep18109.

40. Blango MG, Mulvey MA. Persistence of uropathogenic Escherichia coli in the face of multiple antibiotics. *Antimicrob Agents Chemother*. 2010;54(5):1855-1863. doi:10.1128/AAC.00014-10.

41. Billips BK, Schaeffer AJ, Klumpp DJ. Molecular basis of uropathogenic Escherichia coli evasion of the innate immune response in the bladder. *Infect Immun*. 2008;76(9):3891-3900. doi:10.1128/IAI.00069-08.

42. Garcia-del Portillo F, Finlay BB. The varied lifestyles of intracellular pathogens within eukaryotic vacuolar compartments. *Trends Microbiol*. 1995;3(10):373-380. doi:10.1016/S0966-842X(00)88982-9.

43. Billips BK, Schaeffer AJ, Klumpp DJ. Molecular basis of uropathogenic Escherichia coli evasion of the innate immune response in the bladder. *Infect Immun*. 2008;76(9):3891-3900. doi:10.1128/IAI.00069-08.

44. Emody L, Kerényi M, Nagy G. Virulence factors of uropathogenic Escherichia coli. *Int J Antimicrob Agents*. 2003;22(SUPPL. 2). doi:10.1016/S0924-8579(03)00236-X.

45. Keane WF, Welch R, Gekker G, Peterson PK. Mechanism of Escherichia coli alpha-hemolysin-induced injury to isolated renal tubular cells. *Am J Pathol*. 1987;126(2):350-357. http://www.ncbi.nlm.nih.gov/pubmed/3030115%5Cnhttp://www.pubmedcentral.nih .gov/articlerender.fcgi?artid=PMC1899561. doi:10.1128/jb.172.11.6175-6181.1990.

46. Lee JH, Subhadra B, Son Y-J, et al. Phylogenetic group distributions, virulence factors and antimicrobial resistance properties of uropathogenic *Escherichia coli* strains isolated from patients with urinary tract infections in South Korea. *Lett Appl Microbiol*. 2016;62(1):84-90. doi:10.1111/lam.12517.

47. Ambroz J, Reissbrodt R, Fruth A, Kriz V. High Prevalence of Multidrug Resistance and Random Distribution of Mobile Genetic Elements Among Uropathogenic Escherichia coli (UPEC) of the Four Major Phylogenetic Groups. 2006; 53:158-162. doi:10.1007/s00284-005-0501-4.

48. Sahm DF, Thornsberry C, Mayfield DC, Jones ME. Multidrug-resistant urinary tract isolates of *Escherichia coli*: prevalence and patient demographics in the United States in 2000. *Antimicrob Agents Chemother*. 2001;45(5):1402-1406. doi:10.1128/AAC.45.5.1402.

49. Sillankorva S, Oliveira D, Moura A, et al. Efficacy of a broad host range lytic bacteriophage

against *E. coli* adhered to urothelium. *Curr Microbiol*. 2011;62(4):1128- 1132. doi:10.1007/s00284-010-9834-8.

50. Ramos NL, Sekikubo M, Dzung DTN, et al. Uropathogenic Escherichia coli isolates from pregnant women in different countries. *J Clin Microbiol*. 2012;50(11):3569-3574. doi:10.1128/JCM.01647-12.

51. Galtier M, De Sordi L, Maura D, et al. Bacteriophages to reduce gut carriage of antibiotic resistant uropathogens with low impact on microbiota composition. *Environ Microbiol*. 2016;18(7):2237-2245. doi:10.1111/1462-2920.13284.

52. Vélez Echeverri C, Serna-Higuita LM, Serrano AK, et al. Resistance profile for pathogens causing urinary tract infection in a pediatric population, and antibiotic treatment response at a university hospital, 2011. Colomb Mexica (Cali, colomb.) 2014;45(1):39-44.

53. *Immunity*. 2008;29(2):283-294. doi: 10.1016/j.immuni.2008.06.011.

54. Downey GP, Botelho RJ, Butler JR, et al. Phagosomal maturation, acidification, and inhibition of bacterial growth in nonphagocytic cells transfected with Fc gamma RIIA receptors. *J Biol Chem*. 1999;274(40):28436-28444. doi:10.1074/jbc.274.40.28436.

55. Verbeken G, Pirnay JP, Lavigne R, et al. Call for a dedicated European legal framework for bacteriophage therapy. *Arch Immunol Ther Exp (Warsz)*. 2014;62(2):117-129. doi:10.1007/s00005-014-0269-y.

56. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. 2011;1(2):66-85. doi:10.4161/bact.1.2.15845.

57. Kutter E, Vos D De, Gvasalia G, et al. Phage Therapy in Clinical Practice: Treatment of Human Infections. 2010:69-86.

58. Abul-Hassan H.S., El-Tahan k Massoud B. GR. Bacteriophage therapy of pseudomonas burn wound sepsis. *Ann MBC*. 1990;3(4). http://www.medbc.com/annals/review/vol_3/num_4/text/vol3n4p262.htm.

59. Markoishvili K, Tsitlanadze G, Katsarava R, Morris JG, Sulakvelidze A. A novel sustained-release matrix based on biodegradable poly (ester amide)s and impregnated with bacteriophages and an antibiotic show promise in management of infected venous stasis ulcers and other poorly healing wounds. *Int J Dermatol*. 2002;41(7):453-458. doi:10.1046/j.1365-4362.2002.01451. x.

60. Lavigne R, Darius P, Summer EJ, et al. Classification of *Myoviridae* bacteriophages using protein sequence similarity. *BMC Microbiol*. 2009; 9:1-16. doi:10.1186/1471- 2180-9-224.

61. Adriaenssens EM, Edwards R, Nash JHE, et al. Integration of genomic and proteomic analyses in the classification of the *Siphoviridae* family. *Virology*. 2015; 477:144-154. doi:

10.1016/j.virol.2014.10.016.

62. Krupovic M, Dutilh BE, Adriaenssens EM, et al. Taxonomy of prokaryotic viruses: update from the ICTV bacterial and archaeal virus's subcommittee. *Arch Virol*. 2016;161(4):1095-1099. doi:10.1007/s00705-015-2728-0.

63. Ackermann H. 5500 Phages examined in the electron microscope Brief Review. 2007:227-243. doi:10.1007/s00705-006-0849-1.

64. Ackermann HW. Bacteriophage observations and evolution. *Res Microbiol*. 2003;154(4):245-251. doi:10.1016/S0923-2508(03)00067-6.

65. Bradley DE. Ultrastructure of bacteriophage and bacteriocins. *Bacteriol Rev*. 1967;31(4):230-314.

66. Rakonjac J, Bennett NJ, Spagnuolo J, Gagic D, Russel M. Filamentous bacteriophage: biology, phage display and nanotechnology applications. *Curr Issues Mol Biol*. 2011;13(2):51-76. doi:10.1002/9780470015902.a0000777.

67. Hoffmann-Berling H, Mazé R. Release of male-specific bacteriophages from surviving host bacteria. *Virology*. 1964;22(3):305-313. doi: https://doi.org/10.1016/0042- 6822(64)90021-2.

68. THE BIOLOGY OF FILAMENTOUS PHAGE INFECTION. 2004.

69. Díaz-Muñoz SL, Koskella B. *Bacteria-Phage Interactions in Natural Environments*. Vol 89. 1st ed. Elsevier Inc.; 2014. doi:10.1016/B978-0-12-800259-9.00004-4.

70. Lamont I, Richardson H, Carter DR, Egan JB. Genes for the establishment and maintenance of lysogeny by the temperate coliphage 186. *J Bacteriol*.1993;175(16):5286-5288. doi:10.1128/jb.175.16.5286-5288.1993.

71. Fortier L-C, Sekulovic O. Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence*. 2013;4(5):354-365. doi:10.4161/viru.24498.

72. Tyler JS, Beeri K, Reynolds JL, et al. Prophage Induction Is Enhanced and Required for Renal Disease and Lethality in an EHEC Mouse Model. 2013;9(3). doi: 10.1371/journal.ppat.1003236.

73. Krupovic M, Prangishvili D, Hendrix RW, Bamford DH. Genomics of Bacterial and Archaeal Viruses: Dynamics within the Prokaryotic Virosphere. *Microbiol Mol Biol Rev*. 2011;75(4):610-635. doi:10.1128/MMBR.00011-11.

74. Ogura Y, Ooka T, Itoh T, Sawaguchi A, Nakayama K, Hayashi T. The Defective Prophage Pool of Escherichia coli O157: Prophage – Prophage Interactions Potentiate Horizontal Transfer of Virulence Determinants. 2009;5(5). doi: 10.1371/journal.ppat.1000408.

75. Ventura M, Canchaya C, Pridmore RD, Bru H. The prophages of Lactobacillus johnsonii NCC 533:

comparative genomics and transcription analysis. 2004; 320:229-242. doi: 10.1016/j.virol.2003.11.034.

76. Rabinovich L, Sigal N, Borovok I, Nir-paz R, Herskovits AA. Prophage Excision Activates Listeria Competence Genes that Promote Phagosomal Escape and Virulence. *Cell*. 2012;150(4):792-802. doi: 10.1016/j.cell.2012.06.036.

77. Ventura M, Turroni F, Lima-Mendez G, et al. Comparative analyses of prophage-like elements present in bifidobacterial genomes. *Appl Environ Microbiol*. 2009;75(21):6929-6936. doi:10.1128/AEM.01112-09.

78. Fortier LC, Moineau S. Morphological and genetic diversity of temperate phages in Clostridium difficile. *Appl Environ Microbiol*. 2007;73(22):7358-7366. doi:10.1128/AEM.00582-07.

79. Tomasz M. Mitomycin C: small, fast and deadly (but very selective). 1995.

80. Manuscript A, Structures T. NIH Public Access. 2009;6(617):247-253. doi: 10.1111/j.1743-6109.2008.01122.x.Endothelial.

81. Juul N, Timmerman E, Gevaert K, Christiansen G, Birkelund S. Proteolytic cleavage of the. *Proteomics*. 2007;72(1):4477-4487.

82. Ripp S, Miller R. Dynamics of the psuedolysogenic response in slowly growing cells of Pseudomonas aeruginosa. *Microbiology*. 1998;144(1 998):2225-2232.

83. Ripp S, Miller R V. The role of pseudolysogeny in bacteriophage-host interactions in a natural freshwater environment. *Microbiology*. 1997;143(6):2065-2070. doi:10.1099/00221287-143-6-2065.

84. Cenens W, Mebrhatu MT, Makumi A, et al. Expression of a Novel P22 ORFan Gene Reveals the Phage Carrier State in Salmonella Typhimurium. *PLoS Genet*. 2013;9(2). doi: 10.1371/journal.pgen.1003269.

85. Twort FW. AN INVESTIGATION ON THE NATURE OF ULTRA-MICROSCOPIC VIRUSES. *Lancet*.1915;186(4814):1241-1243.doi:https://doi.org/10.1016/S0140-6736(01)20383-3.

86. Williams AT, Rush ME, Lipner H. Isolation and preliminary characterization of inhibin- f. *Adv Exp Med Biol*. 1979;112(1955):429-435.

87. Hyman P, Abedon ST. *Bacteriophage Host Range and Bacterial Resistance.* Vol 70. 1st ed. Elsevier Inc.; 2010. doi:10.1016/S0065-2164(10)70007-1.

88. Abedon ST. *Chapter 1 Phage Evolution and Ecology*. Vol 67. 1st ed. Elsevier Inc.; 2009. doi:10.1016/S0065-2164(08)01001-0.

89. Mesosomes B, Artifacts MD. Hicrobinlngy Bacterial Mesosomes: Method Dependent Artifacts.

J Bacteriol. 1981:19-22.

90. Chibeu A, Lingohr EJ, Masson L, et al. Bacteriophages with the ability to degrade uropathogenic Escherichia Coli biofilms. *Viruses*. 2012;4(4):471-487. doi:10.3390/v4040471.

91. Nishikawa H, Yasuda M, Uchiyama J, et al. T-even-related bacteriophages as candidates for treatment of Escherichia coli urinary tract infections. *Arch Virol*. 2008;153(3):507-515. doi:10.1007/s00705-007-0031-4.

92. Battaglioli EJ, Baisa GA, Weeks AE, Schroll RA, Hryckowian AJ, Welch RA. Isolation of Generalized Transducing Bacteriophages for Uropathogenic Strains of Escherichia coli □ †. 2011;77(18):6630-6635. doi:10.1128/AEM.05307-11.

93. Morona R, Henning U. Host range mutants of bacteriophage Ox2 can use two different outer membrane proteins of Escherichia coli K-12 as receptors. *J Bacteriol*. 1984;159(2):579-582.

94. Jurczak-Kurek A, Gąsior T, Nejman-Faleńczyk B, et al. Biodiversity of bacteriophages: morphological and biological properties of a large group of phages isolated from urban sewage. *Sci Rep*. 2016;6(October):34338. doi:10.1038/srep34338.

95. Hancock REW, Reeves P. Bacteriophage resistance in Escherichia coli K 12: general pattern of resistance. *J Bacteriol*. 1975;121(3):983-993.

96. Dalmasso M, Strain R, Neve H, et al. Three new Escherichia coli phages from the human gut show promising potential for phage therapy. *PLoS One*. 2016;11(6):1-16. doi: 10.1371/journal.pone.0156773.

97. Morita M, Tanji Y, Mizoguchi K, Akitsu T, Kijima N, Unno H. Characterization of a virulent bacteriophage specific for Escherichia coli O157:H7 and analysis of its cellular receptor and two tail fiber genes. *FEMS Microbiol Lett*. 2002;211(1):77-83. doi:10.1016/S0378-1097(02)00656-0.

98. Chatterjee S, Rothenberg E. Interaction of bacteriophage λ with Its *E. coli* receptor, LamB. *Viruses*. 2012;4(11):3162-3178. doi:10.3390/v4113162.

99. Chibani-Chennoufi S, Sidoti J, Bruttin A, et al. Isolation of Escherichia coli bacteriophages from the stool of pediatric diarrhea patients in Bangladesh. *J Bacteriol*. 2004;186(24):8287-8294. doi:10.1128/JB.186.24.8287-8294.2004.

100. Łusiak-Szelachowska M, Weber-Dąbrowska B, Jończyk-Matysiak E, Wojciechowska R, Górski A. Bacteriophages in the gastrointestinal tract and their implications. *Gut Pathog*. 2017;9(1):44. doi:10.1186/s13099-017-0196-7.

101. Shousha A, Awaiwanont N, Sofka D, et al. Bacteriophages Isolated from Chicken Meat and the Horizontal Transfer of Antimicrobial Resistance Genes. *Appl Environ Microbiol*.

2015;81(14):4600-4606. doi:10.1128/AEM.00872-15.

102. Kudva IT, Jelacic S, Tarr PI, Youderian P, Hovde CJ. Biocontrol of Escherichia coli O157 with O157-specific bacteriophages. *Appl Environ Microbiol*. 1999;65(9):3767-3773. doi:10.1128/aem.70.6.3417-3424.2004.

103. Beheshti Maal K, Delfan AS, Salmanizadeh S. Isolation and identification of two novel Escherichia coli bacteriophages and their application in wastewater treatment and coliform's phage therapy. *Jundishapur J Microbiol*. 2015;8(3). doi:10.5812/jjm.14945.

104. Demerec M, Fano U. Bacteriophage-resistant mutants in escherichia coli. *Genetics*. 1945;30(2):119-136.

105. Abedon ST. The murky origin of snow white and her t-even dwarfs. *Genetics*. 2000;155(2):481-486.

106. Luria_&_Delbruck_1943_Genetics_28-491.pdf.

107. Chater K. The emergence of bacterial genetics. *Trends Genet*. 1991;7(2):68. doi:10.1016/0168-9525(91)90236-J.

108. Brüssow H. Bacteriophage–host interaction: from splendid isolation into a messy reality. *Curr Opin Microbiol*. 2013;16(4):500-506. doi: 10.1016/j.mib.2013.04.007.

109. Drexler H. Bacteriophage T1. In: Calendar R, ed. *The Bacteriophages*. Boston, MA: Springer US; 1988:235-258. doi:10.1007/978-1-4684-5424-6_7.

110. May R. G E N E T I C S T U D I E S O F LYSOGENICITY I N. 1953;(490):51-64.

111. Lambda phage: a complex of operons - An Introduction to Genetic Analysis - NCBI Bookshelf.

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

113. Pankey GA, Sabath LD. Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections. *Clin Infect Dis*. 2004;38(6):864-870. doi:10.1086/381972.

114. Stratton CW. Dead bugs don't mutate: Susceptibility issues in the emergence of bacterial resistance. *Emerg Infect Dis*. 2003;9(1):10-16. doi:10.3201/eid0901.020172.

115. Carlton RM. Phage therapy: past history and future prospects. *Arch Immunol Ther Exp (Warsz)*. 1999;47(5):267-274. doi:10.2217/fvl.15.3.

116. Hyman P, Abedon ST. *Chapter 7 - Bacteriophage Host Range and Bacterial Resistance*. Vol 70. Elsevier Inc.; 2010. doi:10.1016/S0065-2164(10)70007-1.

117. Merril CR, Biswas B, Carltont R, et al. Long-circulating bacteriophage as antibacterial agents

(phage/bacteria/reticuloendothelial system/toxins/antibiotic resistance). *Microbiology*. 1996;93(April):3188-3192.doi:10.1073/pnas.93.8.3188.

118. Health B. *Bacteriophages in Health and Disease*.; 2012. doi:10.1079/9781845939847.0000.

119. Clark JR, March JB. Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. *Trends Biotechnol*. 2006;24(5):212-218. doi: 10.1016/j.tibtech.2006.03.003.

120. Inchley CJ. The activity of mouse Kupffer cells following intravenous injection of T4 bacteriophage. *Clin Exp Immunol*. 1969;5:173-187.

121. Halpern BN. The Role and Function of the Reticulo-Endothelial System in Immunological Processes. *J Pharm Pharmacol*. 1959;11(1):321-338. doi:10.1111/j.2042-7158. 1959.tb12560. x.

151. Edition S. *Microbial Toxins*.; 2017. doi:10.1007/978-94-007-6725-6.

152. Drulis-Kawa Z, Majkowska-Skrobek G, Maciejewska B, Delattre A-S, Lavigne R. Learning from Bacteriophages - Advantages and Limitations of Phage and Phage- Encoded Protein Applications. *Curr Protein Pept Sci*. 2012;13(8):699-722. doi:10.2174/138920312804871193.

153. Scott JR, Arber W, Falkow S, et al. Immunity and Repression in Bacteriophages P1 and P7. 1980; 90:49-65.doi:10.1007/978-3-642-67717-5_3.

154. Kliem M, Dreiseikelmann B. The superimmunity gene sim of bacteriophage P1 causes superinfection exclusion. *Virology*. 1989;171(2):350-355. doi:10.1016/0042-6822(89)90602-8.

155. Gill JJ, Hyman P. Phage Choice,Isolation,and Preparation for Phage Therapy. 2010:2- 14.

156. Foxman B, Barlow R, D'Arcy H, Gillespie B, Sobel JD. Urinary tract infection: Self- reported incidence and associated costs. *Ann Epidemiol*. 2000;10(8):509-515. doi:10.1016/S1047-2797(00)00072-7.

157. Clokie MRJ, Millard AD, Letarov A V., Heaphy S. Phages in nature. *Bacteriophage*. 2011;1(1):31-45. doi:10.4161/bact.1.1.14942.

158. Richard C, Katia R, Dominik M, Alex R. Associations among Antibiotic and and Clinical Escherichia coli Isolates. 2017;8(5):0-13.

159. Lee SH, Onuki M, Satoh H, Mino T. Isolation, characterization of bacteriophages specific to Microlunatus phosphovorus and their application for rapid host detection. *Lett Appl Microbiol*. 2006;42(3):259-264. doi:10.1111/j.1472-765X.2006.01840. x.

160. WOLFES H, FLIESS A, PINGOUD A. A comparison of the structural requirements for DNA

cleavage by the isoschizomers HaeIII, BspRI and BsuRI. *Eur J Biochem*. 1985;150(1):105-110. doi:10.1111/j.1432-1033. 1985.tb08994. x.

161. Canchaya C, Desiere F, McShan WM, Ferretti JJ, Parkhill J, Brüssow H. Genome analysis of an inducible prophage and prophage remnants integrated in the Streptococcus pyogenes strain SF370. *Virology*. 2002;302(2):245-258. doi:10.1006/viro.2002.1570.

162. Battaglioli EJ, Baisa GA, Weeks AE, Schroll RA, Hryckowian AJ, Welch RA. Isolation of generalized transducing bacteriophages for uropathogenic strains of Escherichia coli. *Appl Environ Microbiol*. 2011;77(18):6630-6635. doi:10.1128/AEM.05307-11.

163. King AMQ, Adams MJ, Carstens EB, Lefkowitz EJBT-VT, eds. Family - *Myoviridae*. In: San Diego: Elsevier; 2012:46-62. doi: https://doi.org/10.1016/B978-0-12-384684- 6.00002-1.

164. Piracha ZZ, Saeed U, Khurshid A, Chaudhary WN. Isolation and Partial Characterization of Virulent Phage Specific against Pseudomonas Aeruginosa. *Glob J Med Res*. 2014;14(1):1-8. http://medicalresearchjournal.org/index.php/GJMR/article/view/521/439.

165. Sano E, Carlson S, Wegley L, Rohwer F. Movement of Viruses between Biomes. 2004;70(10):5842-5846. doi:10.1128/AEM.70.10.5842.

166. Brüssow H. Phage therapy: The Escherichia coli experience. *Microbiology*. 2005;151(7):2133-2140. doi:10.1099/mic.0.27849-0.

167. del Rio B, Binetti AG, Martín MC, Fernández M, Magadán AH, Alvarez MA. Multiplex PCR for the detection and identification of dairy bacteriophages in milk. *Food Microbiol*. 2007;24(1):75-81. doi:10.1016/j.fm.2006.03.001.

168. Paneto BR, Schocken-Iturrino RP, Macedo C, Santo E, Marin JM. Occurrence of toxigenic Escherichia coli in raw milk cheese in Brazil. *Arq Bras Med Vet e Zootec*. 2007;59(2):508-512. doi:10.1590/S0102-09352007000200035.

169. Madera C, Monjard??n C, Su??rez JE. Milk contamination and resistance to processing conditions determine the fate of Lactococcus lactis bacteriophages in dairies. *Appl Environ Microbiol*. 2004;70(12):7365-7371. doi:10.1128/AEM.70.12.7365-7371.2004.

170. Samson JE, Moineau S. Bacteriophages in Food Fermentations: New Frontiers in a Continuous Arms Race. *Annu Rev Food Sci Technol*. 2013;4(1):347-368. doi:10.1146/annurev-food-030212-182541.

171. Zuber S, Ngom-Bru C, Barretto C, Bruttin A, Brüssow H, Denou E. Genome analysis of phage JS98 defines a fourth major subgroup of T4-like phages in Escherichia coli. *J Bacteriol*. 2007;189(22):8206-8214. doi:10.1128/JB.00838-07.

172. Brzuszkiewicz E, Thürmer A, Schuldes J, et al. Genome sequence analyses of two isolates from the recent Escherichia coli outbreak in Germany reveal the emergence of a new pathotype: Entero-Aggregative-Haemorrhagic Escherichia coli (EAHEC). *Arch Microbiol*. 2011;193(12):883-891. doi:10.1007/s00203-011-0725-6.

173. Gutiérrez D, Rodríguez-Rubio L, Martínez B, Rodríguez A, García P. Bacteriophages asWeapons Against Bacterial Biofilms in the Food Industry. *Front Microbiol*. 2016;7(June):1-15. doi:10.3389/fmicb.2016.00825.

174. Hayashi T, Makino K, Ohnishi M, et al. Complete Genome Sequence of Enterohemorrhagic Eschelichia coli O157: H7 and Genomic Comparison with a Laboratory Strain K-12. 2001; 22:11-22.

175. Randall Hazelbauer L, Schwartz M. Isolation of the bacteriophage lambda receptor from Escherichia coli. *J Bacteriol*. 1973;116(3):1436-1446.

176. Riede I, Eschbach ML. Evidence that TraT interacts with OmpA of Escherichia coli. *FEBS Lett*. 1986;205(2):241-245. doi:10.1016/0014-5793(86)80905-X.

177. Hallewell J, Niu YD, Munns K, et al. Differing populations of endemic bacteriophages in cattle shedding high and low numbers of escherichia coli O157:H7 bacteria in feces. *Appl Environ Microbiol*. 2014;80(13):3819-3825. doi:10.1128/AEM.00708-14.

178. McLaughlin MR, King RA. Characterization of Salmonella bacteriophages isolated from swine lagoon effluent. *Curr Microbiol*. 2008;56(3):208-213. doi:10.1007/s00284-007- 9057-9.

179. McLaughlin MR, Balaa MF, Sims J, King R. Isolation of Salmonella Bacteriophages from Swine Effluent Lagoons Journal article number J-10632 of the Mississippi Agricultural and Forestry Experiment Station. Mention of a trade name, proprietary product, or specific equipment does not constitute a gu. *J Environ Qual*. 2006; 35:522-528. doi:10.2134/jeq2005.0080.

180. Beutin L, Geier D, Steinruck H, Zimmermann S, Scheutz F. Prevalence and Some Properties of Verotoxin.pdf. 1993;31(9):2483-2488.

181. Ain D. Faith, Technology, and the Afterlife: The Death of Death in the 21st Century. *Conserv Jud*. 2009;61(1-2):21-32. doi:10.1353/coj.0.0003.

182. Calci KR, Burkhardt W, Watkins WD, Rippey SR. Occurrence of male-specific bacteriophage in feral and domestic animal wastes, human feces, and human- associated wastewaters. *Appl Environ Microbiol*. 1998;64(12):5027-5029.

183. Lacqua A, Wanner O, Colangelo T, Martinotti MG, Landini P. Emergence of biofilm- forming subpopulations upon exposure of Escherichia coli to environmental bacteriophages. *Appl Environ Microbiol*. 2006;72(1):956-959. doi:10.1128/AEM.72.1.956-

959.2006.

184. Black River E. Coli Counts (per 100ml). 2005:3-5.

185. Manning PA, Puspurs A, Reeves P. Outer membrane of Escherichia coli K-12: Isolation of mutants with altered protein 3A by using host range mutants of bacteriophage K3. *J Bacteriol*. 1976;127(3):1080-1084.

186. Scholl D, Adhya S, Merril C. Escherichia coli K1's capsule is a barrier to bacteriophage T7. *Appl Environ Microbiol*. 2005;71(8):4872-4874. doi:10.1128/AEM.71.8.4872- 4874.2005.

187. Langenscheid J, Killmann H, Braun V. a FhuA mutant of Escherichia coli is infected by phage T1-independent of TonB. *FEMS Microbiol Lett*. 2004;234(1):133-137. doi: 10.1016/j.femsle.2004.03.019.

188. Oechslin F. Resistance development to bacteriophages occurring during bacteriophage therapy. *Viruses*. 2018;10(7). doi:10.3390/v10070351.

189. Phornphisutthimas S, Thamchaipenet A, Panijpan B. Conjugation in Escherichia coli. *Biochem Mol Biol Educ*. 2007;35(6):440-445. doi:10.1002/bambed.113.

190. Thomassen E, Gielen G, Schütz M, et al. The Structure of the Receptor-binding Domain of the Bacteriophage T4 Short Tail Fibre Reveals a Knitted Trimeric Metal- binding Fold. *J Mol Biol*. 2003;331(2):361-373. doi: https://doi.org/10.1016/S0022- 2836(03)00755-1.

191. Montag D, Hashemolhosseini S, Henning U. Receptor-recognizing proteins of T-even type bacteriophages: The receptor-recognizing area of proteins 37 of phages T4 TuIa and TuIb. *J Mol Biol*. 1990;216(2):327-334. doi: https://doi.org/10.1016/S0022- 2836(05)80324-9.

192. Drexler K, Riede I, Montag D, Eschbach M-L, Henning U. Receptor specificity of the Escherichia coli T-even type phage Ox2: Mutational alterations in host range mutants. *J Mol Biol*. 1989;207(4):797-803. doi: https://doi.org/10.1016/0022-2836(89)90245-3.

193. Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. *Nat Rev Microbiol*. 2010; 8:317. http://dx.doi.org/10.1038/nrmicro2315.

194. Roer L, Aarestrup FM, Hasman H. The EcoKI type I restriction-modification system in Escherichia coli affects but is not an absolute barrier for conjugation. *J Bacteriol*. 2015;197(2):337-342. doi:10.1128/JB.02418-14.

195. Edgar R, Qimron U. The Escherichia coli CRISPR system protects from λ lysogenization, lysogens, and prophage induction. *J Bacteriol*. 2010;192(23):6291-6294. doi:10.1128/JB.00644-10.

196. Touchon M, Rocha EPC. The small, slow and specialized CRISPR and anti-CRISPR of Escherichia and Salmonella. *PLoS One*. 2010;5(6). doi: 10.1371/journal.pone.0011126.

197. Touchon M, Charpentier S, Clermont O, Rocha EPC, Denamur E, Branger C. CRISPR distribution within the Escherichia coli species is not suggestive of immunity- associated diversifying selection. *J Bacteriol*. 2011;193(10):2460-2467. doi:10.1128/JB.01307-10.

198. Cornelissen A, Ceyssens PJ, Krylov VN, Noben JP, Volckaert G, Lavigne R. Identification of EPS-degrading activity within the tail spikes of the novel Pseudomonas putida phage AF. *Virology*. 2012;434(2):251-256. doi: 10.1016/j.virol.2012.09.030.

199. Abedon ST, ed. Phages, ecology, evolution. In: *Bacteriophage Ecology: Population Growth, Evolution, and Impact of Bacterial Viruses*. Advances in Molecular and Cellular Microbiology. Cambridge: Cambridge University Press; 2008:1-28. doi: DOI: 10.1017/CBO9780511541483.004.

200. Abedon ST. Selection for lysis inhibition in bacteriophage. *J Theor Biol*. 1990;146(4):501-511. doi: https://doi.org/10.1016/S0022-5193(05)80375-3.

201. Cornelissen A, Ceyssens PJ, T'Syen J, et al. The t7-related pseudomonas putida phage φ15 displays virion-associated biofilm degradation properties. *PLoS One*. 2011;6(4). doi: 10.1371/journal.pone.0018597.

202. Hsu CR, Lin TL, Pan YJ, Hsieh PF, Wang JT. Isolation of a Bacteriophage Specific for a New Capsular Type of Klebsiella pneumoniae and Characterization of Its Polysaccharide Depolymerase. *PLoS One*. 2013;8(8). doi:10.1371/journal.pone.0070092.

203. Glonti T, Chanishvili N, Taylor PW. Bacteriophage-derived enzyme that depolymerizes the alginic acid capsule associated with cystic fibrosis isolates of Pseudomonas aeruginosa. *J Appl Microbiol*. 2010;108(2):695-702. doi:10.1111/j.1365- 2672.2009.04469.x.

204. Sung BH, Lee CH, Yu BJ, et al. Development of a biofilm production-deficient Escherichia coli strain as a host for biotechnological applications. *Appl Environ Microbiol*. 2006;72(5):3336-3342. doi:10.1128/AEM.72.5.3336-3342.2006.

205. Reisner A, Haagensen JAJ, Schembri MA, Zechner EL, Molin S. Development and maturation of Escherichia coli K-12 biofilms. *Mol Microbiol*. 2003;48(4):933-946. doi:10.1046/j.1365-2958.2003.03490. x.

206. Reisner A, Krogfelt KA, Klein BM, Zechner EL. In Vitro Biofilm Formation of Commensal and Pathogenic. *Society*. 2006;188(10):3572-3581. doi:10.1128/JB.188.10.3572.

207. Park SC, Shimamura I, Fukunaga M, Mori K-I, Nakai T. Isolation of bacteriophages specific to a fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. *Appl Environ*

Microbiol. 2000;66(4):1416-1422. doi:10.1128/AEM.66.4.1416- 1422.2000.

208. Chibani-chennoufi S, Bruttin A, Dillmann M, Bru H. MINIREVIEW Phage-Host Interaction: an Ecological Perspective. 2004;186(12):3677-3686. doi:10.1128/JB.186.12.3677.

209. Petrova MI, van den Broek M, Balzarini J, Vanderleyden J, Lebeer S. Vaginal microbiota and its role in HIV transmission and infection. *FEMS Microbiol Rev*. 2013;37(5):762-792. doi:10.1111/1574-6976.12029.

210. Bolocan AS, Callanan J, Forde A, Ross P, Hill C. Phage therapy targeting Escherichia coli-a story with no end? *FEMS Microbiol Lett*. 2016;363(22):1-5. doi:10.1093/femsle/fnw256.

211. Subhadra B, Kim DH, Kim J, et al. Complete genome sequence of uropathogenic Escherichia coli isolate UPEC 26-1. *Genes and Genomics*. 2018;40(6):643-655. doi:10.1007/s13258-018-0665-5.

212. Chen SL, Hung C-S, Xu J, et al. Identification of genes subject to positive selection in uropathogenic strains of Escherichia coli: a comparative genomics approach. *Proc Natl Acad Sci U S A*. 2006;103(15):5977-5982. doi:10.1073/pnas.0600938103.

213. Billips BK, Yaggie RE, Cashy JP, Schaeffer AJ, Klumpp DJ. A Live-Attenuated Vaccine for the Treatment of Urinary Tract Infection by Uropathogenic *Escherichia coli*. *J Infect Dis*. 2009;200(2):263-272. doi:10.1086/599839.

214. DebRoy C, Sidhu MS, Sarker U, et al. Complete sequence of pEC14_114, a highly conserved IncFIB/FIIA plasmid associated with uropathogenic Escherichia coli cystitis strains. *Plasmid*. 2010;63(1):53-60. doi: 10.1016/j.plasmid.2009.10.003.

215. Cusumano CK, Hung CS, Chen SL, Hultgren SJ. Virulence plasmid harbored by uropathogenic Escherichia coli functions in acute stages of pathogenesis. *Infect Immun*. 2010;78(4):1457-1467. doi:10.1128/IAI.01260-09.

216. Brüssow H. Phage therapy for the treatment of human intestinal bacterial infections: soon to be a reality? *Expert Rev Gastroenterol Hepatol*. 2017;11(9):785-788. doi:10.1080/17474124.2017.1342534.

217. Tyson GW, Banfield JF. Rapidly evolving CRISPRs implicated in acquired resistance of microorganisms to viruses. *Environ Microbiol*. 2008;10(1):200-207. doi:10.1111/j.1462-2920.2007.01444. x.

218. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J Bacteriol*. 1987;169(12):5429-5433. doi:10.1128/jb.169.12.5429-

5433.1987.

219. Abedon ST. Lysis of lysis-inhibited bacteriophage T4-infected cells. *J Bacteriol*. 1992;174(24):8073-8080.doi:10.1128/jb.174.24.8073-8080.1992.

220. Węgrzyn G, Węgrzyn ABT-P in NAR and MB. Genetic Switches During Bacteriophage λ Development. In: Vol 79. Academic Press; 2005:1-48. doi: https://doi.org/10.1016/S0079-6603(04)79001-7.

221. Yanagida M, Suzuki Y, Toda T. Molecular organization of the head of bacteriophage Teven: Underlying design principles. *Adv Biophys*. 1984; 17:97-146. http://www.sciencedirect.com/science/article/pii/0065227X84900261.

222. Verreault D, Moineau S, Duchaine C. Methods for Sampling of Airborne Viruses. *Microbiol Mol Biol Rev*. 2008;72(3):413-444. doi:10.1128/MMBR.00002-08.

223. Neve H, Berger A, Heller KJ. A method for detecting and enumerating airborne virulent bacteriophage of dairy starter cultures. *Kieler Milchwirtsch Forschungsberichte*. 1995;47(May 2016):193-207.

224. Furuse K, Osawa S, Kawashiro J, et al. Bacteriophage distribution in human faeces: Continuous survey of healthy subjects and patients with internal and leukaemic diseases. *J Gen Virol*. 1983;64(9):2039-2043. doi:10.1099/0022-1317-64-9-2039.

225. Qualitative Analysis of DNA template for Sanger Sequencing. :1.

226. William C Summers. Félix d'Herelle and the origins of molecular biology. *B Rev*. 1999:294-295.

227. Smith HW, Huggins MB. Successful Treatment of Experimental Escherichia coli Infections in Mice Using Phage: its General Superiority over Antibiotics. *J Gen Microbiol*. 1982;128(1982):307-318. doi:10.1099/00221287-128-2-307.

228. Smith HW, Huggins MB. Effectiveness of Phages in Treating Experimental Escherichia coli Diarrhoea in Calves, Piglets and Lambs. *Microbiology*. 1983;129(8):2659-2675. doi:10.1099/00221287-129-8-2659.

229. Hang XIE. Prevention of Escherichia Coli Infection in Broiler Chickens With. 2018;(February):5-6.