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IN VITRO STUDY OF EFFECTIVITY OF BACTERIOPHAGES IN THE PREVENTION AND DEGRADATION OF BIOFILMS FORMED BY UROPATHOGENIC *E. COLI* IN URINARY CATHETERS

Marc-Michel Wilson

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, 2018

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine MSc (Med) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

(Signature of candidate)

_____day of

_____2018_____in_____

In memory of

Kerran John Yates

1986 – 2012

PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

1. Oral presentation at the 8th Cross Faculty Postgraduate Symposium, Wednesday 25th October 2017, Auditorium at the School of Public Health, Education Campus, University of the Witwatersrand

2. Oral presentation at the Molecular Biosciences Research Thrust Postgraduate Research Day, Thursday 30th November 2017, Wits Club, West Campus, University of the Witwatersrand

ABSTRACT

As the primary mode of growth of bacterial populations biofilms are a major contributing factor to the globally pertinent issue of antibiotic-resistant infections. Research into the use of bacteriophages to combat bacterial infections was abandoned by most of the world following the discovery of antibiotics and, only recently, in light of the imminent threat to global health that is associated with antimicrobial resistance (AMR), has this alternative started to garner renewed interest on an international scale. This study is aimed at furthering specific understanding in this newly resurgent field, by investigating interactions between bacteriophages and the biofilms that are prevalent in *E. coli* urinary tract infections (UTIs). The study involved isolation of bacteriophages from the environment and scrutiny of their capacity to both degrade existing, and prevent the formation of, uropathogenic *Escherichia coli* (UPEC) biofilms, *in vitro* in urinary catheters. Techniques employed to this end included: multifaceted microbial and viral culture, isolation, and characterization; PCR and DNA sequencing; transmission electron microscopy (TEM); spectrophotometry; and flow cytometry. Isolation and characterization of two biofilm forming uropathogenic *E. coli* strains (UEmw_8 and UEmw_9) were carried out and identification was confirmed morphologically, biochemically, and genetically (using 16S rRNA gene sequencing). The isolation, purification, and description of two novel lytic bacteriophages (vB_EcoP-U8, and vB_EcoX-U9) that are active, with high specificity, against the clinical *E. coli* strains was achieved. The genome of bacteriophage vB_EcoP-U8 has been fully sequenced and annotated. Both bacteriophages were capable of significantly reducing established biofilms formed by their respective bacterial hosts (UEmw_8 and UEmw_9) in catheters *in vitro.* Pre-treatment of catheters with bacteriophage was ineffective in preventing formation of biofilms of either bacterial strain in the catheters over a 24 hour period. Future studies should focus on the specific mechanisms of action of these bacteriophages, with the ultimate goal of creating prevention strategies or treatments for *E. coli*–associated UTIs.

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LIST OF ABBREVIATIONS

AMR – Antimicrobial resistance
AUM – Artificial urine medium
BLAST – Basic Local Alignment Search Tool
bp – Base pairs
BSC – Biological safety cabinet
CAUTI – Catheter associated urinary tract infection
CFU – Colony forming units
CFU/ml – Colony forming units per millilitre
DFR – Drip flow reactor
DNA – Deoxyribonucleic acid
dNTP – Deoxynucleoside triphosphate
EPS – Extracellular polymeric substances
FR – Flow reactor
g – grams
h – hours
Hz – hertz
ICTV – International Committee on Taxonomy of Viruses
kb – kilobase
l – litres
LB – Luria-Bertani
LLQ – Lower left quadrant
LRQ – Lower right quadrant
min – minutes
ml – millilitres
mol – moles
mW – milliwatts
MSU – Midstream urine
MTP – Microtitre plate
NHLS – National Health Laboratory Services
NICD – National Institute for Communicable Diseases
nm – nanometres
nmol – nanomoles
NTC – Non-template control
OD – Optical density
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PI – Propidium iodide
rDNA – Ribosomal deoxyribonucleic acid

RNA – Ribonucleic acid

rpm – Revolutions per minute

rRNA – Ribosomal ribonucleic acid

SG – SYBR Green I

SM – Sodium magnesium (phage buffer)

TEM – Transmission electron microscopy

UA – Uranyl acetate

ULQ – Upper left quadrant

URQ – Upper right quadrant

UPEC – Uropathogenic *E. coli*

UTI – Urinary tract infection

V – volts

µl – microlitres

CHAPTER 1 – INTRODUCTION

1.1 General introduction

1.1.1 Antibiotic resistance and biofilms

Antibiotic resistant pathogens and the infections associated with them are a major global health concern.(1) As the number of cases of infections by multiple drug resistant microbes increases, the need for alternative treatments becomes increasingly apparent and urgent.(2) It has emerged in recent years that one of the primary contributing factors to antimicrobial resistance (AMR) is the way in which bacterial populations naturally grow and survive, i.e. in biofilms.(3) Bacterial biofilms are simply “matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces.”.(4) Bacteria in these biofilms are inherently resistant to both innate defences and chemotherapeutic strategies.(5,6) Biofilm associated cells can differ highly from their planktonic counterparts in several ways including; phenotype, resistance, behaviour and pathogenicity.(4) In the formation of a biofilm, bacterial cells attach to a surface, aggregate, and then secrete a slimy, heterogeneous substance, which forms the immediate surrounding environment for the cells. This secreted layer is highly variable in composition depending on both the constituent species and the environment being colonised.(4) The substance, which is given the generic term ‘extracellular polymeric substances’ (EPS) can be made up of a mixture of any number of polysaccharides, peptides, and nucleic acids.(7)

It has become apparent that combating a bacterial infection involves not simply killing the bacterial cells but also eliminating the protective layers of EPS around the biofilm cells. The mechanisms by which biofilm cells evade strategies to eradicate them, on both biologically inert surfaces and in infected patients, are exceptionally complex, but a great deal is already known. One of the major mechanisms by which biofilms confer resistance to their constituent cells, is that the EPS physically inhibits (or at least hinders) diffusion of drugs to the enclosed bacterial cells, while still facilitating the supply of a constant store of nutrients.(6) Singer et al. posit that varying concentrations of antibiotics have access to different bacterial cells in a population when they are enveloped in (and thus compartmentalized by) the EPS. Such a situation leads to co-location of both sensitive and resistant strains within either a complex (multispecies), or simple bacterial community.(8) The variability in the degree of exposure to antimicrobials in this way leads to some portions of a bacterial population being subjected to extreme levels of selective pressure, while others are hardly affected at all.

It has also been demonstrated that the differences in metabolic activity of various cells within the biofilm matrix directly affect the susceptibility of those cells to antibiotics, with slower growing cells being less affected by specific drugs than faster growing variants of the same

strain.(4) Thus, the protective effects of a biofilm foster a store of hardy living cells even after the planktonic cells have been eradicated by antimicrobial treatments.(5) Indeed, the persistent cells then often serve as a reservoir of infection of the original strain but with phenotypic alterations that are characteristic of biofilm inclusion. These phenomena lead to the development of recurrent and chronic infections (5) even after a given treatment regime has initially seemed to be effective. Cerca et al. have specifically demonstrated that resistant cells within *S. epidermidis* biofilms maintain tolerance to vancomycin and penicillin when removed from a biofilm and cultured into planktonic modes of growth.(9) This indicates that biofilm inclusion imparts not only physical barriers to drugs, but also complex, induced, phenotypic advantages.

1.1.2 Bacteriophages

The established processes requisite for development of new drugs that are active against bacterial infection are both costly and time consuming.(1) In addition, while effective antibiotics can still be created by these conventional procedures, emergence of strains resistant to new drugs is inevitable due to the very nature of the mechanisms responsible for inception and transfer of AMR.(10) While this is the case, there exists presently, a non-chemotherapeutic agent for combating bacterial infection that holds much promise. A naturally occurring group of organisms that are active with species- and even strain level-specificity against bacteria (as opposed to antimicrobial compounds which act across broad classes of bacteria), can enable the circumvention of some of the problems inherently associated with currently available antibiotics, or even yet to be developed ones. To this end, the specific group of viruses that infect and kill bacteria is the perfect candidate.

Named "bacteriophages" by Felix d'Herelle, one of the two independent discoverers of these organisms,(2) this class of viruses has been used for the prevention and treatment of bacterial infections since before the discovery of classical antibiotics.(2) In parts of Eastern Europe and the former Soviet Union, bacteriophage therapy has been continuously studied and developed since its first application and description by d'Herelle.(2) Most notably, much work and research done has been carried out at the Eliava Institute in Tbilisi, Georgia, and the Hirszfeld Institute of Immunology and Experimental Therapy in Poland.(11) Notwithstanding the continued study and application in small European phage-study loci, following the discovery of antibiotics and the heightened treatment efficacy they afforded, interest in bacteriophages (or simply phages) as an option to combat microbial infection waned drastically in the western world from the 1940's onwards.(10) The dangers associated with the ongoing emergence of antibiotic resistant bacterial strains, as mentioned above, has led globally to a renewed interest in bacteriophage therapy.(12) One of the greatest reasons for the surge in interest in the use of bacteriophages in the fight against bacterial infection, besides their ubiquity, high specificity, and safety (2), is that the mechanisms by which

phages kill bacteria are completely different from those employed by antibiotics.(11) Because of this, and armed with a growing knowledge of the mechanisms of AMR development, it is thought that the exploitation of bacteriophages could greatly reduce the looming threat of AMR. Many reviews detailing the history of bacteriophage therapy and of its potential use have been published in recent years.(2,10,11,13)

Bacteriophages have two possible life cycles, namely:

i) the lysogenic cycle; in which infecting phages incorporate their genes into host bacterial genomes and thus propagate in tandem with the host replication cycles, and

ii) the lytic cycle; in which large numbers of virus are replicated inside the host cell by corruption and exploitation of their cellular reproductive machinery, leading to cellular lysis and consequently death of the host cells.(12)

While the exact mechanisms of adherence, infection and subsequent bacterial killing cannot be assumed to be the same for all phages, in most cases cell death has been shown to be caused by cellular lysis mediated by phages in the lytic cycle.(2) In animal tissues where bacterial infection is occurring, introduced therapeutic phages multiply and accumulate at the site of infection until the bacterial population diminishes and the viruses can no longer replicate without their hosts' replication machinery, and thus the added advantage of "autodosing" is conferred.(14) The in-depth process by which cell lysis takes place must be further investigated for species-specific bacteria/bacteriophage interactions, but it is known that in many cases a group of proteins called "phage lytic enzymes" or "lysins", is involved.(12) Knowledge of these enzymes' functions has led to development of the idea of using lysins themselves as therapeutic agents, rather than whole phage, partly to circumvent immune responses to phage coat proteins. Investigations, however, into the viability of such an approach and methods of delivery of such agents without whole phage can only be carried out where cellular lysis of distinct host bacterial species, by specific bacteriophages has been shown to be induced *in vitro.* The demonstration of such species- and strain-specific interactions is an ongoing area of study and is the first step toward facilitating the desired downstream investigation into development of therapeutics, clinical trials, and eventually, real-world applications of phage and/or their lysins targeted against specific drug resistant infections.

1.1.3 Biofilms in UTI

It has been established and expounded above, that the occurrence of biofilms and the emergent phenomenon of drug resistant bacteria are intrinsically linked. Furthermore, it is known that biofilms are involved in many hospital-acquired infections, especially those

relating to in-dwelling medical devices.(15,16) With over four million hospital acquired infections a year occurring in Europe and well over one million a year in the US,(15) it is obvious that nosocomial diseases in particular pose a substantial threat to world health. In humans, urinary tract infections (UTIs) are one of the most common bacterial conditions,(17) and they are the most prevalent type of hospital acquired infection,(15) and they account for the second largest number of bloodstream infections in the hospital setting.(16)

In the majority of cases, the bacterium *Escherichia coli* is responsible for UTIs.(17) The strains of *E. coli*, that have the capacity to cause infection in the urinary tract are termed uropathogenic *Escherichia coli* (UPEC). In South Africa the infection trends are in line with those seen across the globe. Habte et al. indicated that in their 2009 retrospective analysis of laboratory reports of urine samples over a one year period in South African hospitals, they found a high culture-positive rate for uropathogens, with UPEC being the most commonly identified organism.(18) They also indicated a "very high" level of amoxicillin and co-trimoxazole resistance. The prevalence of UTI, specifically nosocomial and catheter associated urinary tract infections (CAUTI), and their strong link to drug resistance arise from a set of complex and varied factors as described in the literature.(19–23)

Two of the most addressed and thoroughly researched of these causative factors are:

a. Catheter usage and design:

The very use of inserted extraneous devices for therapeutic functions, exacerbated by their antiquated and inappropriate design, and the obstacles to development/use of alternatives,(21,24,25) plays a major role in development of CAUTI. The effects of catheter usage and design have been addressed considerably in investigations into, and reviews of, the relationship between UTI and catheterization,(21–25) but it is not an issue that falls directly within the scope of this research project. It is mentioned briefly here as it is an aspect of importance in the overall topic and has some overlap with biofilm formation, which is of primary concern in this text.

b. Bacterial biofilms in CAUTI:

By far the most important factor leading to the high number of CAUTI and the difficulty in treating them is the biofilm mode of growth.(22,26–30) As mentioned previously, this problem is not wholly distinct from, but is rather compounded by the materials and designs of catheters commonly in use. In comparison with the body's interior surfaces, catheters are far more conducive to microbial colonisation and biofilm formation, primarily because of the mechanisms by which biofilms develop on abiotic surfaces. Upon insertion of a catheter, host body fluids coat the surfaces of the device, creating a layer of proteins, polysaccharides, ions and other organic substances.(23,26) The inner and outer synthetic catheter surfaces, thus coated with

a layer of organic nutrients, is amenable to bacterial adhesion but is lacking in the first line defences to bacterial adherence afforded by the epithelial mucosa.(31) Thus a pivotal aspect in the genesis of AMR, i.e. efficient adherence of invading planktonic bacterial cells, is increased greatly by the use of catheters. Trautner & Darouiche state that “The pathogenesis of CAUTI is related to the susceptibility of inert catheter material to microbial colonization”.(31) Stickler asserts that: “The latex and silicone used in the manufacture of catheters are especially susceptible to bacterial colonization”.(26) In addition, Fenely et al. warn that the catheters often damage the urinary tract epithelia, attenuate blood flow to it, and decrease lubrication to those body surfaces in contact with the catheter.(25) The process by which biofilms grow in catheters has been described in detail by Stickler and by Soto.(27,30) Briefly, initially colonizing bacteria attach to a surface reversibly, they then surround themselves in secreted EPS. Following this they attach irreversibly and continue to secrete EPS as the matrix becomes more complex. Nutrients can then be passed through the matrix of EPS in channels formed between the enveloped cells.(4) This allows for multiplication of the infecting cells and the biofilm mass grows. As previously discussed, the cells at both the attachment site and throughout the biofilm mass (although to a lesser degree than at the attachment site) are then shielded from antibiotics by the slimy biofilm layer. Cellular signalling and quorum sensing is thus also facilitated.(28,32,33) Cellular reproduction occurs and the ongoing environmental manipulation by deposition of EPS constituents, plays an important role in bacterial gene exchange(7) and thus expression within biofilms. Cells in the biofilm can then “undertake” varying tasks/specialisation.(34) Some metabolically less active cells, deep within the biofilm and close to the attachment surface, known as the “persister cells” remain firmly attached.(34) New cells then begin to attach at sites on the surface distal to the original site of attachment. Among these, in the clinical setting, are often antibiotic resistant cells selected for by the use of antibiotics which may have been effective at removing other more superficial biofilm cells.(9) The cells growing above the persister cells become “disperser cells” and often break off from the main biofilm mass becoming deposited elsewhere as the biofilm grows and spreads along the attachment surface and upwards. Resistant cells capable of gene transfer are thus propagated through the growing infection site.

Thus it is evident that the fight against AMR is tightly linked to combating the formation of biofilms by bacterial pathogens. This sentiment is reinforced by Holmes et al. who, while focusing on understanding the mechanisms of AMR with the aim of highlighting methods to evade it, indicate a primary need for pursuing technologies that function to disrupt biofilm formation.(35)

It has been shown by Chibeu et al. that established UPEC biofilms can be eradicated *in vitro* using specific phages.(17) The current study focuses on determining whether similar results can be achieved by the use of phages that are isolated from the local environment and applied to UPEC strains infecting patients in South Africa. Further, it must be determined whether pre-treatment of catheters with these environmentally isolated phages can be used to prevent formation of UPEC biofilms on such surfaces. These goals are important because the previously highlighted specificity of host-phage interactions rules out generalization of previous results across all UPEC strains. Moreover, such enquiry is a necessary prerequisite and starting point for developing and maintaining a local repository of phages for further research into phage mediated therapeutic applications. Following the results from such investigations, research into the mechanisms of phage-induced bacterial killing *in vitro* may be useful. Specifically, future studies comparing the effects of single bacteriophage suspensions, bacteriophage cocktails, and phage lytic enzymes would be pertinent to this end. Such knowledge could then inform research into effective phage-based therapies.

1.2 Literature review

1.2.1 Introduction

Antimicrobial resistance is promulgated by exposure to antibiotics in agriculture, the environment, and the medical setting.(35) At present, hospitals are the primary site of focus for research into the mechanisms of development and spread of AMR infections, as they inadvertently but unavoidably serve as a point of confluence of pathogens, selective pressure against those pathogens in the form of therapeutics, and immunocompromised persons. In addition, the hospital setting is, in comparison small, controlled, well understood, and far less complex than the external environment. It is for these reasons that the majority of information that is used to guide strategies to combat AMR is generated and collected at hospitals.(8) This being the case, it is noted that UTI are among the most common of all hospital infections.(36) It follows then that UTIs serve as an appropriate target of investigation for enquiry into the nature of infections that are closely associated with the hospital setting. Further, the literature indicates definitively that both the incidence and complexity of UTI is increased in catheterized patients(37–39) with the likelihood of infection increasing proportionally to the duration of catheterization.(22,26,29) It is thus evident that CAUTI provide a highly pertinent model infection type for investigation into the mechanisms of bacterial infections with a high likelihood of development of AMR.

1.2.2 Progression of research prompting investigations into the use of bacteriophages to combat UPEC biofilms in CAUTI

Doolittle et al. showed in 1995 that T4 phage could infect and lyse *E. coli* cells in a biofilm, a finding that was contrary to the commonly accepted viewpoint held at that time; that bacterial cells in biofilms were, unlike their planktonic counterparts, immune to phage attack.(40) A year later the same authors reported the use of fluorescent probes to visualize infecting T4 phage within biofilms grown on glass slides.(41) This showed that phages were able to not only lyse bacteria located within a biofilm, but also to some extent to then gain access to the other EPS shielded cells for further infection. During the study, the authors had difficulty with one of their *E. coli* strains, which formed biofilms very poorly on glass.(41) This was an indication that in future work the bacteria under scrutiny would need to fulfil both the criteria of: i) capacity for biofilm growth on a specific type of surface and, ii) susceptibility to attack by the phage of interest.

In 1998, based on the work of Doolittle and his associates, Hughes et al. demonstrated strain specific phage lytic activity against *Enterobacter agglomerans* (subsequently renamed *Erwinia herbicola*) biofilm cells.(6) This study demonstrated that both the bacteriophages and their biofilm-degrading lytic enzymes are both exceptionally specific. The results showed that phages capable of killing the cells of their biofilm-forming hosts could not degrade biofilms of different bacterial strains.

Corbin et al., building upon and citing the work of both Doolittle et al. and Hughes et al., investigated the use of phage T4 for the control of *E. coli* K12 biofilms grown on glass.(42) Their work demonstrated the effects of changes in phage concentration and media parameters.(42) This highlighted conclusively the exceptional sensitivity of the host-phage relationship to almost all environmental factors. That is, the way in which even a specific host and specific phage interact is altered under differing conditions, making generalizations about these types of interactions almost impossible without rigorous controls.

Sutherland et al. reviewed the then-current knowledge of interactions between bacteriophages and biofilms in 2004.(43) They stated that notwithstanding the evidence that phages can in fact penetrate biofilms and reduce viable cell counts under certain conditions, the data supporting the idea that phages may, in future, be used for the control of clinically relevant biofilms was “still scanty”. They further suggested that if such a possibility existed, “…the technology for this has not yet been successfully developed.”(43) Despite these assertions, with the ever increasing threat of AMR, and the expanding understanding of the role played by biofilms in this plight, in combination with the increasing knowledge that phages can, in certain instances overcome their specific hosts’ defences, efforts to gather knowledge about and evidence for development of phage-mediated biofilm therapies continued.

At this point in time, it having already been established that biofilms could be susceptible to phage attack and that the surface material for the biofilm is an important consideration in such instances, a shift towards more focused studies was seen. Research was narrowed in scope to consider biofilms of specific microbes, conditions, and surfaces that were of clinical importance.

In 2006 Curtin and Donlan then showed that the growth of *Staphylococcus epidermidis* biofilms could be significantly decreased in hydrogel coated catheters by pre-treatment of the catheters with phages proven to have lytic activity against that species, and even more so when supplementing the growth media with divalent cations.(16) Much like the uropathogenic gram-negative *E. coli* strains, the gram-positive *S. epidermidis* is a component of the normal human flora, and is a common causative agent of nosocomial infection, especially on in-dwelling devices.(9) The work of Curtin and Donlan focused on a capacity for prevention of biofilms by pre-treatment and did not measure capacity of the phages to degrade established biofilms. It involved however, neither the isolation of phages nor of their bacterial hosts from the environment but relied on previously established bacteria–bacteriophage specificity. The methodology and techniques used have helped establish those used by many authors following the study.(17,44,45)

Subsequently, in 2010 Fu et al., basing their study directly on the work and methods of Curtin and Donlan's study, investigated the use of phages that were active against *Pseudomonas aeruginosa* to combat the biofilms formed by that species in hydrogel coated Foley catheters.(44) They demonstrated that the phages were capable of reducing formation of biofilms on the catheters by pre-treatment. They also demonstrated that the phages were capable of reducing viable cell counts within established biofilms. This study was important in that it also showed emergent resistance to phage following pre-treatment and described re-growth of phage treated biofilms after 24 hours (h). In addition, the authors showed that far greater efficacy for prevention of biofilms is seen with the use of phage cocktails, as opposed to single phage species. The same year, following this work, Carson et al. showed that previously characterized phages from a commercially available lytic bacteriophage cocktail were capable of effectively eradicating biofilms of their specific hosts, grown in Foley catheters, and that the phages were also capable of preventing biofilm formation by pre-treatment of the catheter surfaces with phages.(45) Two gram-negative bacteria, both known to often be involved in CAUTI were used in this study, namely: *Proteus mirabilis* and *Escherichia coli*. The study again involved no isolation of phages from the environment, and the bacterial pathogens were not clinical isolates but were instead commercially available cultures. The results were exceptional and indeed to our knowledge no other study to date has been able to demonstrate such high levels of decrease of biofilm with the use of single phage preparations for these strains or any other. In the light of the previous work the results obtained seem contrary to what has been established as possible with a single phage

species, but it is noted that these interactions are exceptionally specific for each single phage-host system.

So, with mounting evidence for the feasibility of development of effective phage treatments or pre-treatments for catheter associated UTI, a shift towards less generalised research once again occurred. In 2012, Chibeu et al. carried out a study that built upon, but differed markedly from the previous research in that it focused on the interactions between clinically isolated *E. coli* and phages isolated from the environment.(17) The authors characterized 123 clinically isolated community acquired UTI UPEC samples from a 'community health service' and a 'community health centre' in Montreal Canada and a further 130 UPEC isolates from a Canadian national surveillance study (CANWARD). The study characterized both the UPEC strains and the isolated phages and tested the capacity of three of the phages to degrade one of the UPEC trains. This study, while using clinically relevant strains, assessed effects on biofilm formation only in microtitre plates, a surface material differing substantially from Foley catheter surface composition. The UPEC strains isolated were also identified by phenotypic methods only in this study.

1.2.3 Bacterial strains, media and growth conditions

In vitro modelling of specific conditions associated with *in vivo* phenomena is the basis of useful and applicable laboratory research. The extent to which a modelled system is similar to the real world scenario it is intended to simulate depends on the researchers' ability to clearly identify and isolate the critical variables. In turn, the applicability of the results, in general rely largely on the identification and validation of which parameters and variables are to be modelled, controlled for, and measured, and which are only incidental. In terms of biofilm related studies, Crusz et al. assert that: "Laboratory methods for culturing surface attached biofilms are varied and their use depends upon the question being asked".(46) Many different biofilm cultivation models have been developed and described in the literature. All of these fulfil some specific control requirement such as, ease of measurement of biofilm characteristics or hydrodynamic conditions.(47) A full review of these systems is outside of the scope of this study, but a comprehensive list of existing models for *in vitro* biofilm analyses has been compiled and tabulated by Lebeaux et al.(48)

To adequately study the characteristics of UPEC biofilms in urinary catheters, in the laboratory, as they would occur in patients in hospitals, the *in vitro* system must as closely as possible approximate the variables affecting this type of growth *in vivo*. The most important parameters to consider in this endeavour, as identified by Dohnt et al., are: i) uropathogenic organisms, ii) urine composition, iii) urine volume/flow rate, iv) catheter composition, v) body temperature, and vi) source of infection.(47) Based on achieving analogous conditional outcomes for these defined parameters, the authors have developed a catheter based *in vitro*

continuous flow system.(47) This model system was validated by comparisons of generated data with *in vivo* CAUTI data, by the same authors.

Exploring these parameters in terms of *in vivo* phenomena with reference to the literature gives a clear understanding of what a biofilm cultivation system should achieve for a study such as this one:

i) Uropathogenic organisms:

Of vital importance in the modelling of biofilm formation by uropathogenic microorganisms in catheters, is the choice of microbe itself. Stickler asserts that during initial stages of urinary catheterization, single species infection predominates and is commonly effected by either: *Staphylococcus epidermidis, Enterococcus faecalis, Escherichia coli* or *Proteus mirabilis.*(26) Indeed, in the introductory portion of this text it was established that *E. coli* are the primary causative agent of CAUTI. But when catheterization is prolonged, multispecies infections begin to arise(49) with the majority of UTI biofilms comprising populations of at least two species and up to five.(27) Given then that multispecies biofilms predominate, it is obvious that the longer any apparatus is setup, the greater the chance of inadvertent contamination of the system due to increased sampling and reagent replacement. This makes long-term catheterization much more difficult to simulate *in vitro* than short-term catheterization. Thus shorter experimental procedures are desirable. The applicability and validity of the data generation and collection methodologies comes into play. Because initial infection spurs on later multispecies infection *in vivo,*(50) investigations into methods for combating the initial colonizing species are a priority.(23)

When the overall rationale for the methodologies considered are based on combating AMR, prior published data on capacity for biofilm formation is of key interest in determining the species to study. It has been demonstrated that *E. coli* are the third most likely organism to be isolated from either single- or multi-species biofilms in CAUTI.(50) In addition, it has been shown that the vast majority of UTI derived *E. coli* isolates (98.1%) are capable of forming biofilms, with 34.7% being strong biofilm formers.(51)

The implications of available reports on specific organisms' capacity for biofilm growth are however fairly ambiguous. For example, if "most commonly isolated species from biofilms" are described, as in Macleod & Stickler (50), ambiguity arises from whether these species are actually biofilm formers, or just cells that thrive by exploiting conditions within existing biofilms. The condition could also exist that these cells are just more easily recoverable/cultivable than the other multi-organism biofilm

cohabiting species. The question as to whether any of these considerations actually matter when biofilm formation is considered only as a measure of capacity for AMR development remains unresolved.

It has thus been established that uropathogenic *E. coli* are the:

- most common causative agent of all UTI
- most frequent initial colonizing microorganism of urinary catheters
- third most isolated organism from UTI biofilms, and
- capable of strong biofilm formation

In light of this, it is asserted that UPEC strains are the ideal model organisms for studying biofilm forming uropathogenic bacteria in catheter flow reactor systems. The uropathogenic type strain *E. coli* CFT073 (ATCC 700928) has been demonstrated to be biofilm forming in previous studies.(52–54)

ii) Urine composition:

Because metabolic activity and growth rate parameters of *E. coli* are dependent on environmental conditions and nutrient availability,(55) presumably the best way to study the activity of uropathogenic *E. coli* would be in urine (the environment in which they persist). But urine composition between individuals and in individuals is variable over time.(56) This natural variability of the composition of urine can affect the collection of consistent and accurate data for the uropathogens under scrutiny. Thus, an artificial urine medium (AUM) of consistent composition that is based on an "average" urine composition is desirable for investigations involving uropathogens, within physiological ranges *in vitro*. Brooks and Keevil described such a growth medium.(57) Chutipongtanate & Thongboonkerd found that in comparison to five other published AUMs, the Brooks and Keevil formulation was the best suited for physiologically accurate study involving *in vitro* cell culture analyses.(58) In addition, Lehman and Donlan used the same AUM in their two species biofilm analyses and carried out a validation procedure comparing the medium to urine, confirming its applicability for use as a urine analogue in such studies.(59)

iii) Urine volume / flow rate:

The flow rate can be controlled in the catheter flow reaction system described, by the use of a variable speed peristaltic pump. This is a standard procedure utilized by researchers carrying out similar experiments in which biofilm growth is induced in catheters *in vitro.* (44,47,59)

iv) Catheter composition:

The composition and coatings of the catheters used in such experiments are selected based upon the purpose of the analysis, noting that catheters of identical composition to those used in the modelled environment are selected to maintain validity of results.

v) Body temperature:

As an aspect of a system modelled to mimic the environment in which CAUTI develop, the temperature of the system should approximate that of the average human physiological temperature of 37 °C.(47) The temperature range for *E. coli* growth is from 21-37 °C.(55) To maintain the required temperature of the flow reactor system, placement of the entire apparatus in an incubator would be optimum. Clearly the size limitations of the apparatus, maintaining sterility of components coming into contact with the cultures and media, and the types of available incubators play a role. Researchers such as Curtin and Donlan, Carson et al. and others carry out similar biofilm flow reactor experiments at 37 °C. (16,45)

vi) Source of infection:

This parameter, as described by Dohnt et al., refers to the fact that *in vivo* infection usually spreads distally from a single point of contamination. In fact it has been demonstrated that the process of placement of the catheter is the primary source of CAUTI.(39) The controlled flow reactor setup described above ensures that pathogens of interest move initially in one direction, from the batch culture through the catheter to the collection vessel, with no other possible route of entry for microbes.

Curtin and Donlan in 2006 used a modified drip flow reactor (mDFR) functionally identical to the flow reactor described by Dohnt et al., except that it allowed for cultivation of biofilms in four catheter segments at a time rather than one, by taking advantage of the sealed glass slide chambers of an existing drip flow device and modifying them for catheter segment insertion. (16) Following this, Carson et al.,(45) Fu et al.,(44) and Lehman and Donlan (59) used analogous modified drip flow reactor setups in similar experiments to model various effects on cultivated biofilms. A multichannel peristaltic pump is needed for the use of such a reactor, and although more replicates can be run simultaneously no other advantage is achieved by this addition.

1.2.4 Preliminary biofilm assay

One of the most frequently used *in vitro* biofilm assay models is the microtitre plate (MTP) based system.(60) Most MTP-based biofilm assays use the method described by O'Toole et al.(61) Briefly, the method consists of a plate assay that is amenable to rapid biofilm

quantification by macroscopic methods that can reliably isolate mutants that are unable to form a biofilm on an abiotic surface. Besides the simplicity of the method of biofilm detection in relation to complex imaging techniques, other advantages also exist. Such advantages include that they are inexpensive, easy to use, take little time, and use small amounts of reagents.(60) These assays are extensively used and cited in the literature but their applicability is limited to screening procedures as biofilm formation is dependent on surface material.(62) Indeed, Coenye & Nelis warn in reference to MTP based models that: "…they are closed systems and the conditions in which biofilms are formed and/or tested are more often than not very different from the *in vivo* situation."(60) In addition to the limitations described, in terms of repeatability and thus standardization, Naves et al. have demonstrated that *E. coli* biofilm growth in microtitre plates is entirely procedurally dependent.(63)

1.2.5 Bacterial characterization

The selection of uropathogenic microorganisms for investigations into CAUTI should be based on attaining valid comparisons with previous work available in the literature. Following this, the characterization of environmentally isolated microbes centres on achieving a high degree of certainty that the selected isolates are actually members of the desired taxonomical group. Historically the endeavour has its roots in phenotypic and morphological analyses, but it has rapidly expanded with the advances in all aspects of molecular biology. With each new method comes the disruption of the "settled" classifications and assignments based on the previous methods. Newer more powerful evaluation methods are continuously being developed, including 16S ribosomal gene analysis, whole genome comparison methods, MALDI-TOF mass spectrometry, and others. So the question now is shifting from "what species is being dealt with" to "to what degree of certainty must classification be made". Considering the immense complexity of the problems concerning bacterial taxonomy only very briefly touched upon above, and based on recommendations by researchers addressing these issues, it is noted that taxonomical identification should be based on a 'polyphasic approach' which combines a variety of morphological, phenotypic (biochemical) and genotypic criteria that include biochemical testing and 16S rRNA gene sequence analyses.(64,65)

1.2.6. Bacteriophage isolation and characterization

The initial step in analyzing the usefulness of phages for combating biofilm-forming pathogens is the effective isolation of those phages. It is well established that bacteriophages are the most abundant of all known organisms.(66) This notwithstanding, phages are exceptionally specific and "have an obligate requirement for a host".(67) Their distribution is therefore largely dependent on the localization of the specific bacteria that they infect. Since *E. coli* and other bacteria are abundant in sewage, as are the phages infecting them, sewage

plants are an ideal source for the sampling of phages.(68) Some of the major principles underlying the goals of isolation of bacteriophages from any source, as described by Adams, are that the phage preparation obtained should be “free of bacteria, molds, and insoluble debris”.(69) There are a multitude of descriptions and reviews of the general and specific methods involved in the isolation of bacteriophages to this end, dating from the time of the discovery of phages, until the present day.(70–74) To date however, the parameters of these methods are still not standardized or consistent in yield/titre of phage.(75) This is unsurprising and largely due to the fact that the titre of phages in a purified sample are as dependent on the number of specific phages in the starting material and the physical characteristics of those phages, as they are on the methods used. Nonetheless, the most commonly used techniques include centrifugation, filtration, ultrafiltration, precipitation with Polyethylene Glycol (PEG), ultracentrifugation in cesium chloride (CsCl) gradients, and dialysis.(75)

These techniques, and combinations of and amendments to them, have been analysed and compared by various authors including Bourdin et al., Kleiner et al., Castro-Mejía et al., and Bonilla et al.(74–77) All steps or variations of them are essential, except for CsCl gradient ultracentrifugation, which is reported by some authors as dispensable except in cases where extremely high purity of yield is needed, as in the case of isolation for direct application in phage therapy.(76,77) Following isolation of phages, assays investigating their usefulness can be carried out, but without knowledge of the characteristics of the phages themselves the results would remain useless. Fortier & Moineau assert that: “When a particular phage is first isolated, some basic characterization must be conducted in order to properly identify the phage and to determine its affiliation regarding other known phages. Electron microscopy, host range analysis, and genome analysis are among the most common methods for phage identification”.(78)

1.2.7 Biofilm catheter assays

Different techniques are often employed for biofilm quantification and characterization after treatment, depending on the exact experimental data being sought. For instance, if the primary inquest is into the ability of the phages to kill the cells within the biofilm matrix, different techniques would need to be employed than if it is to determine the effect on the actual EPS material independently of cell death. Distinct parameters would need to again be measured if it is to determine how much biofilm the remaining cells produce after treatment with phages. Different techniques still would be useful for investigation into the overall effect on both the biofilms and the cells that produce them. This idea is exceptionally important in that it puts an emphasis on what is being measured and if the generated data actually relate to the hypotheses.

The literature indicates definitively that a common method for determining cell viability of phage treated biofilm cells is plate counting, as used by Curtin & Donlan, Macleod & Stickler, Fu et al., Carson et al. and others.(16,44,45,50) It is noted that the problems associated with the indirect nature of the plate counting technique can be easily overcome by creating calibration plots by comparison with turbidity measurement by spectrophotometry as used by Carson et al.(45) This technique relates cell viability to overall biomass and thus also to biofilm thickness, and overcomes the problem of only live cells being measured by plate counts. However, a major problem with this technique is that it assumes that all live cells within the biofilm are cultivable to the same extent as planktonic cells of the same strain, i.e. that all of the live cells remaining in the biofilm will grow on a plate. Barbesti et al. and Cerca et al. clearly state that microbes in different environments can exist in a "viable but not culturable" (VNC) state.(9,79) It also negates the obvious point of variability of EPS production (and thus turbidity) of different biofilm-dwelling populations. The phenotypic diversity of cells and differing growth rates within different levels of biofilms is well established.(19,80) Furthermore, it has been shown that the efficacy of applied antimicrobial agents against certain bacteria is directly related to those organisms' rate of cellular growth, and this very fact has been suggested to be causal to the AMR seen within biofilm cells.(4) Thus the idea that persister cells, remaining after treatment with bacterial killing agents, are cultivable to the same extent as their planktonic counterparts is erroneous. Scanning electron microscopy (SEM) visualization of biofilm mass in conjunction with spectrophotometry, has been described by Djeribi et al. but the method is complex and again involves culture of the cells following biofilm formation.(39) Furthermore dehydration and gold coating of the biofilm before visualization is required and it is noted that both the dehydration and coating steps have the capacity to disrupt the integrity of the biofilm.(4) Cerca et al. described and analyzed a flow cytometric methodology for the measurement of live and dead cells in solubilised *S. epidermidis* biofilms, using SYBR Green (SG) and propidium Iodide (PI).(80) This methodology affords the direct measure of cell viability by the combined use of differentially permeant nucleic acid dyes, and in combination with plate counts, can be used to determine the number of VNC cells. This combination can adequately quantify cell-killing capacity of antimicrobials but, unlike turbidity measurements, it affords no measure of effects on EPS.

Determination of the effects of any antimicrobial substance/organism on biofilm populations is therefore fairly complex, in that the detection and measurement of any changes must quantify effects on EPS and on the number of both live cells and dead cells. Thus, an ideal quantification scheme of the effects of phage on biofilm mass and biofilm associated cells is a direct viability assay in combination with a measurement of EPS and a measure in the change in percentage of dead cells.

1.3 Justification for the study

As outlined in section 1.2.2, research into the use of bacteriophages to combat biofilms formed by uropathogenic species has steadily progressed from broad general enquiries of phage-biofilm interactions on glass slides and polystyrene plates towards ever more specific interactions. This narrowing investigative scope is in reality a movement towards ever-closer *in vitro* model approximations of the *in vivo* biofilm infection condition. Ultimately *in vivo* trials will succeed these investigations. Greater understanding, better experimental controls, and improving methods facilitate this ongoing shift. At this point in time *in vitro* criteria have in different studies reached the key landmarks of; i) studying clinically relevant isolated bacterial strains as opposed to those that are commercially available, ii) searching for novel lytic bacteriophages and, iii) focusing on biofilms grown on surfaces that are of clinical importance rather than generic materials chosen for ease of use (like glass slides or microtitre plates). It is hoped that the combination of these key areas in a single study will provide relevant information on controlling biofilms generated on the surfaces they are associated with in the clinical setting, while also uncovering information about the characteristics of the naturally occurring bacteriophages that combat the bacteria that generate those biofilms. The search for a methodological framework that can accurately detect the effects of phages on biofilms could also be addressed in such a study by the implementation of three different biofilm/cell quantification techniques, each with its own advantages and flaws, in combination. As such the study involves plate counts, turbidity measurements and a flow cytometric cell viability assay, with the hope of achieving a holistic view of effects rather than the narrower perspective gained by each technique in isolation.

1.4 Aim and objectives

1.4.1 Aim

The present study aims to further the progression of research into the use bacteriophages to combat UPEC biofilms in urinary catheters by closer approximation of conditions, strains, and surfaces expected *in vivo*, in combining these three parameters in a single study. It thus involves investigating the effects of environmentally isolated lytic bacteriophages in both the degradation and prevention of *in vitro* biofilm formation caused by clinically derived UPEC isolates on urinary catheters.

1.4.2 Objectives

The objectives of the study are**:**

1. To isolate bacteriophages from the environment that are active against clinically isolated uropathogenic *E. coli* strains.

2. To determine if the isolated bacteriophages are effective in the reduction (degradation) or elimination of established biofilms in urinary catheters *in vitro*.

3. To determine if the isolated phages are effective as a preventative measure for biofilm formation in urinary catheters *in vitro*.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Control strains, media and buffers

Lyophilized uropathogenic *E. coli* (ATCC 700928 - strain designation CFT073) was purchased from ATCC (USA) through LGC Standards (South Africa) as the UPEC positive control strain. The *E. coli* control strain was reconstituted and propagated in #3 Nutrient Broth as per the supplier's instructions. Artificial urine medium (AUM) was prepared as per the recipe of Brooks and Keevil,(57) modified slightly as by Chibeu et al.(17) and adjusted to pH 5.80 ± 0.2. (Table A1, Appendix A). All further work was carried out in AUM or LB broth. Bacteriophages were propagated and stored in sodium magnesium (SM) phage buffer.

2.2 Sampling, bacterial isolation and culture conditions

All overnight cultures of *E. coli* were grown either in AUM or LB broth adjusted to pH 6.5. AUM was used for propagation of all isolated bacterial strains. Thirty-eight midstream urine (MSU) samples from patients at the Charlotte Maxeke Johannesburg Academic Hospital suspected of having hospital or community acquired UTIs were obtained from the NHLS Microbiology Laboratory. Preliminary bacterial identification was carried out by cell culture on Uriselect™ 4 Chromogenic Medium (Bio-Rad Laboratories, Johannesburg, South Africa). The urine samples were inoculated directly onto Uriselect™ 4 plates, using the urine streak plating method, and were then incubated overnight at 37 °C. Plates subsequently exhibiting smooth pink colonies were considered to be harboring possible uropathogenic *E. coli* (UPEC) strains. As UPEC strains account for more than 80% of uncomplicated UTIs,(81) in this study all *E. coli* strains isolated from patients with suspected UTIs were designated UPECs. Of the 38 screened urine samples, 15 possible UPEC strains were indicated. Single colonies from each of the preliminarily identified *E. coli* cultures on the chromogenic medium plates were transferred onto MacConkey agar (Sigma-Aldrich, Germany) plates using the quadrant streak method, followed by overnight incubation at 37 °C. Confirmation of identification was attained for each isolate using morphological analysis via light microscopy and Gram staining, and Microscan analysis using the Siemens Microscan Walkaway96SI and LabPro™ Software. The Microscan analysis on the samples was carried out as per the manufacturer's instructions. Only samples thus further biochemically confirmed (over 98% confidence) as being *E. coli* isolates were accepted. One sample was omitted from the study due to Microscan analysis indicating it as a possible Enterohaemorrhagic *E. coli* (EHEC), leaving a total of 14 *E. coli* strains. The 14 biochemically confirmed *E. coli* isolates were preliminarily designated UEmw_1 to 14 for further use in the study; all others were excluded from further analyses. Representative colonies from each MacConkey purity plate were then picked and inoculated into Microbank™ cryopreservation tubes (Pro-Lab Diagnostics) and frozen at -75 °C (Labex

Ultra Low Freezer) for long-term storage. For further work all bacterial isolates were grown in AUM or in standard LB broth.

2.3 Preliminary biofilm assay

The assay was carried out five times, in duplicate, and the averages of the results obtained across all replicates for each strain were used. The selected strains were tested for their propensity/capacity to form biofilms in the standard 96 well plate biofilm assay using crystal violet stain, as described by O'Toole et al.*,*(61) modified slightly according to the method used by Chibeu et al.(17) Firstly, a bead from each of the 14 UPEC sample Microbank stocks was inoculated into 10 ml of AUM in sterile 15 ml tubes (Sterilin Ltd, Parkway, Peny-fen, Newport, United Kingdom). These were then incubated for 48 h at 37 °C. (Each sample was streaked onto a MacConkey agar plate and incubated overnight at 37 °C to confirm purity of the stock cultures at this stage). 1:100 dilutions of each bacterial sample where then made in sterile 5 ml microcentrifuge tubes (Sterilin Ltd) by adding 50 µl of each culture to 4.95 ml sterile-filtered AUM. Two hundred microlitres of each sample were then added to each of six wells of a sterile 96 well microtitre plate. Negative controls containing only 200 µl sterile AUM were included in the plate assay. The plates were then incubated for 48 h at 37 °C without agitation. Following incubation, the fluid was carefully removed from each well using a multichannel pipette. The wells were then gently rinsed twice with phosphate buffered saline (PBS) in order to remove any remaining planktonic bacteria, again using a multichannel pipette. Two hundred microlitres of a staining solution of 0.1% (w/v) crystal violet (in dH_2O) was then added to each well and allowed to adhere to the biofilms for 15 min at room temperature. The staining solution was then decanted and the plates rinsed in a water bath (containing sterile dH_2O) to remove any unbound dye.

Two hundred microlitres of a freshly prepared de-staining solution of 80% (v/v) ethanol and 20% (v/v) acetone was then added to each well to solubilize the bound stain. Biofilm growth was confirmed and quantified via spectrophotometry, with optical density (OD) readings taken at 595 nm (Bio-Rad iMark™ Microplate Reader). Isolates were considered to be positive for biofilm formation in stained samples registering an OD_{595} equal to, or greater than, three times the value of those obtained from the negative control wells as defined by Chibeu et al.(17)

The strains were ranked in terms of greatest to least propensity to form biofilms based on the optical density measurements. The two strains with the greatest tendency to form biofilms that also exhibited susceptibility to infection and lysis by the crude phage lysate (as elucidated in the host range susceptibility assay described below - section 2.5.2) were then used as propagation strains for phage culture.

In addition, the propensity of the bacteria to form biofilms on catheter segments was compared with their individual propensity to do so in the microtitre plates. This was done using the method of Patel et al.(82) (slightly amended to allow for catheters rather than dental unit water lines) and turbidity measurement of biomass rather than plate counting as per the method of Sauer et al.(83) To achieve this comparison, under sterile conditions in a Class II biological safety cabinet (BSC), sterile silicone coated latex Foley catheters were cut into 2.5 cm segments and each of these was halved lengthwise. The sectioned catheter pieces were then placed in the 5 ml microcentrifuge tubes with the remaining 2.6 ml of each of the diluted (1:100) overnight bacterial sample cultures, and controls, in AUM. The samples were then also incubated for 48 h at 37 °C and the resulting biofilms scraped off with swabs. The swabs and catheter segments were then placed in 5 ml sterile dH_2O, vortexed and the OD_{595} measured (Jenway 6300 spectrophotometer). This added test for assessment of the applicability of the results from the microtitre plate assay was only carried out in duplicate and the results of this check can be found in appendix B (Figure B1).

2.4 Bacterial genetic characterization

2.4.1 DNA extraction

For downstream amplification of the 16S gene, genomic DNA of bacterial strains designated UEmw_8 and UEmw_9 (the strains that showed the highest propensity to form biofilms in the 96 well plate biofilm assay described above) were extracted from fresh 3 h cultures of the respective strains grown in AUM. The DNA isolation was carried out using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) as per the manufacturer's instructions: a microbead from each of the frozen bacterial stocks was inoculated into 1.5 ml sterile AUM and placed in an incubator at 37 °C for 48 h. The stocks were then diluted to 1:10 in sterile AUM by adding 150 µl of each 48 h sample into 1350 µl AUM in sterile 1.5 ml microcentrifuge tubes. The inoculants were then incubated for 3 h at 37 °C, to get to an approximate concentration of 10^9 cells for each, confirmed using MacFarland standards. From each of the exponential phase *E. coli* samples, cells were then collected via low speed centrifugation at 3000 × *g* for 5 min (Beckman Coulter Microfuge® 16). All subsequent transfers, additions, and dilutions were carried out in sterile conditions in a Class II BSC. The pellets were re-suspended in 200 µl PBS. 15 µl of lysozyme solution (10 mg/ml in Tris-HCl, pH 8.0) was then added and the tubes incubated for 15 min at 37 °C. Following incubation of each sample, 200 µl of tissue lysis buffer and 40 µl Proteinase K solution was added and the preparation was immediately mixed by rapid repeated inversion of the tube. The samples were then incubated for 10 min at 70 °C. One hundred microlitres of isopropanol was then mixed into each tube. Tube contents were then transferred to High Pure filter tubes, which were placed in the High Pure collection tubes. The tube assemblies containing the sample were centrifuged for 1 min at 8000 × *g*. The filter tubes were then removed from the

collection tubes and the liquid and collection tubes were discarded. Enzyme inhibitors were then removed by the addition of 500 µl Inhibitor Removal Buffer to the filter tube. The filter tubes were placed in new collection tubes and the samples centrifuged again at 8000 × *g* for 1 min. The flow-through and collection tubes were once again discarded, and the filter tubes placed in new collection tubes. Each sample was then washed by the addition of 500 µl Wash Buffer followed by another centrifugation step at 8000 × *g* for 1 min, after which the flow-through was again discarded. The wash step was then repeated. The collection tube and filter tube assembly was then spun at 13000 × *g* for 10 s to remove any residual wash buffer. The collection tube was then discarded and the filter tube placed in a new sterile 1.5 ml microcentrifuge tube. Elution of the nucleic acid was then carried out by the addition of 200 µl pre-warmed elution buffer to the filter tube, which was left to stand in the BSC for 5 min to maximize elution efficiency. The tube was then centrifuged for 1 min at 8000 × *g*. The purity and yield of the extracted DNA was checked via spectrophotometry using a Nanodrop (Thermo Scientific NanoDrop 2000) spectrophotometer.

2.4.2 PCR

The 16S rRNA gene was amplified from the prepared genomic DNA using a MyCycler™ Thermal Cycler (Bio-Rad). The 16S rRNA gene was amplified using universal primers that were chosen for amplification of the entire bacterial 16S gene with an expected size of ±1540 bp.

They were forward primer: **4F** (5'-TTGGAGAGTTTGATCCTGGCTC-3') (84)
and reverse primer : **BAKR** (5'-AAGGAGGTGATCCAGCCGCA-3').(85)

Prior to gene amplification, each of the universal primers was aligned and analyzed in relation to the published 1542 nucleotide sequence of the *E. coli* CFT073 16S rRNA gene, available at the NCBI nucleotide database (Accession number - AE014075 REGION: 3858590 – 3860131).

Alignment and analysis of applicability of the universal primers was carried out using Geneious (version 10.0.9) genetic sequence data analysis software and the Sequence Manipulation Suite available at: http://www.bioinformatics.org/sms2/.

The negative control for the amplification reaction was a non-template control reaction mixture (NTC).

The cycling conditions for the reaction are shown below in Table 1.

Table 1. Cycling conditions for bacterial 16S rRNA gene amplification PCR

Step	Temperature (°C)	Time (min)	Number of Cycles
Initial denaturation and Taq polymerase activation	95	5	1
Denaturation	95	1	30
Annealing	54	1	30
Elongation	72	1.5	30
Final Elongation	72	10	1
Hold	4	∞	1

2.4.3 Electrophoresis

Agarose gel electrophoresis of each PCR product was carried out in a 2.0% (w/v) agarose gel prepared using 1 g SeaKem LE Agarose (Lonza, Rockland, Me, USA) added to 50 ml of 1 × TBE buffer, and microwaved until fully dissolved. Visualization was facilitated by pre-staining of the gel by the addition of 1 × (v/v) concentration GelRed® dye (Biotium, Hayward, Ca, USA) to the molten agarose (5 µl of 10 000 × stock solution of GelRed in 50 ml gel) before pouring the warm gel solution into the gel casting cassette. Once the gel set, it was placed in the electrophoresis tank with 1 × TBE buffer, filled to cover the surface of the gel. 10 µl of each DNA amplicon was added to 2 µl loading dye in sterile 200 µl PCR tubes, with the same ratio of 1 kb DNA ladder (Promega, USA) and loading dye being prepared as molecular weight marker in the gel. The comb was removed and 10 µl of each reaction mixture was pipetted into a separate well in the gel. Electrophoresis was run at 80 V with constant current. Viewing and photography of the gel was carried out using a Bio-Rad Gel Doc.

2.4.4 Sequencing

2.4.4.1 DNA clean up

Following confirmatory visualization of the 16S rDNA in the gel, the remainder of each amplicon was cleaned up for sequencing using the Biospin PCR Purification Kit (Bioer Technology Co., China), as per the manufacturer's instructions. The procedure was carried out to remove any residual dNTPs and unbound primers in the eluted samples. Briefly, the process was carried out as follows: The remaining PCR amplicon was transferred to a 1.5 ml microcentrifuge tube. Two volumes of binding buffer (92 µl) was added to each volume (46 µl) of amplicon. The mixture was then pipetted into the spin column provided. The column was then centrifuged for 1 min at 6000 × *g* and the flow-through discarded. Six hundred and fifty microlitres of wash buffer was then added to the column and it was re-centrifuged at 12000 × *g* for 1 min. This washing/centrifugation step was repeated once before an additional, final 12000 × *g* centrifugation was carried out for 1 min. The spin column was transferred to a new 1.5 ml microcentrifuge tube and 50 µl of elution buffer was added. The column was then left

to stand for 1 min at room temperature. The tube assembly was then centrifuged at 12000 × *g* for 1 min. The purity and yield of the cleaned up DNA was checked via spectrophotometry (Thermo Scientific NanoDrop 2000).

2.4.4.2 PCR cycle sequencing

The cleaned up DNA was then subjected to dideoxy-nucleotide chain termination PCR for variable chain elongation of the 16S rRNA gene. Each elongation-terminating nucleotide species is labeled with a different fluorophore, thus facilitating sequence determination by the detection of these labeled bases in the generated strands of varying length. A combination of 6 different primers was used to ensure a high chance of overlap of the fragments spanning the whole 16S rRNA gene i.e. the conserved and variable regions. The positive control for the cycle sequencing PCR was the pGEM 3Zf (+) plasmid. This plasmid is used as a positive control for the overall sequencing preparation and analyzer reaction to highlight possible procedural errors that could potentially affect the outcome or quality of the sequencing data.

In addition to the previously described primer pair used for the amplification of the entire gene (i.e. 4F and BAKR), four other 16S universal primers with different binding sites along the bacterial 16S rRNA gene were used. The use of 6 primers allowed for amplification of a range of overlapping fragments from which a contiguous sequence (contig) could be constructed. The primers were all purchased from Applied Biosystems/Life Technologies (LTC Tech, Johannesburg, South Africa). The sequences of the primers are shown below in Table 2.

Table 2. Universal primers used in the 16S rDNA sequencing PCR reaction

Primer	Sequence (5'->3')	Source/Reference
Forward Primers		
A17F	GTTTGATCCTGGCTCAG	(86)
4F	TTGGAGAGTTTGATCCTGGCTC	(84)
5F	GGCGATGTGGTCCTTAAGGT	NHLS*
Reverse Primers		
810R	GGCGTGGACTTCCAGGGTATCT	(87)
534R	TTACCGCGGCTGCTGGCA	(88)
BAKR	AAGGAGGTGATCCAGCCGCA	(85)

*Primer 5F is an NHLS in-house designed primer used for routine diagnostic 16S rRNA gene sequencing at the Department of Infection Control of the NHLS, Johannesburg, South Africa

A master mix was prepared for all primer reactions, with 6.4 µl of the 80 nmol/ml stock of each primer being added to each reaction tube. The amount of DNA and dH_2O used per reaction varied depending on the yield of DNA from each sample gene amplification and clean up (Table 5). The ratio used was calculated such that a total of 30 ng of DNA was added to each reaction tube with a total volume of 9.4 µl of sample and dH_2O. This involved

the dilution of eluted samples to concentrations of 5 ng/µl in elution buffer from the DNA clean up kit, then addition of the dilute sample to the dH_2O.

The binding sites of the selected primers can be seen mapped to the CFT073 16S rRNA gene sequence below in Figure 1.

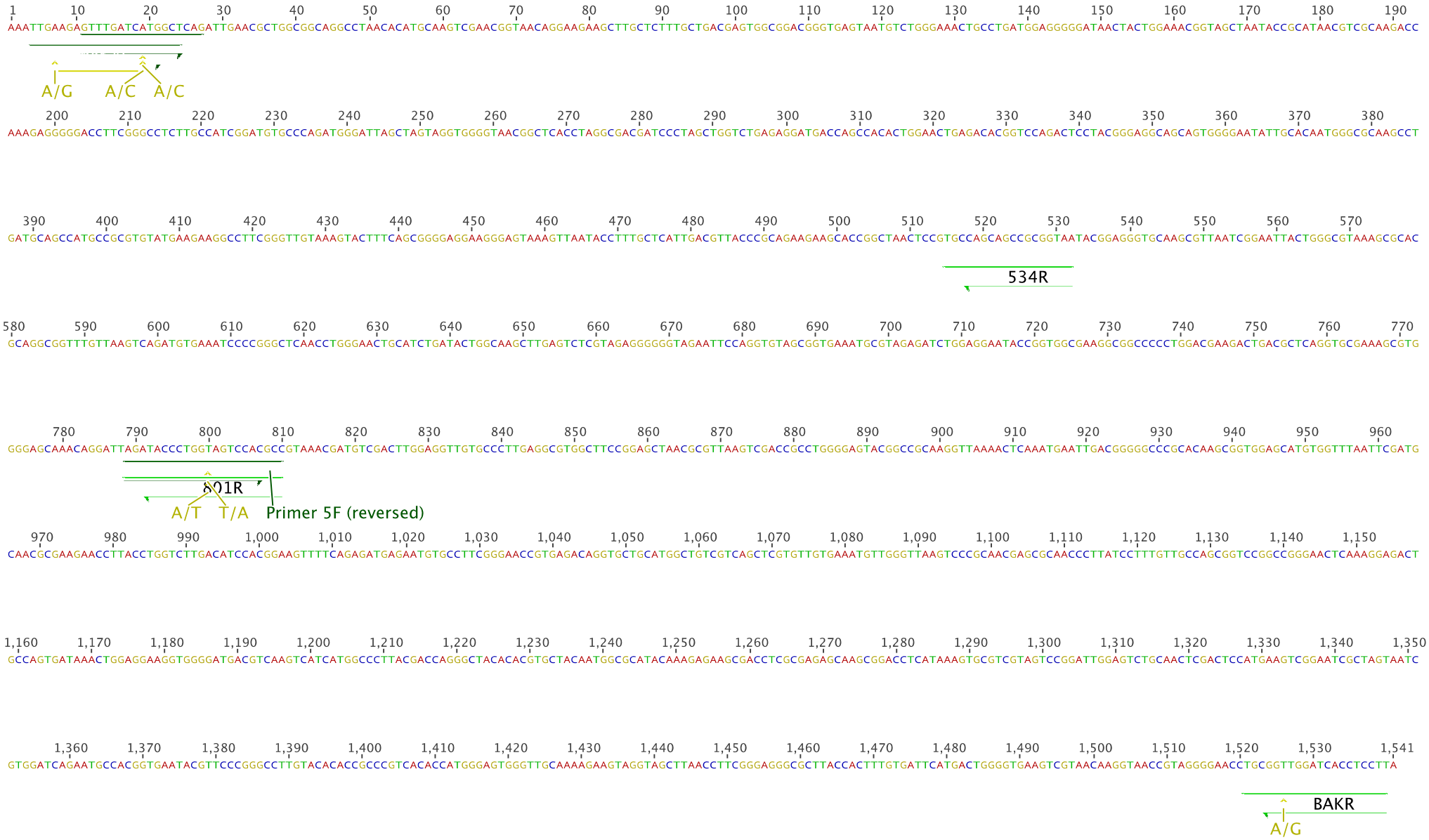


Figure 1. Binding sites of the universal primers used in this study for the *E. coli* 16S rRNA gene sequencing reaction.
The primer binding sites are indicated by dark green arrows for forward primers and light green arrows for reverse primers. The yellow letters in these primer sites indicate where primer mismatches might occur. (Such mismatches are common with the use of universal primers as discussed in section 4.3).

Table 3 lists the volumes of amplicon from the 16S rRNA gene amplification for each sample, that were used in the sequencing reactions.

Table 3. DNA and dH_2O volumes used in the bacterial 16S rDNA cycle sequencing PCR

Bacterial Strain	DNA Volume (µl)	dH_2O Volume (µl)	Final DNA Concentration (ng/µl)
CFT073	7.4	2.0	1.5
UEmw_8	2.9	6.5	1.5
UEmw_9	3.0	6.4	1.5

The positive control for the cycle sequencing PCR reaction contained 1.5 µl pGEM 3Zf (+) Plasmid, 3 µl of BigDye Terminator V3.1 Ready Reaction Mixture, 4 µl BigDye Terminator V3.1 Buffer, 5 µl M13 (-21) Primer, and 6.5 µl dH_2O.

Table 4 indicates the time and temperature conditions used for the sequencing reactions.

Table 4. Cycling conditions used for the bacterial 16S rDNA cycle sequencing PCR

Reaction Step	Temperature (°C)	Time (s)	Number of Cycles
Initial denaturation and Taq polymerase activation	96	60	1
Denaturation	96	10	30
Annealing	50	5	30
Elongation	60	240	30
Hold	4	-	1

2.4.4.3 Isopropanol purification of cycle sequencing products

Following the cycle sequencing PCR reaction, 80 µl of 75% (v/v) isopropanol was pipetted into each sample well of a sterile bar-coded 96-well Optical Reaction Plate (Applied Biosystems, Foster City, CA, USA). The entire 20 µl sample volume for each reaction was then transferred to a different well, aspirating to mix. The microtitre plate was then covered with a rubber 96-well plate cover. The plate containing the samples was then incubated in the dark at room temperature for 30 min. The plate was then centrifuged (Eppendorf Centrifuge 5810R, Merck Millipore) at 2000 × *g* for 45 min at room temperature. After removal of the plate from the centrifuge rotor, the rubber cover was taken off and the plate placed upside down on paper towel on the rotor platform. The plate was then re-centrifuged for 1 min at 700 rpm to remove the isopropanol. The plate was then left to air dry for 5 min. Following drying, the samples were re-suspended using HiDi formamide (Applied Biosystems, UK). The plate was then centrifuged at 500 rpm for 30 s to ensure that the re-suspended samples were all in the bottom of the wells as required by the analyzer. The plate containing the samples was then placed in the DNA analyzer (Applied Biosystems | Hitachi 3500 Genetic Analyzer) for nucleotide quantification.

2.4.4.4 Bioinformatics

Bioinformatic analysis was carried out on the gene sequence results from the DNA analyzer using Geneious (version 10.0.9) genetic sequence data analysis software, and 16S Biodiversity Tool, nucleotide BLAST on NCBI database and the Sequence Manipulation Suite available at: http://www.bioinformatics.org/sms2/.

Multiple sequence alignment of each of the sequenced fragments from the sequence analyzer was used to build a contiguous consensus sequence for each sample and control using Geneious multiple sequence alignment tool (version 10.0.9). The assembled consensus sequence of each sample and control was then aligned to the published 16S rRNA gene sequence of the control UPEC strain CFT073. The sequence obtained for the sequencing reaction control plasmid (pGEM 3Zf (+)) was compared to the known sequence of the plasmid to confirm fidelity of the analyzer reaction and read data.

2.5 Bacteriophage isolation

2.5.1 Preliminary isolation

Preliminarily treated sewage water was obtained from Johannesburg Water's Northern Wastewater Treatment Works. The sewage was collected in 2 L Schott bottles and stored at 4 °C for no more than one week before processing. The initial isolation of bacteriophage was carried out using the centrifugation and polyethylene glycol (PEG) precipitation methodologies as described by Bourdin et al.(76) Two litres of sewage water was transferred into sterile 500 ml polypropylene centrifuge bottles (Beckman Coulter) and solid debris was removed via centrifugation at 4500 *x g* for 45 min at 4 °C (Beckman Coulter Avanti® J-E Centrifuge) using a JA-10 fixed angle aluminium rotor. The supernatant was then recovered and the pellet discarded. The supernatant was then subjected to overnight precipitation with PEG 6000 (Merck KGaA, Darmstadt, Germany) at a concentration of 10% w/v and 0.5 M NaCl (Merck KGaA, Darmstadt, Germany), with constant low speed stirring at 4 °C. The precipitated bacteriophage was then collected by centrifugation at 11000 × *g* for 15 min at 4 °C. The pellet was then re-suspended in phage buffer to obtain 2 × 20 ml samples (in 50 ml sterile centrifuge tubes) of concentrated phage. Extraction of the bacteriophage from the resulting solution was then carried out by the addition of 20 ml of chloroform and gentle inversion for 20 min. The phases were then separated via low speed centrifugation at 800 rpm for 5 min at 4 °C. The upper, aqueous, phase from each tube was then collected carefully using a Pasteur pipette, and the lower phase discarded. The obtained phage suspension from each tube was then combined in a single sterile falcon tube and then centrifuged at 4000 *x g* for a further 15 min at 4 °C to remove any remaining debris. The resulting bacteriophage

suspension was then filtered using Millex® 0.22 µm pore size syringe filters (Merck Millipore Ltd, Tullagreen, Carrigtwohilll, Co. Cork, IR) and 20 ml Norm-Ject® syringes (Sigma Aldrich, Germany). The phage suspension was stored at 4 °C. In addition, 500 µl of the lysate was aliquoted into each of two 1.5 ml microcentrifuge tubes with a few drops of glycerol, which were then snap frozen in liquid nitrogen. The frozen crude bacteriophage stocks were placed in the freezer at -75 °C for long-term storage.

2.5.2 Crude bacteriophage lysate host range susceptibility

All 14 UPEC isolates were tested for susceptibility to phage infection and lysis using the soft agar overlay technique described by Adams.(69) Each UPEC isolate was cultured at 37 °C for 24 h overnight. 100 µl of each of the overnight cultures was inoculated into 1 ml cation supplemented LB broth (LB supplemented with 10 mM $MgSO_4$ and 10 mM CaCl and sterile filtered), and subsequently cultured for 3 h at 37 °C. LB plates were pre-warmed to 37 °C and 5 ml of liquid soft agar (7 g/l) LB solution was aliquoted into pre-warmed 5 ml microcentrifuge tubes (Sigma Aldrich, Germany) which were then placed back into a water bath set at 48 °C to prevent the soft agar solution from solidifying. 500 µl of each 3 h UPEC sample was then added to the 4.5 ml of soft agar and swirled to mix. The contents of each soft agar-bacteria tube were then poured onto the bottom agar layer on the pre-warmed agar plates. The plates were then placed into an incubator at 37 °C for 20 min to set and to allow for initiation of bacterial lawn growth. Following the incubation period 10 µl of isolated bacteriophage sample was carefully dropped into the centre of each plate. The plates were then placed inverted in the incubator at 37 °C for 24 h. Following this, the plates were removed from the incubator and examined for plaques caused by lysis of the bacteria on the bacterial lawns. The 14 UPEC strains were then ranked in order of susceptibility to lysis by the bacteriophage crude preparation, qualitatively - depending on the presence and the appearance of the plaques formed.

2.6 Bacteriophage purification

2.6.1 Plate enrichment

UPEC strains UEmw_8 and UEmw_9 were the selected propagation strains for bacteriophages on the basis of susceptibility to phage lysis and capacity for biofilm formation. 10 ml of each of these two isolates was cultured in AUM for three h in 15 ml sterile centrifuge tubes. One millilitre of phage lysate was added to each of the 3-hour cultures, which were then left to grow overnight at 37 °C for 24 h.

Each tube containing bacteriophage-bacterial host preparation was then subjected to centrifugation at 4000 × *g* for 5 min (Eppendorf Centrifuge 5804 R) to remove any residual bacteria and cellular debris. The supernatant was then filtered through a 0.22 µm pore size syringe filter. The soft agar overlay method was carried out as described above, by dropping 10 µl of each of the cultured, filtered bacteriophage samples onto the centre of plates containing lawns of UEmw_8 and 9 respectively. The plates were then placed inverted in the incubator at 37 °C for 24 h. A section of agar harboring a resulting plaque from each bacterial lawn was then excised from the plates using sterile pipette tips and each was re-suspended in 1.5 ml SM phage buffer in sterile 1.5 ml microcentrifuge tubes. The tubes containing the plaque overlay sections were then each vortexed for 5 min and then centrifuged at 4000 × *g* for 5 min (Eppendorf Centrifuge 5417C). The soft agar overlay method was then repeated, this time allowing 10 µl of the excised plaque phage preparation to infect 500 µl of bacterial culture for 30 min at 37 °C before combining the phage-bacteria mix with 4.5 ml of soft agar, and then plating – rather than dropping phage directly onto the bacterial lawns. This amended method allowed for avoidance of picking a central plaque at the spotting area that may contain several species of phage. The plates were then incubated overnight and distinct plaques located. Single plaque isolation, re-suspension in SM buffer, and re-plating, was repeated three more times in each propagation strain in order to secure a distinct single-species phage population from each.

For ease of reference to the specific bacteriophages, the two unique viruses were given preliminary designations according to the naming convention set out by Kropinski et al.(89) without designations for indication of tail morphology (which was added after microscopic analysis).

2.6.2 Bacteriophage concentration and ultracentrifugation

The phage suspensions thus derived from a single distinct virus for each sample were then concentrated in liquid culture by inoculating 1.5 ml of each plate purified lysate into 2 l of UEmw_8 and UEmw_9 cultures respectively, in order to obtain adequate volumes of high titre phage for purification. The liquid cultures were incubated overnight at 37 °C in the dark with low speed shaking (50 rpm). Following this culture step, the 2 distinct phage lysates were again isolated and concentrated using the same centrifugation-PEG process described above (section 2.5.1) for the raw sewage. Next, 4 ml of each high titre phage lysate in SM buffer was aliquoted into 2 sterile 10 ml thick-walled polyallomer tubes and balanced. The tubes were then placed in a Type 70.1 Ti rotor (Beckman Coulter) and centrifuged at 25 000 × *g* for 1 h at 4 °C (Beckman Coulter Optima™ L-80 XP Ultracentrifuge) to pellet the virus. The majority of the supernatant was carefully removed by gentle pipetting. Ammonium acetate washing of the phage suspension was then carried out: 4 ml of 0.1 M ammonium acetate solution was added to each tube. The tubes were balanced and the ultracentrifugation repeated under the

same conditions. The supernatant was again removed and 4 ml of 0.1 M ammonium acetate solution was added to each tube followed by a final identical ultracentrifugation step. The supernatant was carefully pipetted into 50 ml centrifuge tubes and stored at 4 °C and the viral pellet in each tube was re-suspended in sterile 0.1 M ammonium acetate and stored in 1.5 ml microcentrifuge tubes at 4 °C.

Since the virus infecting UEmw_8 was not cultivable to a high enough concentration in liquid culture, as determined in the results of the succeeding phage enumeration step, a plate concentration procedure was carried out as described by Summer et al.(90) Firstly the calculated phage titre from the plate count was used to estimate the number of plaque forming units of phage (and thus the volume of the prepared lysate) necessary for confluent lysis of a bacterial lawn of UEmw_8. The volume and concentration was then checked by soft agar overlay to confirm its accuracy. Ten soft agar overlay plates were then prepared by infecting each with equal volumes of the phage lysate such that all ten yielded almost complete lysis. Onto each of the ten plates, 8 ml of SM buffer was pipetted using a Pasteur pipette. The plates were then left to stand in a Class II BSC at room temperature for 4 h to allow phage desorption into the buffer from the plaques on the plate surfaces. Following this incubation period, the phage-containing buffer was collected via a 5000 µl automatic pipette, while gently tilting the plate to one side. The high titre phage lysate from all ten plates was combined and stored in two 50 ml centrifuge tubes. The tubes were then centrifuged at 4000 × *g* for 5 min at 4 °C. (Eppendorf Centrifuge 5804 R) to remove any residual viable bacteria and cellular debris. The supernatant was then filtered through a 0.22 µm pore size syringe filter. The ultracentrifugation and ammonium acetate wash as described above was then carried out. Phage enumeration of the high titre lysate was then carried out as described below.

2.6.3 Bacteriophage enumeration

Once a pure lysate of each phage species was thus obtained each was serially diluted into 15 sterile 200 µl microcentrifuge tubes, by successive transference of 10 µl phage lysate into 90 µl SM buffer and then pipetting 10 µl of each subsequent dilution into 90 µl of SM, providing a tenfold dilution in each subsequent tube. Ten microlitres of each dilution was then inoculated into 500 µl of an exponential growth-phase culture of the respective host strain in cation supplemented LB media. The phage-bacteria preparations were then incubated for 30 minutes at 37 °C to allow infection of host bacteria by the phages. 500 µl of each phage-bacteria sample was then mixed with 4.5 ml soft agar that had been maintained at 48 °C in a water bath in 5 ml centrifuge tubes. The entire soft agar – phage – bacteria mixture of each dilution was then poured over pre-warmed (to 37 °C) LB agar plates. The plates were then placed in an incubator at 37 °C for 24 h. The number of plaques per plate was counted on plates harboring between 30 and 200 plaques such that the phage titre could be determined.

2.6.4 Final bacteriophage host range determination

The 14 UPEC isolates were again tested for susceptibility to phage infection and lysis, this time using the two distinct bacteriophages active against UEmw_8 and UEmw_9 as enriched and isolated as described above. The soft agar overlay spot test technique described by Adams was used as before.(69) Plates containing the bacterial lawns were divided into two halves and each half marked with P8 or P9 indicating which phage was being tested. A 5 µl drop of dilute high titre phage lysate was dropped onto the corresponding marked side of the bacterial lawns of each of the 14 strains (Figure C1, Appendix C).

2.7 Bacteriophage characterization

2.7.1 Transmission Electron microscopy

The phage isolates obtained from the ultracentrifugation purification were stained using negative staining with 2% (w/v) uranyl acetate (UA). The single-droplet method was used, as described by Ackerman.(68) Fifteen microlitres of the 2% (w/v) aqueous solution of UA was mixed with 15 µl of each phage sample on a piece of parafilm in a sterile Petri dish. One drop of the mixture was then carefully pipetted onto a lacey carbon TEM (holey-carbon) grid for each sample while holding the grid in place with microscopy tweezers (PTFE-Coated stainless steel tweezers, Sigma-Aldrich, USA). The mixture was left to adhere to the grid for ±20 s and then the majority was wicked off by touching filter paper to the side of the grid. The grids were left to air-dry at room temperature in a closed sterile Petri dish in the dark. The phages were then examined and photographed via transmission electron microscopy (Tecnai T12 Transmission Electron Microscope at the Wits Microscopy and Microanalysis Unit, Johannesburg; a JEOL JEM-1011 Electron Microscope at the NHLS, Johannesburg; a Tecnai T12 Transmission Electron Microscope at the National Institute for Communicable Diseases (NICD), Sandringham, Johannesburg) to confirm bacteriophage morphology. The two unique viruses were given designations based on host species, morphology, and tail features after visualization, according to the naming convention set out by Kropinski, Prangishvili and Lavigne.(89) The virus infecting UEmw_8 is referred to vB_EcoP-U8 and that infecting UEmw_9 is designated vB_EcoX-U9.

2.7.2 Bacteriophage genetic characterization

2.7.2.1 Bacteriophage genome extraction

The purified bacteriophages lysates of vB_EcoP-U8 and vB_EcoX-U9 were subjected to genomic nucleic acid extraction using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) as per the manufacturer's instructions.

Briefly 200 µl of each purified phage solution was added to a sterile nuclease free microcentrifuge tube. To this mixture 200 µl of working solution containing binding buffer and 50 µl of freshly prepared proteinase K was then added. The solution was incubated for 10 min at 72 °C, and 100 µl of binding buffer was added. The entire solution was then pipetted into a high pure filter tube in a high pure collection tube. The tube assembly containing the sample was then centrifuged for 1 min at 8000 × *g*. The collection tube with the flow through was discarded and the filter tube placed in a new collection tube. 500 µl of "inhibitor removal buffer" was added to the filter tube. Centrifugation for 1 min at 8000 × *g* was again carried out and the collection tube and flow through discarded. The filter tube was placed into a new collection tube and 450 µl of wash buffer was added and the assembly was again subjected to centrifugation for 1 min at 8000 × *g*. The wash-centrifugation step was then repeated. The tube assembly was then centrifuged for 10 s at 13 000 × *g* to remove any residual wash buffer and the collection tube again discarded. The filter tube was then placed in a sterile nuclease free microcentrifuge tube and 50 µl of elution buffer was added. The tube assembly was then centrifuged for 1 min at 8000 × *g*. The filter tub was discarded and the tube containing the viral DNA stored at 4 °C.

The extraction of vB_EcoX-U9 nucleic acids was unsuccessful with repeated attempts using the High Pure Viral Nucleic Acid Kit, so further attempts at extraction of the nucleic acid were undertaken with different viral nucleic extraction kits namely:

- Direct-zol™ RNA Miniprep (Zymo Research)
- PureLink™ Genomic DNA Mini Kit (invitrogen)
- QIAmp® DNA Blood Midi Kit (Qiagen)
- RNeasy® Protect Animal Blood Kit (Qiagen)

Still further attempts at extraction of vB_EcoX-U9 nucleic acids were made using the MagnaPure automated Total Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) and a Tris-EDTA-SDS lysis buffer - chloroform/isopropanol method adapted from McCarthy & Romanowski.(91) The manual extraction procedure and all other kit methods were also carried out on phage vB_EcoP-U8 for comparison so that the highest quality and quantity of phage DNA would be obtained.

The resulting purity and yield of the vB_EcoP-U8 DNA was measured by Nanodrop spectrophotometry (Thermo Scientific NanoDrop 2000). Electrophoresis of the DNA was carried out on a 0.8% (w/v) agarose gel stained with 1 × (v/v) concentration gel red (5 µl of 10 000 × stock solution in 50 ml gel).

2.7.2.2 Bacteriophage sequencing

The pure vB_EcoP-U8 phage nucleic acid was then diluted in PCR grade nuclease free dH_2O before being sent to the NICD Sequencing Core Facility (NSCF) for next generation sequencing (NGS). One hundred times coverage was used in the sequence analysis. De novo assembly of a contiguous sequence (contig) from the read data was carried out. The contig was then subjected to BLAST on the NCBI website for comparison with published phage genome sequences. Gene and protein analysis and annotation was done using both Prokka(92) and PHAge Search Tool (PHAST)(93).

2.8 Biofilm catheter assays

Sterile 100% silicone coated latex Foley balloon catheters were used for the biofilm prevention and degradation assays. The method used was a modified version of that described by Curtin and Donlan(16) and that used by Chibeu et al.(17) Biofilms were grown in the lumina of 25 cm long segments of catheters using a variable speed Millipore Model - XX8000230 (Millipore Corporation, Massachusettes, USA) peristaltic pump to control flow rate of media through the catheters. The components of this modified flow reactor (FR) setup, including all piping and connections, were sterilized prior to the assays using ethylene oxide gas sterilization. Each of the two bacterial strains was assayed successively using the same method, and experimental and culture conditions, with the sterile piping and connections being replaced between each run to prevent contamination. A 200 ml batch culture of the bacterial strain in AUM, and a sterile medium reservoir containing 50% diluted AUM were prepared. The culture was then pumped through the apparatus for 2 h at a flow rate of $1ml.min^{-1}$ in order to irrigate the catheter segments. The segments were then irrigated for a further 22 h using the diluted AUM at a flow rate of 0.5 ml/min to allow the establishment of a biofilm. All experimental work done with the catheters was carried out at 37 °C by keeping the entire apparatus in a Series 25 Incubator Shaker (New Brunswick Scientific Co., Inc, Edison, NJ, USA). The incubator was set to ‘no shaking’ and was wiped down with 70% (v/v) ethanol and 1.5% (w/v) sodium hypochlorite solution between each run. A simple schematic diagram of the flow reactor apparatus used is shown below in Figure 2.

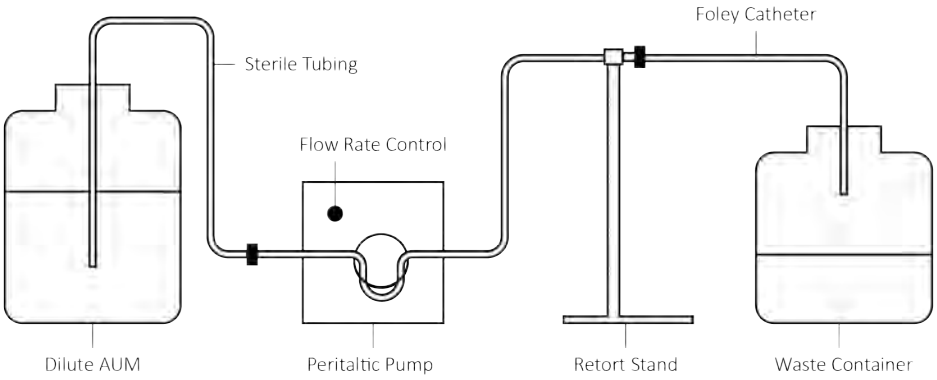


Figure 2. Schematic of the flow reactor configuration for the biofilm degradation and prevention assays.
The entire FR was housed in a top-opening flatbed incubator to maintain a consistent temperature of 37 °C throughout all the runs.

2.8.1 Biofilm degradation assay

After the irrigation period, catheter sections were removed from the FR and moved to a class II BSC. For the positive biofilm controls, two 2.5 cm sections were then cut from the catheter segment, using a sterile surgical blade at standardized positions along its length. Each section was then cut vertically into halves. Each half was washed gently with phosphate buffered saline (PBS) in order to remove cellular debris and planktonic cells. The sections were then vortexed for 15 s in 5 ml freshly prepared PBS (pH 7.2) followed by sonication in a water bath sonicator (Transsonicator 460/H, Elma, Germany) at 35 kHz for 10 min. Vortexing for an additional 15 s, followed by a further 5 min of sonication and then 15 s of further vortexing was carried out. For two of the catheter segment slices the percentage of live to dead cells was determined using a flow cytometric cell viability assay described below (section 2.9). For the other two slices the obtained cell suspension was subjected to spectrophotometric analyses at OD_{595} to record the relative biofilm and cellular composition based on the relationship between optical density and biomass. One hundred microlitres of each sample was also spread onto LB agar plates in duplicate and incubated for 24 h at 37 °C. The resulting bacterial colonies were then counted and recorded for determination of the number of viable cultivable cells from each suspension. The negative controls were prepared by the identical process with the exception that the catheter sections were initially irrigated with only dilute AUM instead of bacterial cultures.

The remaining 20 cm catheter section containing the cultivated biofilm was then treated with dilute phage appropriate for each bacterial strain. Phage lysate diluted to 10^7 pfu/ml in SM buffer from each high titre phage isolate was gently injected into each catheter segment

lumen using a sterile 20 ml syringe, having covered one end of the catheter segment with parafilm to prevent spillage. As with the positive and negative biofilm controls, 2.5 cm slices of the phage containing segments were then removed at 2, 4, 8, and 24 h intervals. Each was processed and subjected to plate count and spectrophotometric assays as described above and flow cytometric analysis described below (Section 2.9) to determine the effects of the phage on the established biofilm and constituent cells. In addition, haemocytometer counting with Trypan blue exclusion viability staining was attempted using light microscopy as an additional direct cell viability assay. This assay was abandoned as the live cells were found to be highly motile and could thus not be counted reliably because they moved around on the grid.

2.8.2 Biofilm prevention assay

In determining the capacity of the isolated phages to prevent establishment of biofilms, the catheters were filled with the dilute (10^7 pfu/ml) phage lysate as prepared previously in SM buffer before being coupled to the FR for irrigation with bacterial samples. The catheter segments containing the dilute phage samples were each incubated for 1 h at 37 °C, before the solution was removed. The non-treatment control catheter was filled with sterile AUM for 1 h before irrigation with UPEC. The apparatus was then set up as before and irrigation of the catheter with bacterial culture and AUM was carried out. In this case, 2.5 cm slices of the catheter segment were removed at 2, 4, 8 and 24 h intervals during the irrigation period. Each catheter slice was washed, sonicated and vortexed as described for the degradation assay. Flow cytometric, plate count and spectrophotometric analyses were carried out as per the method described above for the degradation assay. The resulting data was compared with that of the non-pretreated segments processed in the same way but without prior incubation with phage, in order to determine the overall efficacy of phage pre-treatment in the prevention of biofilm establishment.

2.8.3 Statistics

Both the plate count and absorbance data obtained from the biofilm prevention and degradation assays (run in triplicate) were graphed for visual representation using Numbers for Mac (Version 3.6.2) with error bars indicating standard error of the mean for each incubation time. Statistical analysis was carried out using IBM SPSS Statistics Subscription Package (Version 1.0.0.800). Plate count data was transformed to normal distribution by the use of a “value = log(CFU+1)” transformation, thus ensuring that actual zero counts were presented as zeros in the logarithm.

For the absorbance measurements negative values represented a ‘less than the negative control’ value and as such were considered to have no greater numerical significance than an

actual absorbance of zero (the negative control being the cut-off for no biofilm or cells present in the samples - negative values being meaningless below the threshold). Thus all zero and negative data in the absorbance assays were presented as zero. To attain normal distribution of the absorbance data, they were also transformed by the following transformation: value = log((absorbance × 10^3)+1).

For all assays the hypothesis that; “bacteriophage treatment or pre-treatment significantly decreased biofilms (cells or biomass) compared to the untreated controls” was tested using a one-way analysis of variance (ANOVA). This was done by testing the effect of the independent variable, namely “condition” (treatment or control) on the dependent variable (either plate count or absorbance). The analysis measured the effect of condition across all sampled values throughout the entire incubation period to test for a global significance of difference between cell viability and absorbance data of the treatments vs. the controls. In addition, the data generated from the treatment and control samples at the final time point (24 h) in isolation, for each assay was compared by one-way ANOVA to measure the effect at the end of treatment compared to untreated values at that same time point. Finally, the change in biofilm levels over time was determined by comparing the means of the initial sample values and the means of the final values. This was done for controls and treatments. The statistical information gained thus facilitated insight into whether the independent variable; namely the condition (either “treatment” or untreated “control”) significantly affected the dependent variable (the count or absorbance data), both overall and by the end of treatment.

2.9 Flow cytometry

2.9.1 Nucleic acid double stain viability assessment

Flow cytometric analysis was carried out on the biofilm cell suspensions isolated from the catheter segments of both the degradation and prevention biofilm assays. The method used was a nucleic acid double staining (NADS) procedure as outlined by Barbesti et al.(79) adapted as per the method described by Cerca et al.(80) The cell suspensions were assayed using a Beckman Coulter Gallios Flow Cytometer, with acquisition recorded using the Gallios built-in acquisition software using log amplification for all data. Data analysis was carried out using DeNovo FCS Express Version 6 for Mac OS X. The cell suspensions prepared in each assay were stained with 10 µg/ml propidium iodide (PI) and 10 × concentration of SYBR Green I (SG), incubated at room temperature for 20 min in the dark prior to analysis. Three millilitres of each sample was used in each flow tube. Excitation of both dyes was stimulated by the 22 mW, 488 nm OPSL Sapphire (Blue Diode) laser. SG emission, peaking at 521 nm, was measured in the fluorescence channel 1 (FL1). PI, having a peak emission (when bound to DNA) of ~617 nm, was measured in the fluorescence channel 3 (FL3). Compensation for spectral overlap of emission was carried out using the automatic compensation tool in FCS Express. To facilitate compensation single stain controls for both PI and SG, and a universal

negative of unstained heat-treated cells (boiled for 10 min), were recorded along with the samples for every run.

2.9.2 Statistics

Percentages of live and dead cells were determined in each analysis by automatic gating of populations based on single stained controls with compensation adjusted using the DeNovo FCS Express (Version 6) automatic compensation tool. For graphical representation the data were presented in bar graphs indicating the percentages of the total measured cell count events that were gated as either dead cells and live cells. Error bars indicate standard error of the means for each condition (treatment or control) of each strain. One-way ANOVA was carried out as described above for the absorbance and cell count data, done using IBM SPSS Statistics Subscription Package (Version 1.0.0.800), to determine the global significance of difference between untreated control values and the treatment values, and again at time = 24 h only, to compare the effect of treatment (or pre-treatment) with phage to the controls only at the end of the incubation.

CHAPTER 3 – RESULTS

3.1 Bacterial isolation and culture

In order to obtain 15 confirmed UPEC isolates, 38 urines samples were tested. Thus 39.5% of the urine samples assayed contained cultivable *E. coli* strains. No other single bacterial species was represented as highly in the isolates from the UTI-suspected urines. Other possible uropathogenic species recorded during the screening process included gram-positive and gram-negative bacteria, occurring as both single- and multi-species colonies. Bacterial species present in the urine samples included; *Citrobacter koseri, Klebsiella pneumoniae*, *Enterococcus faecium, Staphylococcus epidermidis, Acinetobacter baumannii* and *Proteus mirabilis.*

Only putative *E. coli* strains were then further scrutinized biochemically by Microscan analyses. One *E. coli* sample (derived from urine sample 28) was indicated as sorbitol-fermentation negative and flagged as a possible strain of *E. coli* O157:H7. It was therefore omitted from further analyses in the study. A total of 14 UPEC strains were carried forward to the preliminary biofilm assay. Microscan analyses print outs can be found in Appendix E.

3.2 Preliminary biofilm assay

The preliminary biofilm assay ranked UPEC strains most likely to form biofilms and to what extent each would do so under physiological conditions. Figure 3 shows the levels of biofilm formed by each strain by measurement (absorbance at 595 nm) of the CV stained EPS and biofilm embedded cells remaining bound to microtitre plate well walls after washing away the planktonic cells.

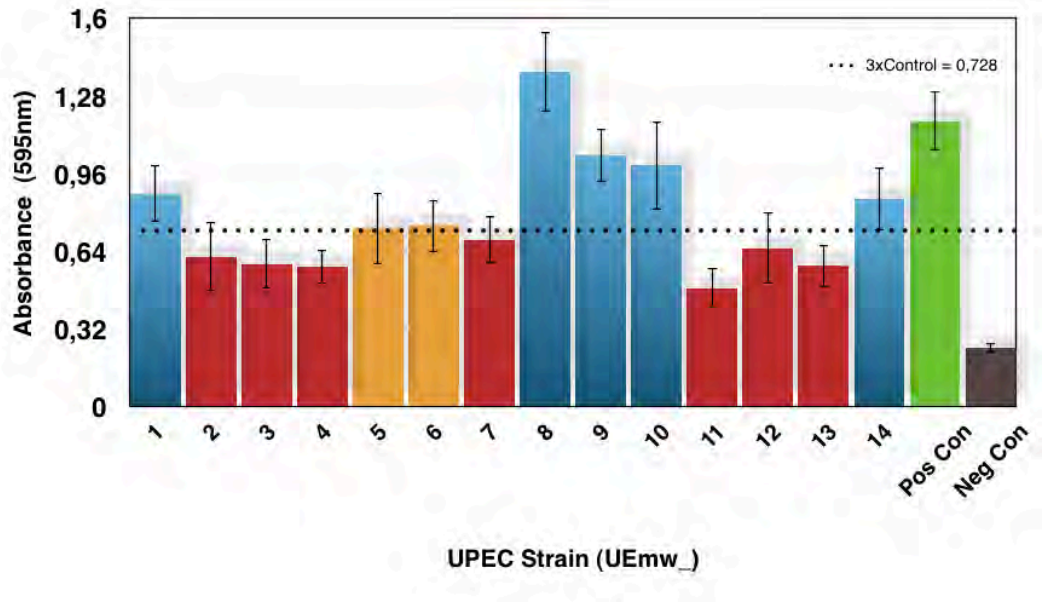


Figure 3. Graph of average absorbance values for CV stained UPEC strains in repeat microtitre plate assays.
Strains with absorbance values consistently greater than 3 times the value (i.e. A_{595}= 0.728) obtained for the negative control (sterile AUM) were considered strong biofilm formers. The dashed reference line on the graph indicates this cut-off. Error bars indicate standard error for all replicate measures. Blue bars show significant biofilm forming strains and red bars indicate non/low biofilm formers. The strains represented by orange bars hover around the cut-off value. UEmw strains 1, 8, 9, 10 and 14, were the greatest biofilm forming strains of the 14 tested, with UEmw_8 consistently producing the highest values.

UEmw_8 was significantly better at forming high levels of EPS (thick biofilms), than any other strain assayed in the microtitre plate assay. UEmw strains 5 and 6 had means slightly above the “3 x negative control” cut-off, but as such represent the lowest levels of biofilm production that could be considered significant. UEmw strains 1, 9, 10 and 14, consistently formed thick biofilms but the produced biofilm levels were not significantly differentiable from each other across repeat analyses. The additional information obtained in the bacteriophage host range susceptibility assays (Section 3.5.1) thus allowed for determination of the most appropriate strains to carry forward in the study.

3.3 Bacterial genetic characterization

3.3.1 DNA extraction

Table 5, below, indicates the efficiency of the genomic DNA extraction procedure, as analysed using a Nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000). The A260/280 ratio was within the accepted range of between 1.7-2.0 for pure DNA, and the A260/230 ratio was a little lower than the desired ±2.0. (94) These deviations indicate a small degree of contamination relating to higher than desirable amounts of co-eluted components

from the extraction kit (like phenol or chaotropic salts), or from pH levels differing from the optimum,(95) but little to no protein contamination and negligible RNA contamination.(96) The purity and yield was sufficient for PCR amplification of the gene of interest.

Table 5. Purity and yield of genomic DNA extracted from sample and control bacterial strains

Sample	DNA concentration/yield (ng/µl)	A260/280	A260/230
UEmw_8	29.9	2.07	1.64
UEmw_9	10.2	1.99	1.72
CFT073	11.7	1.90	1.66

3.3.2 PCR

The 16S rRNA genes were amplified from the extracted genomic DNA by PCR using primers 4F and BAKR. The reactions resulted in a single band corresponding to fragments of around 1500kb each when the amplicons were run on a 2% (w/v) agarose gel, as can be seen in Figure 4 (section 3.3.3).

3.3.3 Electrophoresis

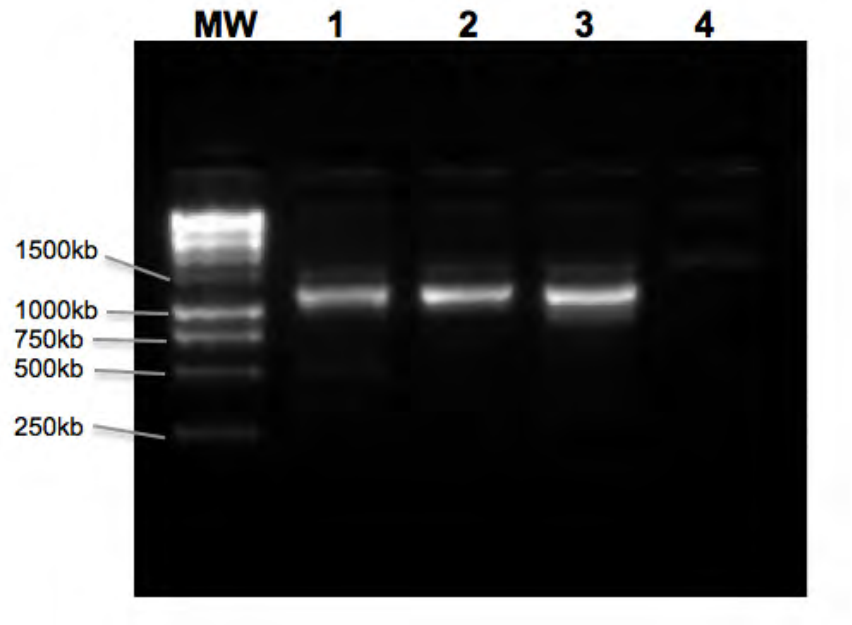


Figure 4. Agarose gel showing electrophoretic migration of the 16S rDNA amplicons.
GelRed stained 2% (w/v) agarose gel indicating PCR amplification products from primers 4F and BAKR. Lanes: MW - Molecular weight marker (1kb ladder, Promega) 1 - CFT073, 2 – UEmw_9, 3 – UEmw_8, 4 – NTC. The presence of a band corresponding to fragments ±1500bp in size for each strain can be seen.

3.3.4 Sequencing

3.3.4.1 DNA clean up

The PCR reactions yielded amplicons of suitable quality for the 16S rDNA sequencing following cDNA clean up as indicated by the spectrophotometric analysis shown in Table 6.

Table 6. Purity and yield of PCR amplified 16S rDNA of sample and control bacterial strains

Sample	A260/280	A260/230	DNA concentration/yield (ng/µl)
UEmw_8	1.83	1.64	102.4
UEmw_9	1.89	1.72	98,8
CFT073	1.84	1.61	40.5

Amplicons were diluted in elution buffer (from the Biospin PCR Purification Kit) to ensure a concentration of 1.5 ng/µl of amplicon per sample in the cycle sequencing reactions, according to the concentrations of DNA obtained as indicated in table 6.

3.3.4.2 PCR cycle sequencing

The sequencing reaction positive control (pGEM 3Zf(+) plasmid DNA) yielded a 1077 base DNA fragment. The highest quality read data (fidelity/reliability of each base identification as marked by the analyzer) was achieved between bases 19 and 968 of the sequence. The sequence was trimmed to the 950 base sequence of highest read quality, which was then subjected to a nucleotide BLAST through the Geneious™ software interface. The sequence was found to have 99.8% pairwise identity (946 to the portion of the published pGEM 3Zf(+) plasmid (Accession number: X65306) sequence to which it corresponded. This alignment can be found in Appendix D. The expect value (E-value) of 0 indicated that the probability of seeing such sequence similarity with the actual plasmid sequence by chance is so low that it is negligible. The sequencing reaction parameters and sequence analyzer were thus accepted as having been run successfully with no detectable operational errors.

The data obtained from the sequencing procedure for each sample were sets of contiguous nucleotide base sequences (contigs) from the fragments generated by differential elongation of the six different primers. These contigs were then assembled into consensus sequences for each of the two UPEC strains, and the control strain *E. coli* CFT073. The obtained consensus sequences were of varying length and read quality as indicated in Table 7 below. The sequences were also subject to nucleotide BLAST against the NCBI 16S ribosomal sequences (Bacteria and Archaea) for typing.

Table 7. Quality, length, and identity with 16S type strain, of sequenced fragments

Strain/Organism	Sequence length (bases)	Read Quality (%)*	Identity to *E. coli* 16S sequence by BLAST (%)**	E. Value
UEmw_8	1528	92.1	99	0
UEmw_9	1547	92.2	99	0
CFT073	1543	93.5	99	0

*Quality of the sequence read data indicates the certainty of base identity across the entire fragment/consensus sequence

**All BLAST data revealed 99% sequence identity with numerous strains of closely related species for the *E. coli* consensus sequences.

The Geneious Biodiversity Tool was used to analyze the consensus sequences attained and the graphical representations of the outcomes are shown below. Both the manual nucleotide BLAST on the NCBI website and the Biodiversity Tool analysis were able to discriminate the 16S rRNA sequences to the Family level for the *E. coli* isolated sample sequences and the CFT073 sequence.

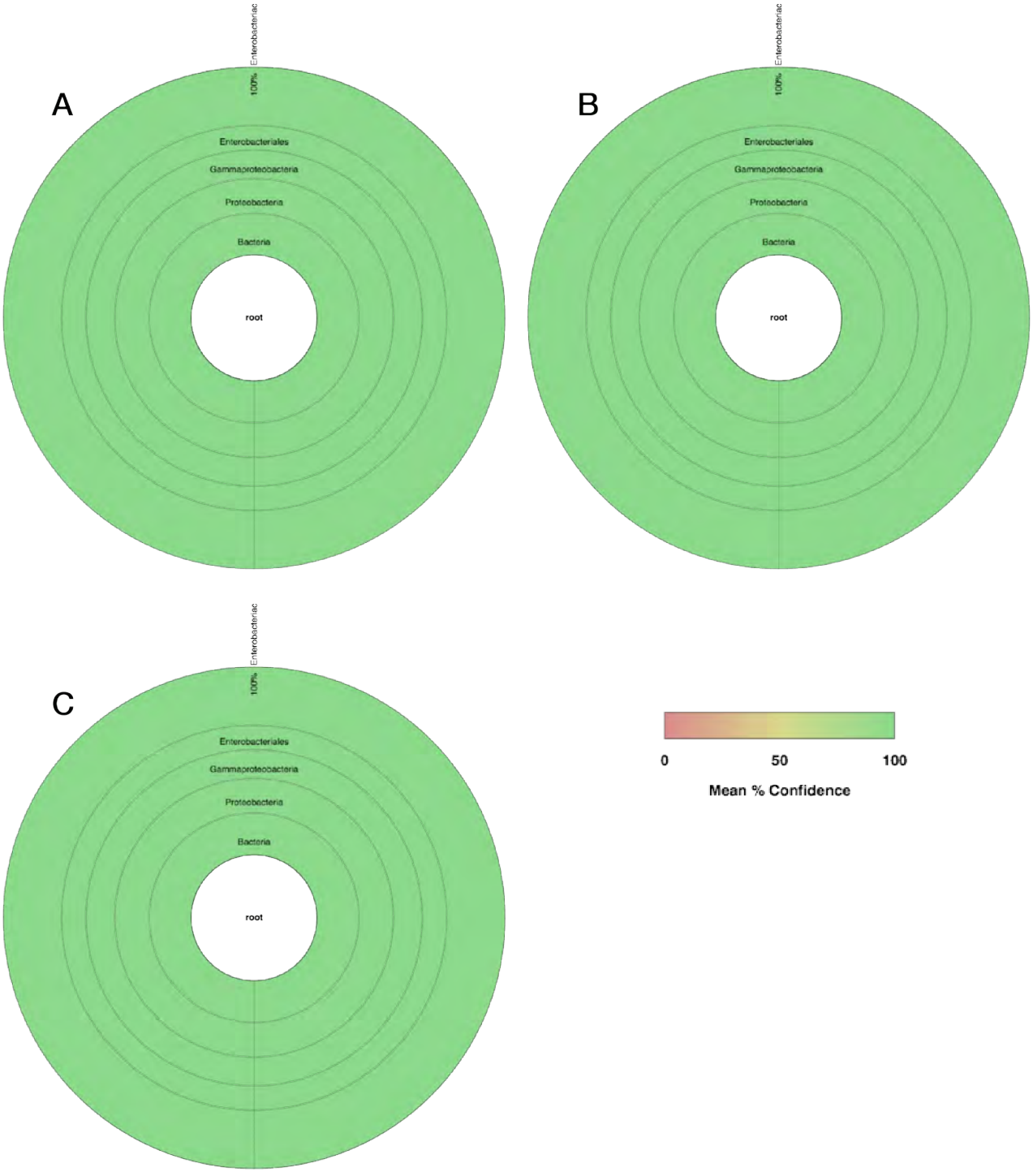


Figure 5. Pictograms indicating results of biodiversity analysis of the consensus sequences derived from sequencing of amplified UEmw_8, UEmw_9 and CFT 073 16S rRNA genes.

A - Biodiversity analysis pictogram for UEmw_8 16S rRNA gene. B - Biodiversity analysis pictogram for UEmw_9 16S rRNA gene. C - Biodiversity analysis pictogram CFT 073 16S rRNA gene. The Geneious biodiversity tool indicated 100% confidence up to the genus level of Enterobacteriaceae for all three consensus sequences.

For ease of comparison, graphical representation of the multiple alignments of the *E. coli* sample strain sequences and the *E. coli* control strain (CFT073) 16S rRNA gene reference sequence is presented below as a single alignment in Figure 6.

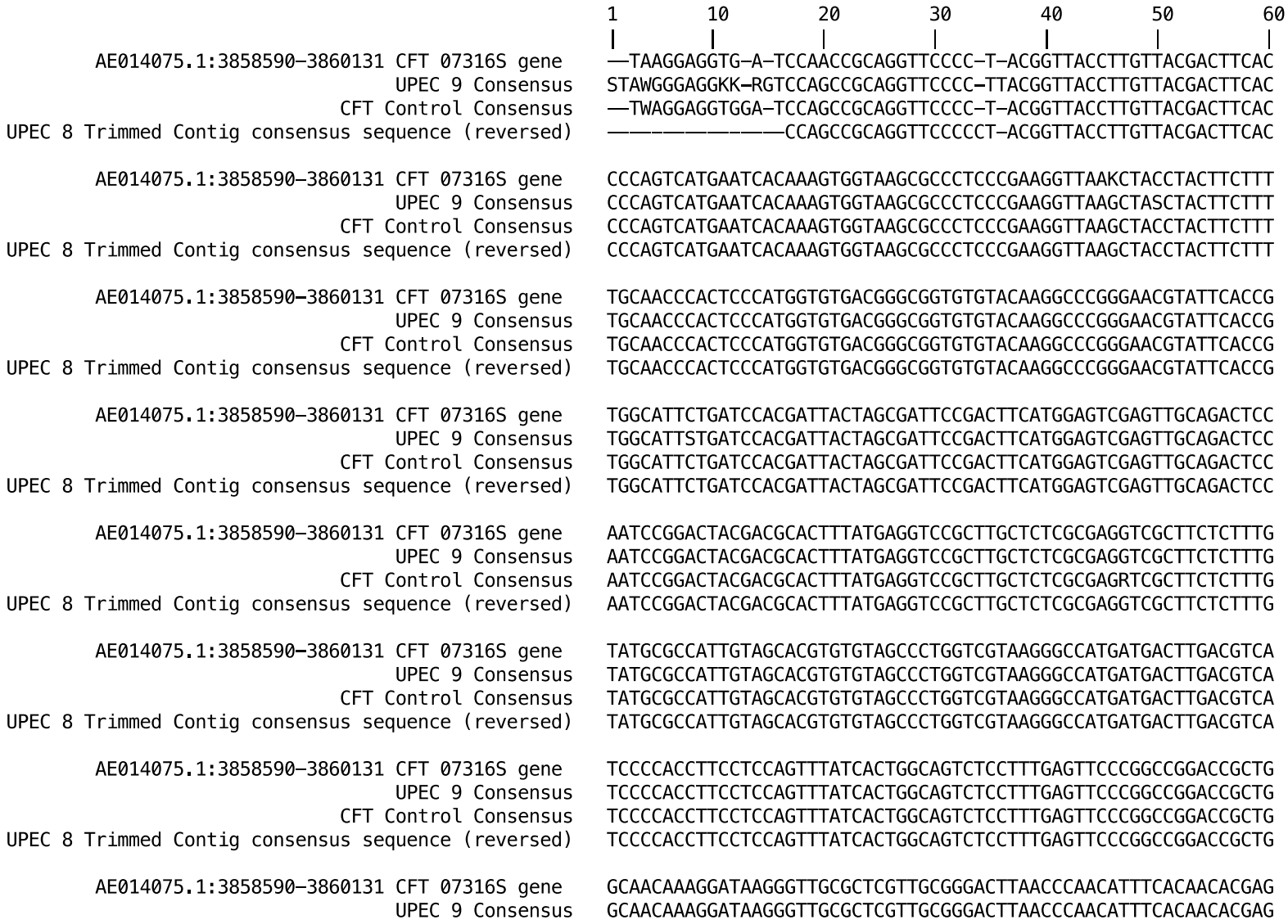


Figure 6. Consensus sequences of *E. coli* 16S rRNA genes mapped to reference 16S rDNA sequence.
CFT073 is labelled “CFT control consensus”, UEmw_8 is marked “UPEC 8 Trimmed Contig consensus sequence” and UEmw_9 is marked as “UPEC 9 Consensus”.

```
UPEC 8 Trimmed Contig consensus sequence (reversed)   GCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAG

        AE014075.1:3858590-3860131 CFT 07316S gene     CTGACGACAGCCATGCAGCACCTGTCTCACGGTTCCCGAAGGCACATTCTCATCTCTGAA
                                   UPEC 9 Consensus   CTGACGACAGCCATGCAGCACCTGTCTCACGGTTCCCGAAGGCACATTCTCATCTCTGAA
                                CFT Control Consensus CTGACGACAGCCATGCAGCACCTGTCTCACGGTTCCCGAAGGCACATTCTCATCTCTGAA
UPEC 8 Trimmed Contig consensus sequence (reversed)   CTGACGACAGCCATGCAGCACCTGTCTCACGGTTCCCGAAGGCACATTCTCATCTCTGAA

        AE014075.1:3858590-3860131 CFT 07316S gene     AA-CTTCCGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACA
                                   UPEC 9 Consensus   AANCTTCCGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACA
                                CFT Control Consensus AA-CTTCCGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACA
UPEC 8 Trimmed Contig consensus sequence (reversed)   AA-CTTCCGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACA

        AE014075.1:3858590-3860131 CFT 07316S gene     TGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTAC
                                   UPEC 9 Consensus   TGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTAC
                                CFT Control Consensus TGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTAC
UPEC 8 Trimmed Contig consensus sequence (reversed)   TGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTAC

        AE014075.1:3858590-3860131 CFT 07316S gene     TCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTC
                                   UPEC 9 Consensus   TCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTC
                                CFT Control Consensus TCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTC
UPEC 8 Trimmed Contig consensus sequence (reversed)   TCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTC

        AE014075.1:3858590-3860131 CFT 07316S gene     CAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCT-CCCCAC
                                   UPEC 9 Consensus   CAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCT-CCCCAC
                                CFT Control Consensus CAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCT-CCCCAC
UPEC 8 Trimmed Contig consensus sequence (reversed)   CAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTNCCCCAC

        AE014075.1:3858590-3860131 CFT 07316S gene     GCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTC
                                   UPEC 9 Consensus   GCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTC
                                CFT Control Consensus GCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTC
UPEC 8 Trimmed Contig consensus sequence (reversed)   GCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTC

        AE014075.1:3858590-3860131 CFT 07316S gene     CAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCC--TCTACGAGACTCA
                                   UPEC 9 Consensus   CAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCC--TCTACGAGACTCA
                                CFT Control Consensus CAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCC--TCTACGAGACTCA
UPEC 8 Trimmed Contig consensus sequence (reversed)   CAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCCCCTCTACGAGACTCA

        AE014075.1:3858590-3860131 CFT 07316S gene     AGCTTGCCAGTATCAGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAA
                                   UPEC 9 Consensus   AGCTTGCCAGTATCAGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAA
                                CFT Control Consensus AGCTTGCCAGTATCAGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAA
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        AE014075.1:3858590-3860131 CFT 07316S gene    CAAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTAT
                                   UPEC 9 Consensus   CAAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTAT
                                CFT Control Consensus   CAAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTAT
UPEC 8 Trimmed Contig consensus sequence (reversed)   CAAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTAT

        AE014075.1:3858590-3860131 CFT 07316S gene    TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATGAGCA
                                   UPEC 9 Consensus   TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATGAGCA
                                CFT Control Consensus   TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATGAGCA
UPEC 8 Trimmed Contig consensus sequence (reversed)   TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATGAGCA

        AE014075.1:3858590-3860131 CFT 07316S gene    AAGGTATTAACTTTACTCCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTC
                                   UPEC 9 Consensus   AAGGTATTAACTTTACTCCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTC
                                CFT Control Consensus   AAGGTATTAACTTTACTCCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTC
UPEC 8 Trimmed Contig consensus sequence (reversed)   AAGGTATTAACTTTACTCCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTC

        AE014075.1:3858590-3860131 CFT 07316S gene    TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCT
                                   UPEC 9 Consensus   TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCT
                                CFT Control Consensus   TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCT
UPEC 8 Trimmed Contig consensus sequence (reversed)   TTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCT

        AE014075.1:3858590-3860131 CFT 07316S gene    GCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGAC
                                   UPEC 9 Consensus   GCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGAC
                                CFT Control Consensus   GCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGAC
UPEC 8 Trimmed Contig consensus sequence (reversed)   GCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGAC

        AE014075.1:3858590-3860131 CFT 07316S gene    CAGCTAGGGATCGTCGCCTAGGTGAGCCTTTACCCCACCTACTAGCTAATCCCATCTGGG
                                   UPEC 9 Consensus   CAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACTAGCTAATCCCATCTGGG
                                CFT Control Consensus   CAGCTAGGGATCGTCGCCTAGGTGAGCCTTTACCCCACCTACTAGCTAATCCCATCTGGG
UPEC 8 Trimmed Contig consensus sequence (reversed)   CAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACTAGCTAATCCCATCTGGG

        AE014075.1:3858590-3860131 CFT 07316S gene    CACATCCGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTA
                                   UPEC 9 Consensus   CACATCCGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTA
                                CFT Control Consensus   CACATCCGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTA
UPEC 8 Trimmed Contig consensus sequence (reversed)   CACATCCGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTA

        AE014075.1:3858590-3860131 CFT 07316S gene    TTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCAC
                                   UPEC 9 Consensus   TTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCAC
                                CFT Control Consensus   TTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCAC
UPEC 8 Trimmed Contig consensus sequence (reversed)   TTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCRGGCAGTTTCCCAGACATTACTCAC
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        AE014075.1:3858590-3860131 CFT 07316S gene    CCGTCCGCCACTCGTCAGCAAAGCAGCAAGCTGCTTTCTGTTACCGTTCGACTTGCATGT
                                   UPEC 9 Consensus   CCGTCCGCCACTCGTCAGCAAAGCAGCAAGCTGCTTCCTGTTACCGTTCGACTTGCATGT
                                CFT Control Consensus   CCGTCCGCCACTCGTCAGCAAAGCAGCAAGCTGCTTYCTGTTACCGTTCGACTTGCATGT
UPEC 8 Trimmed Contig consensus sequence (reversed)   CCGTCCGCCACTCGTCAGCRAARCAGCAAGCTGYTTCCTGTTACCGTTCGACTTGCATGT

        AE014075.1:3858590-3860131 CFT 07316S gene    GTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCAAACTCTTCAATTT
                                   UPEC 9 Consensus   GTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCAGGATCAAACTCTCCAAA??
                                CFT Control Consensus   GTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCAGGATCAAACTCTCCAA???
UPEC 8 Trimmed Contig consensus sequence (reversed)   GTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCAGGATCAAACTCTC------
```

3.4 Bacteriophage isolation

3.4.1 Bacteriophage lysate host range susceptibility

Lawns of each bacterial strain were grown on cation supplemented LB agar plates overnight and subsequently each treated with 10 µl of the prepared phage lysate. The resulting plates were then examined for the presence of plaques / zones of bacterial clearance, where phage lysis of host bacteria had occurred. The lytic activity was qualitatively assessed by size, shape, and clarity of the plaques. Large clear circular plaques were indicative of high susceptibility to phage activity, and turbid plaques being indicative of lysogenic infection. Groups of small plaques are seen as indication of slower infecting phages and infection by different phage species within the crude lysate, effective against the same host. These aspects of plaque morphology were considered as indication of degree susceptibility to phage lytic activity and a hierarchy of susceptibility to the phages present in the crude lysate was created as expressed below in table 10.

Table 8. Hierarchy of UPEC strain phage susceptibility by scrutiny of plaque formation

Rank of susceptibility to phages present in crude lysate	Strain	Description of plaques / lysis pattern
1	UEmw_3	Excellent lysis, one large clear central plaque
2	UEmw_4	Excellent lysis, one large clear central plaque
3	UEmw_8	Excellent lysis, one clear central plaque
4	UEmw_11	Good lysis, some patches of distinct plaques but with bacterial growth between
5	UEmw_5	Good lysis, almost clear central plaque, more opaque than 3,4
6	UEmw_9	Good lysis, large central plaque but slightly less clear than some others
7	UEmw_12	Good lysis, some distinct plaques but with some bacterial growth between
8	UEmw_7	Slight lysis, groups of small plaques with bacteria growing between
9	UEmw_10	Slight lysis, small distinct plaques
10	UEmw_6	Slight lysis, groups of small plaques
11	UEmw_13	Slight lysis, small distinct turbid plaques
12	UEmw_1	Slight lysis, groups of small turbid plaques
13	UEmw_14	No visible plaques
14	UEmw_2	No visible plaques

UEmw_8 and UEmw_9 were chosen for further analyses as both were relatively susceptible to phage attack and were previously shown to have a high propensity to form thick biofilms in the microtitre plate biofilm screening assay

3.5 Bacteriophage purification

3.5.1 Plate enrichment

The viruses were preliminarily named vB_Eco-U8 and vB_Eco-U9, as per the convention described by Kropinski et al.,(89) to indicate that they are *E. coli*-infecting viruses specific to strains UEmw_8 and Uemw_9 with no designation of viral family based on tail morphology yet. The plaques formed on lawns of each propagation strain demonstrate the lytic activity of each virus for its specific host, as can be seen in Figure 7 below.

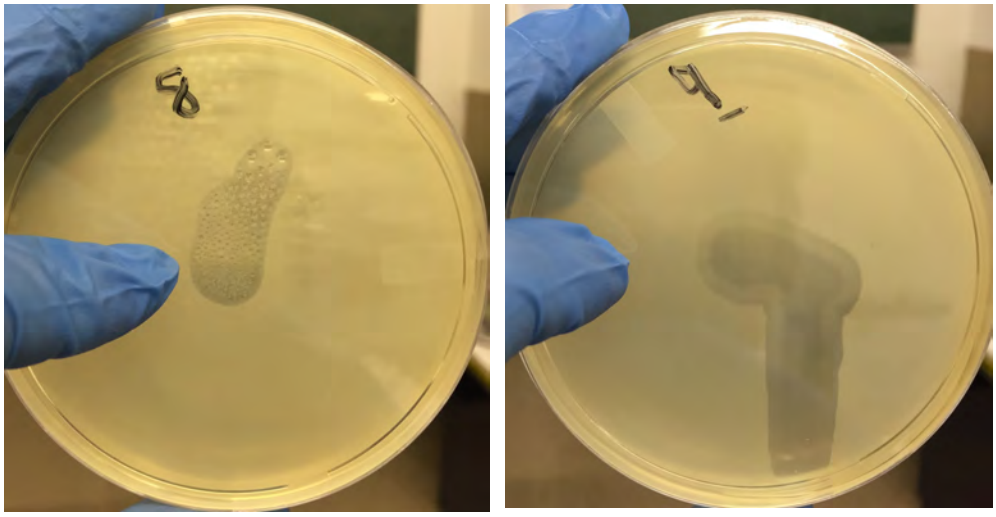


Figure 7. Photos of plaques formed on bacterial lawns of the *E. coli* propagation strains by purified phages, showing their capacity for lysis of their respective hosts.
Lytic activity of vB_Eco-U8 against UEmw_8 (left) and vB_Eco-U9 UEmw_9 (right).

3.5.2 Bacteriophage concentration and ultracentrifugation

The liquid batch culture concentration and ultracentrifugation procedure yielded an inadequate concentration of vB_Eco-U8, with the sample never clearing as would be expected with lysis of the constituent bacteria. Clearance of the liquid media was however seen with UEmw_9 culture and adequate vB_Eco-U9 high titre lysate was attained by ultracentrifugation. Bacteriophage vB_Eco-U8 thus needed to be amplified using a plate culture concentration method as described in section 2.6.2.

3.5.3 Bacteriophage enumeration

For bacteriophage vB_Eco-U8 the phage titre determination following the ultracentrifugation procedure yielded 120 plaques on the 10 × dilution plate when 10 µl of phage lysate was inoculated into 500 µl bacterial culture and mixed with 4.5 ml top agar for the bacterial lawn. The calculated titre of 1.2×10^5 pfu/ml was too low for further analyses (TEM and genetic characterization) so plate concentration was carried out to increase phage numbers. For vB_Eco-U8, following plate concentration protocol, 150 plaques were counted on 10^9 × dilution plate. Thus the titre calculated was 1.5×10^{13} pfu/ml. For bacteriophage vB_Eco-U9, titre from the ultracentrifugation step yielded 114 plaques on 10^7 × dilution plate. Thus the titre calculated was calculated at 1.14×10^{11} pfu/ml.
Figure 8 below shows the plaques formed on the lawns of each bacterial strain following treatment of with bacteriophages for the titre calculations.

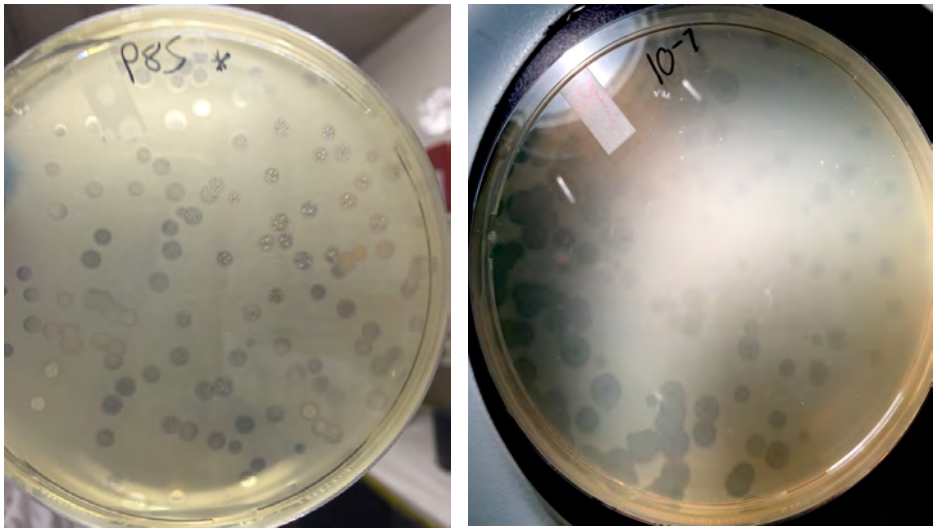


Figure 8. High titre phage-induced plaques on phage enumeration plates.
Phage titre calculation plates for vB_Eco-U8 on UEmw_8 lawn (left) and vB_Eco-U9 on UEmw_9 bacterial lawn (right).

3.5.4 Final bacteriophage host range determination

The host range of the two bacteriophage samples was then assessed by treating each of the 14 UPEC strains with each virus. Five microlitres of purified high titre phage lysate was dropped onto lawns of each of the bacterial strains and incubated overnight. Extremely high specificity of the viruses was seen with no plaques or signs of lysis on any bacterial lawns except in the propagation strains. A single clear plaque on the vB_Eco-U8-treated UEmw_8 lawn was seen, and another clear plaque on the vB_Eco-U9-treated UEmw_9 plate. Images of host range determination plates can be seen in Figure C1 in Appendix C.

3.6 Bacteriophage characterization

3.6.1 Transmission electron microscopy

Transmission electron microscopy facilitated the examination of the morphological features of the isolated bacteriophages at magnifications of between 20 000 and 100 000 ×. The micrographs below shown that the bacteriophage active against UPEC strain UEmw_8, that is vB_Eco-U8, is a tailed bacteriophage with a short noncontractile tail. As such it has been classified as belonging to the order *Caudovirales* and the family *Podoviridae*. The further designation vB_EcoP-U8 indicates the family to which it belongs. vB_EcoP-U8 virions exhibit tails with lengths between 10.3 nm and 12.3 nm and characteristic icosahedral heads measuring between 39.0 nm – 48.1 nm in diameter.

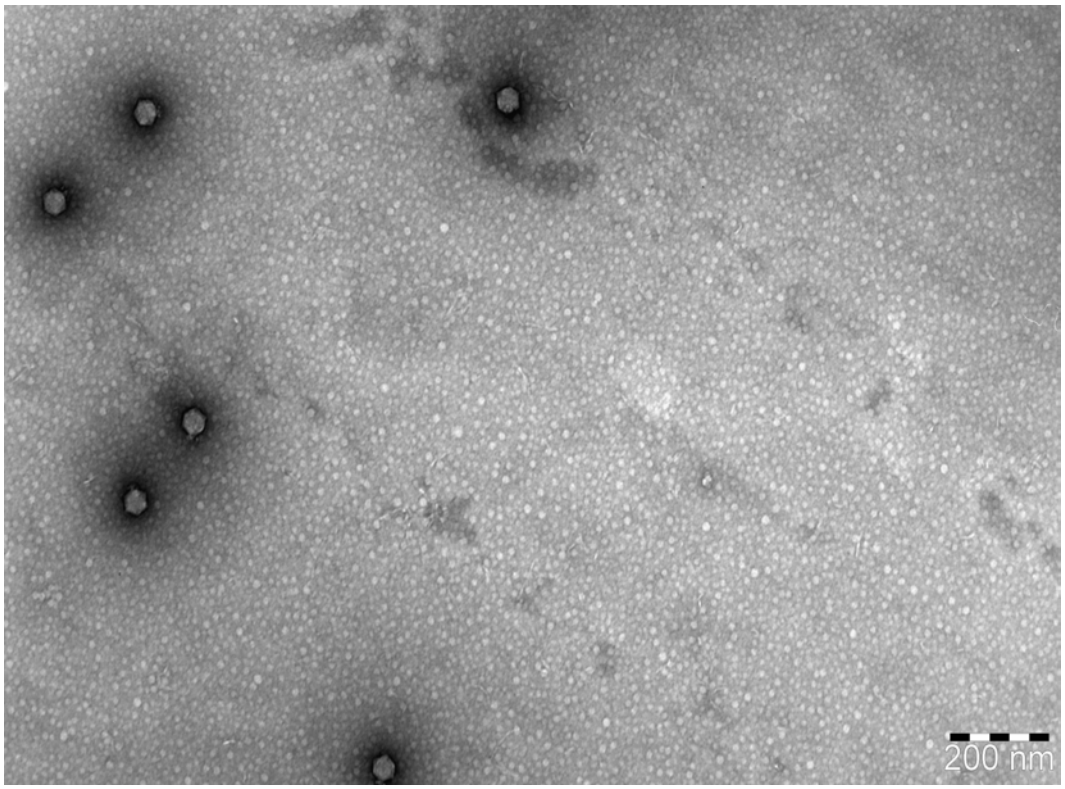


Figure 9. Transmission electron micrographs of vB_EcoP-U8 virions at varying magnifications.
Figure 9a (above). Electron micrograph showing six negative stained vB_EcoP-U8 virions. The consistency in size and morphology is apparent even at low magnification.

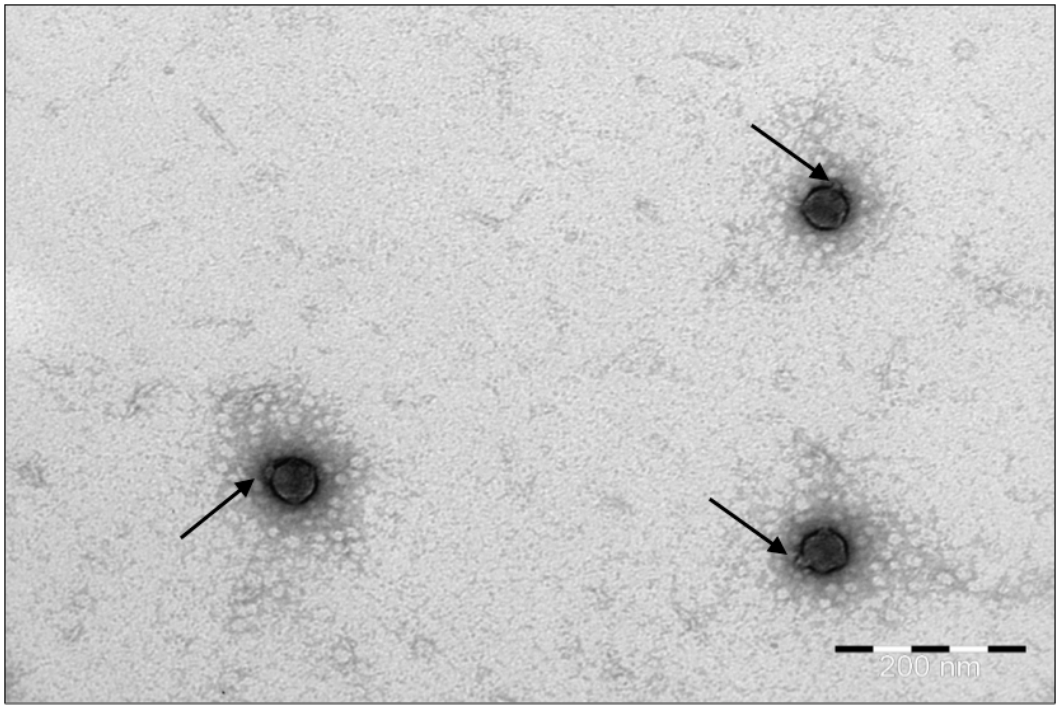


Figure 9b. Electron micrograph of isolated vB_EcoP-U8 virions showing icosahedral shaped capsids and the short non-contractile tails (indicated by arrows) characteristic of members of the *Podoviridae* family bacteriophages.

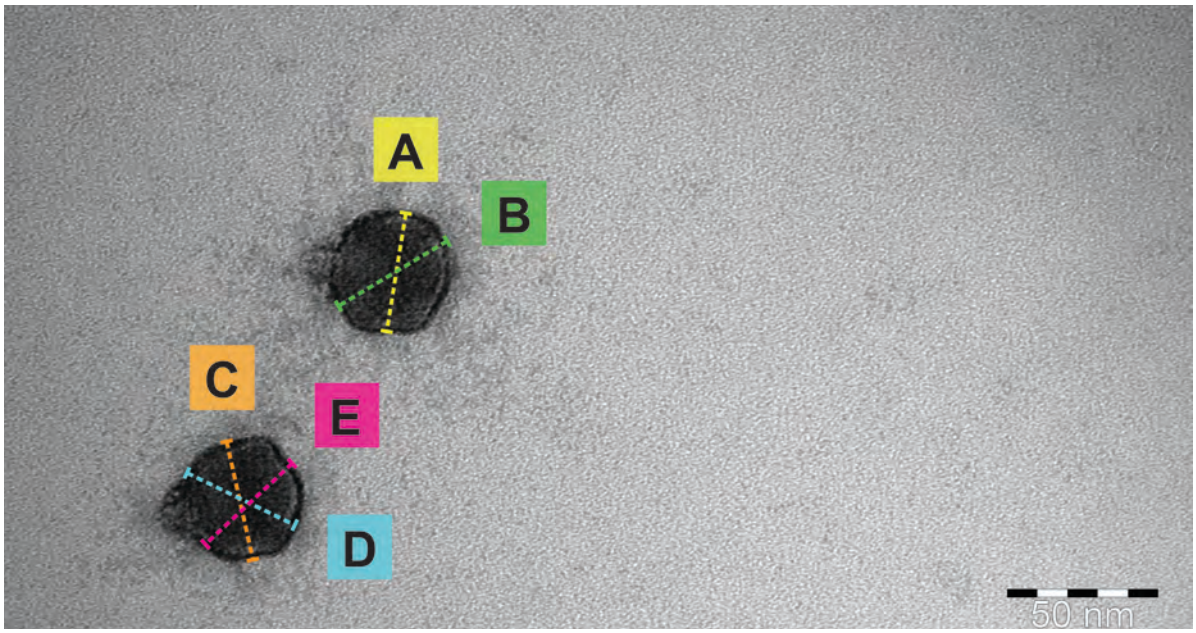


Figure 9c. Transmission electron micrograph showing capsid diameters. Measurements: A - 41.5nm; B - 39.4nm; C - 40.2nm; D - 39.9nm; E – 39.6

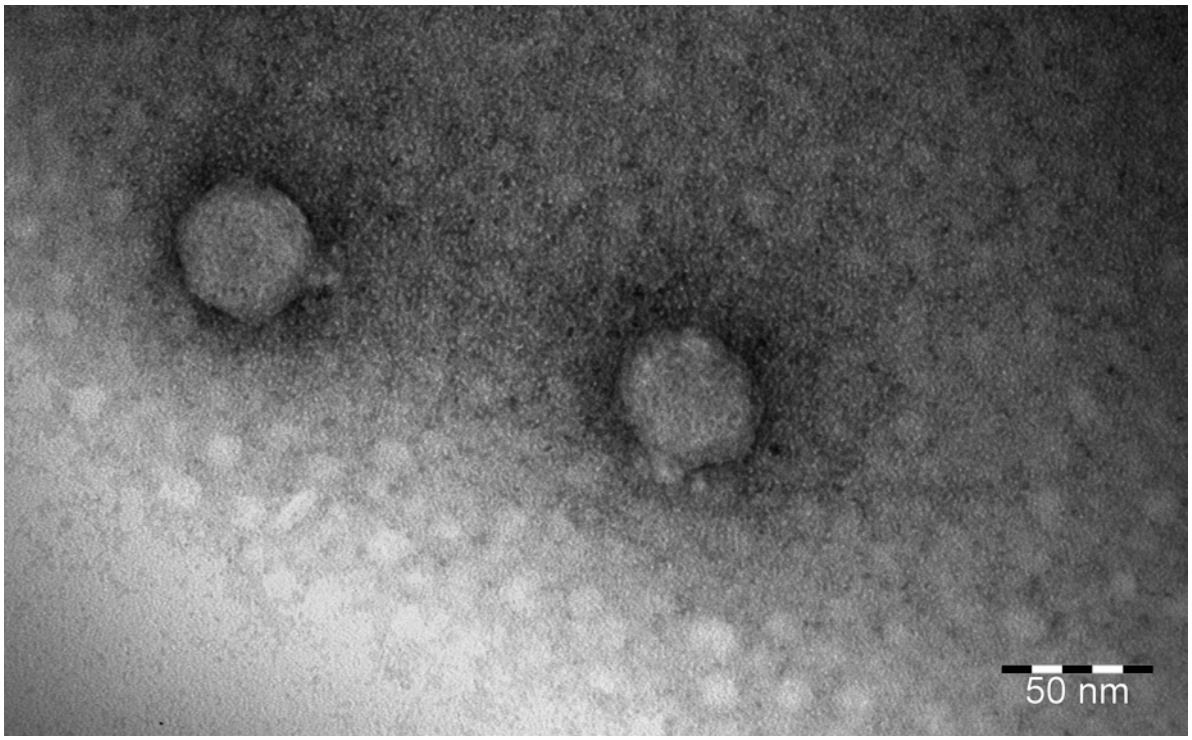


Figure 9d. Transmission electron micrograph showing the icosahedral capsids and short non-contractile tails of vB_EcoP-U8 that are characteristic of the podoviridae.

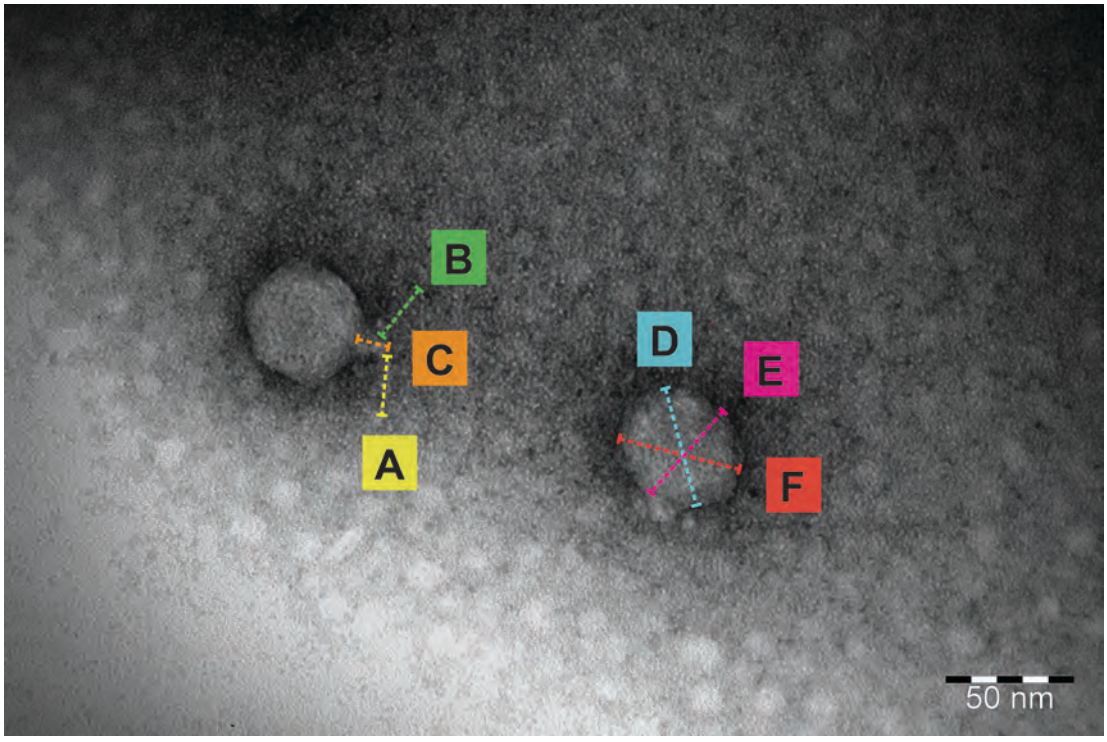


Figure 9e. Transmission electron micrograph showing the short non-contractile tails (C – 12.3nm), tail fibres (A - 23.2nm and B - 23.1nm), and capsid sizes: D – 47.4nm; E – 42.7nm; F – 48.1nm.

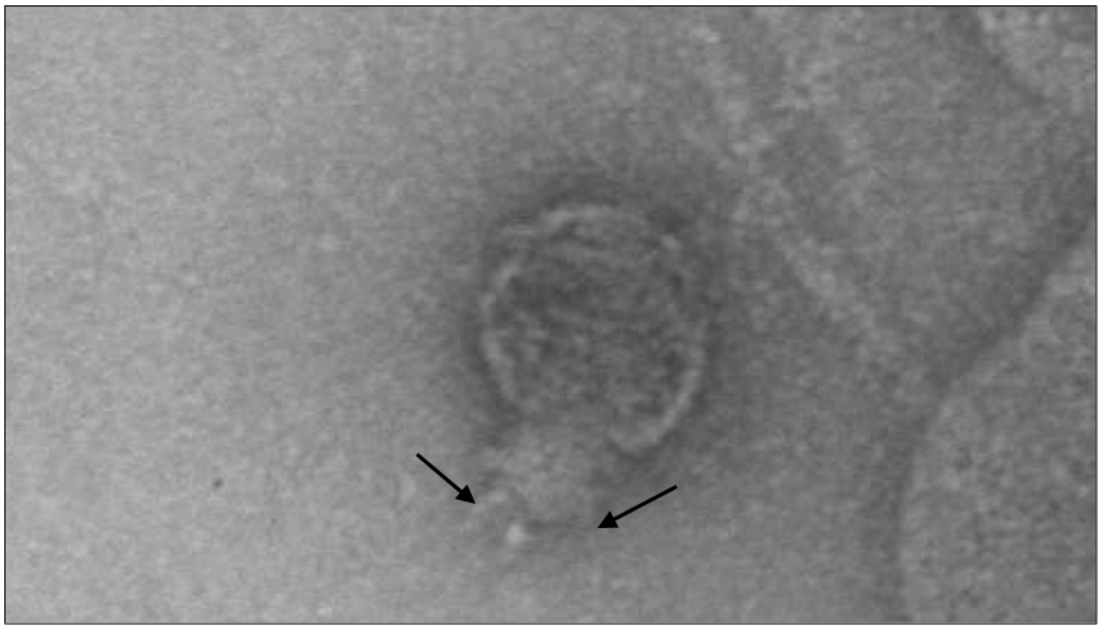


Figure 9f. Transmission electron micrograph showing a highly magnified and cropped image of vB_EcoP-U8 capsid and tail with possible protruding tail fibres (scale bar is omitted due to image cropping).

Although clear images of the bacteriophage active against UPEC strain UEmw_9 were obtained via TEM, morphological classification of the virus proved exceptionally difficult. Morphological features to our knowledge not described in the literature were observed and after much deliberation and consultation with various authorities it was decided that the order and family of the phage was not defined conclusively, and the designation of the virus was left at vB_EcoX-U9 with the X indicating that the virus family to which this phage belongs is yet to be determined. The micrographs below indicate a capsid of ±40 nm in diameter. The capsid however is surrounded by what seems to be a lipid layer that extends the diameter of the particle to on average ±60 nm, and sometimes as large as 70 nm. There are only five families of enveloped bacteriophages and of these only one - the Cystoviridae share morphological similarities with the observed virus although they are bigger than the current virus, having enveloped virion sizes of around 85 nm.(97,98) In some images the phage appears to be tailed, but for the most part it seems to be circular with the lipid envelope, with an icosahedral capsid, and un-tailed. Figures 10g and 10f show a number of the phages bundled together with some of the lipid layers completely removed, others partially covering the capsids and others still completely intact. In these micrographs the the icosahedral shape of the capsids is clearly visible where the lipid layer has been separated from the capsid.

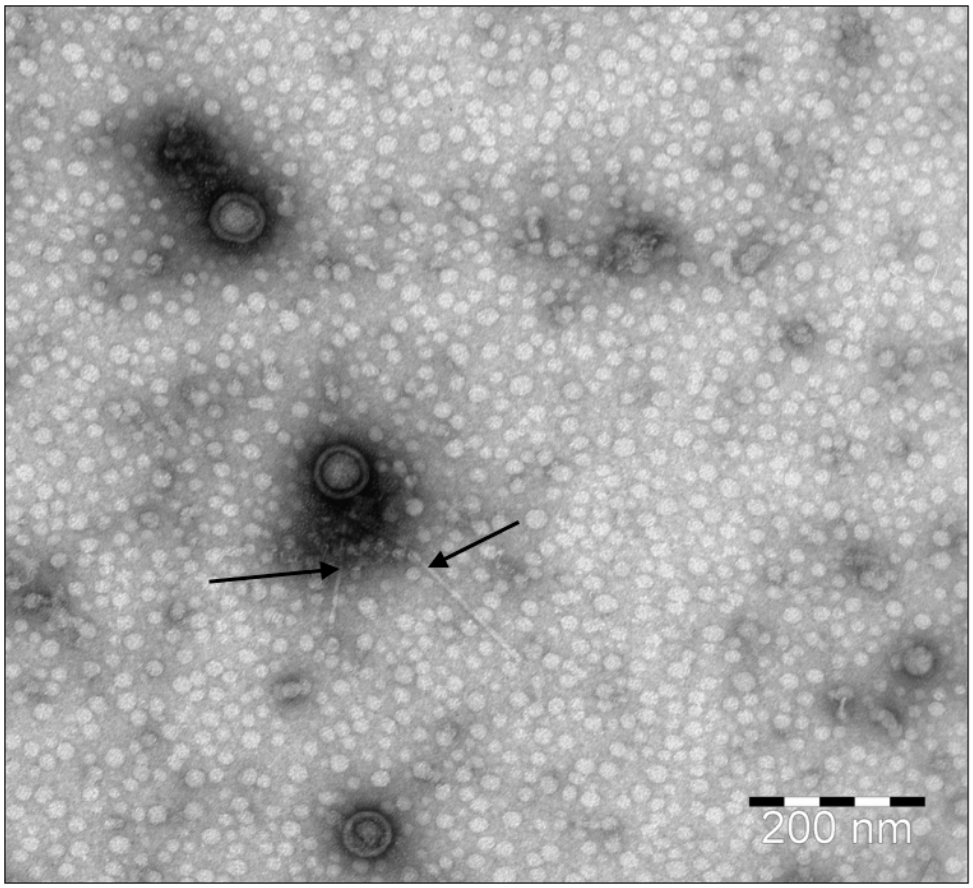


Figure 10 a-i. Transmission electron micrographs of vB_EcoX-U9 virions at varying magnifications.

Figure 10a. TEM micrograph indicating three vB_EcoX-U9 particles, the centremost one with two long tail-like projections seemingly protruding from it.

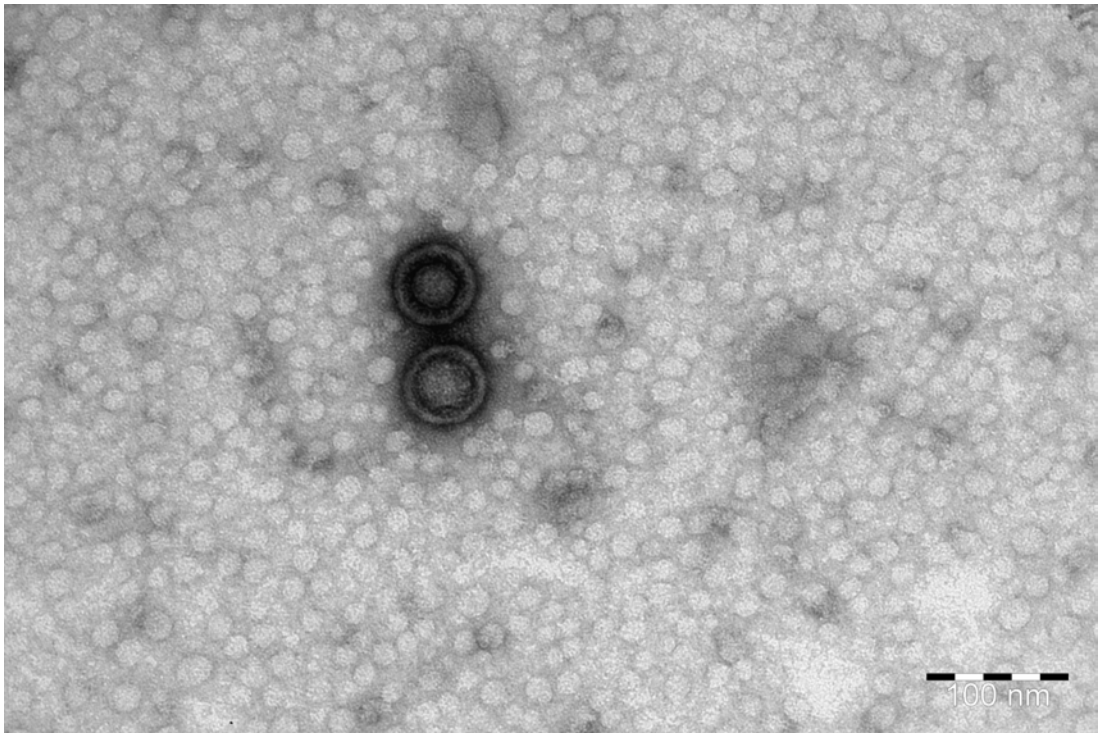


Figure 10b. Micrograph showing two vB_EcoX-U9 phages in close proximity, with the icosahedral (shape somewhat obscured) capsids visible and encapsulated in a circular envelope, no distinct tails are seen on either of the phage particles.

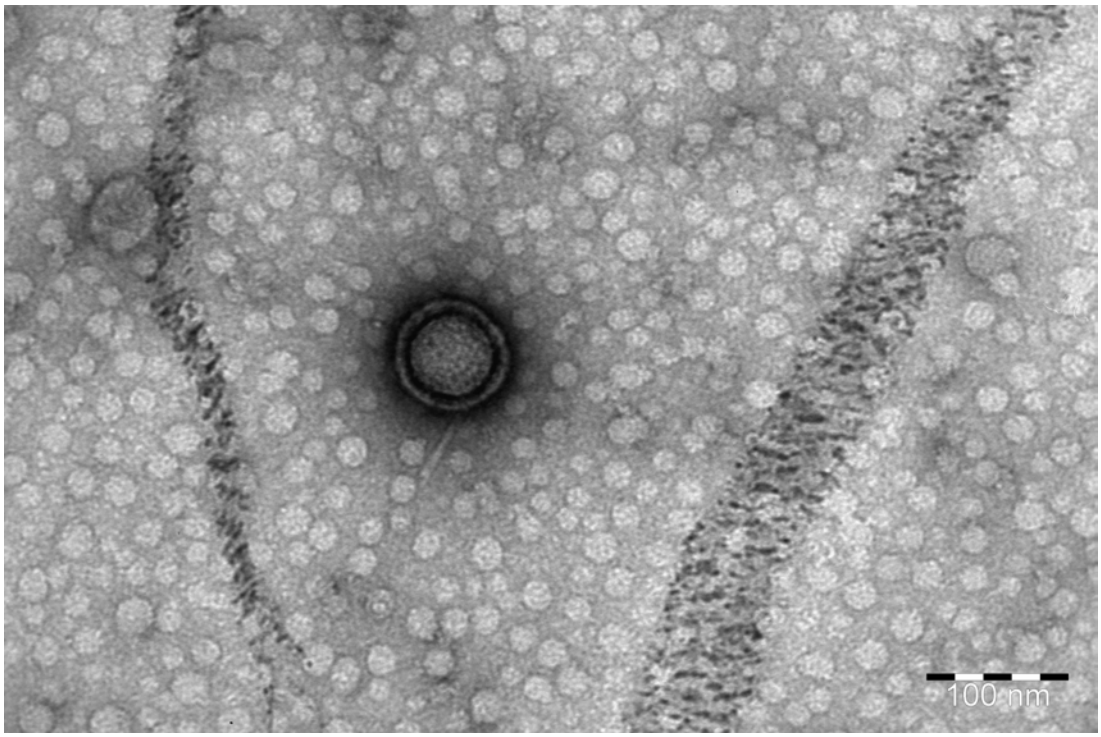


Figure 10c. A negative stained vB_EcoX-U9 virion with the capsid definitively encapsulated by the distinctive envelope.

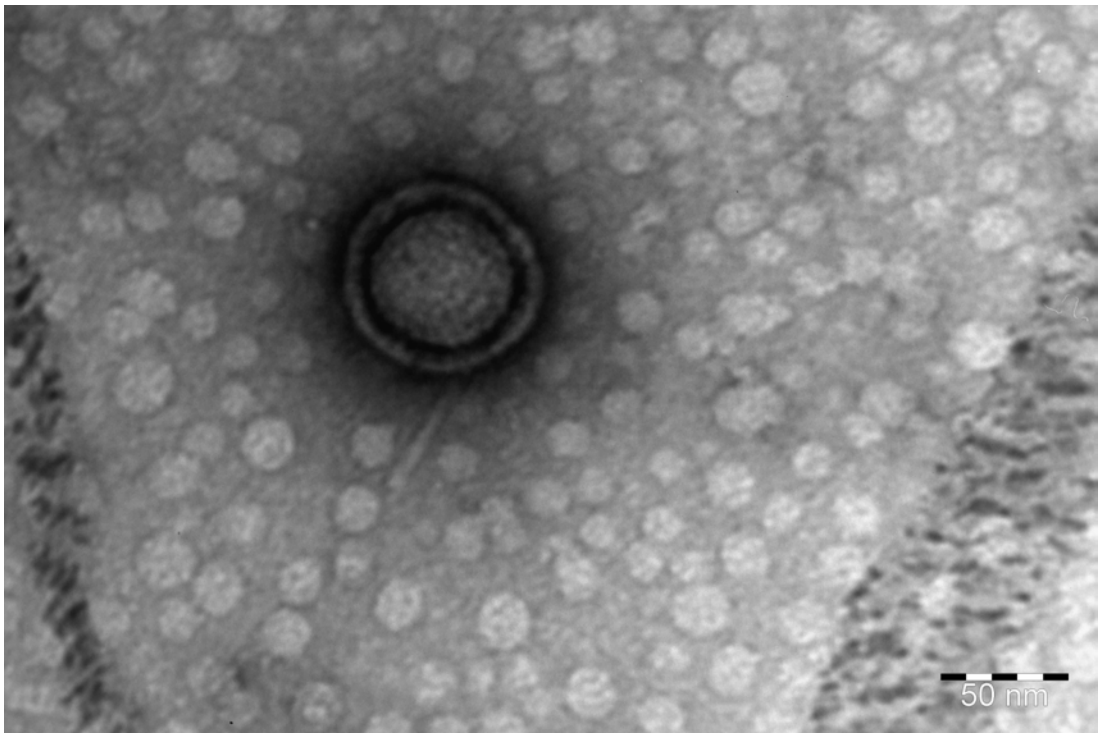


Figure 10d. A higher magnification of the same viral particle seen in 8c above, showing the capsid clearly nested in an envelope layer. Again no tail is present.

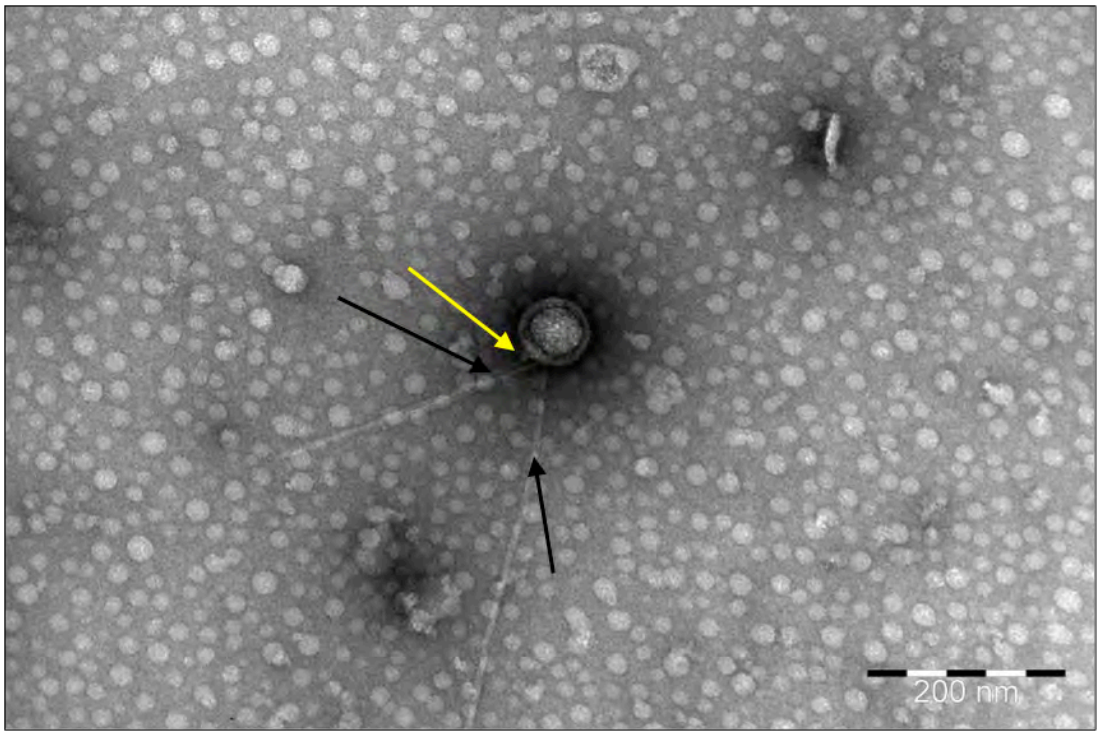


Figure 10e. Transmission electron micrograph showing tail-like protrusions extending from the vB_EcoX-U9 head (black arrows) with some pinching or extension of the envelope that is surrounding the capsid (yellow arrow).

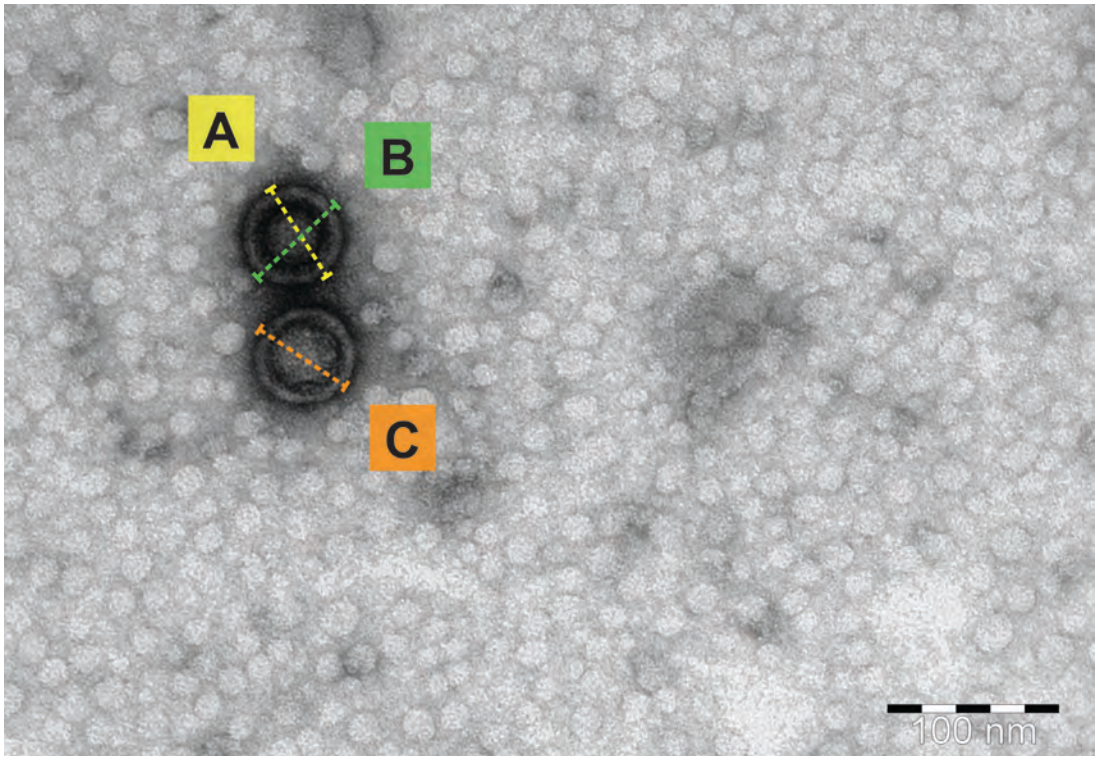


Figure 10f. Transmission electron micrograph showing enveloped phage particle diameters. Measurements include envelopes A - 60.6nm; B - 61.5nm; C - 60.6nm.

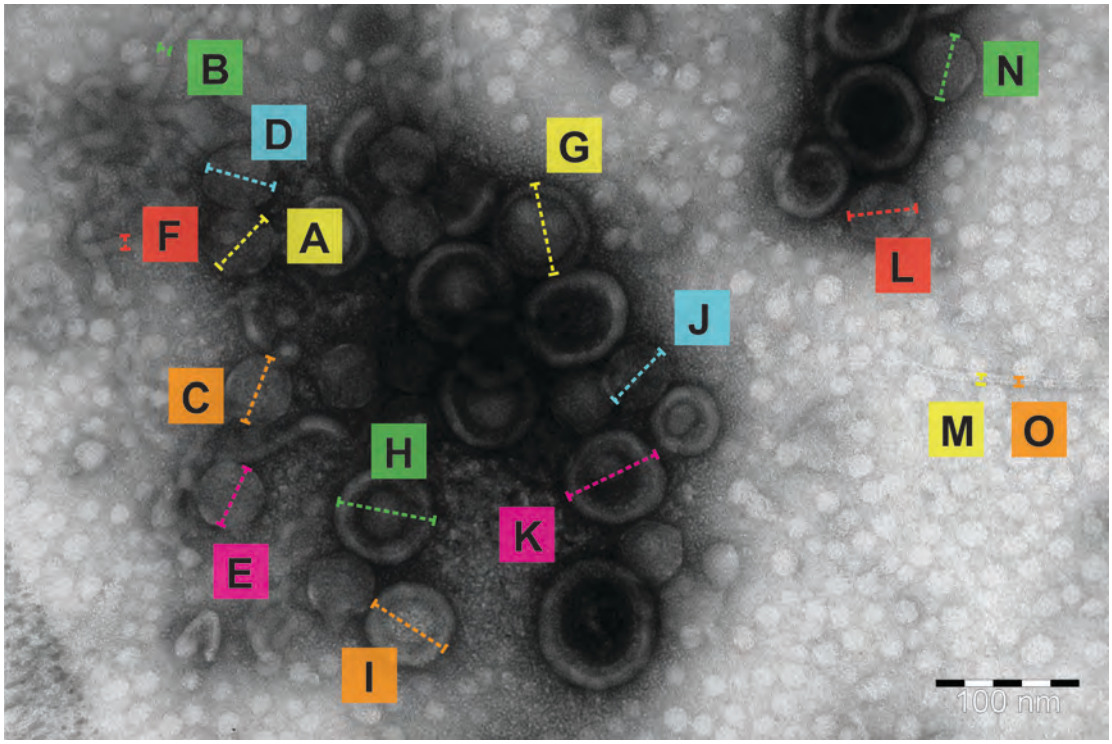


Figure 10g. Micrograph showing the sizes of various vB_EcoX-U9 phage particles with and without the observed envelopes. Non-encapsulated capsids: A – 46.8nm; C – 48.0nm; D – 48.7nm; E - 43.4nm; J – 46.8nm; L – 48.1nm; N – 45.9nm. Enveloped virions: G – 63.7nm; H – 68.2nm; I – 58.5nm; K – 67.4nm; M – 7.7nm. Membrane/envelope width: B – 8.0nm; F – 10.0nm. Miscellaneous observed fibres width: M – 7.7nm; O - 5.4nm

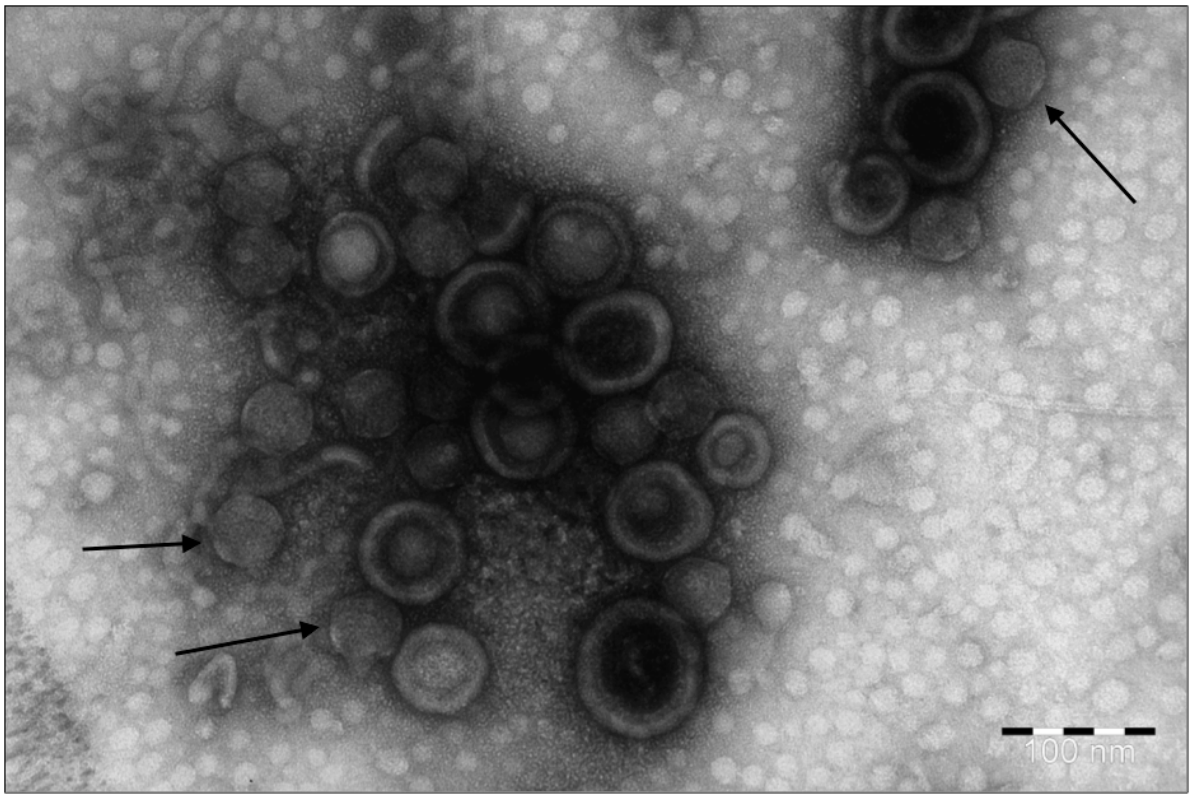


Figure 10h. The same group of vB_EcoX-U9 bacteriophages seen in figure 10g, without the size markers, allowing better visualization of the opening/unfolding/peeling away of the envelope around some virions. The icosahedral capsids (indicated by arrows are easier to distinguish when the envelope is absent.

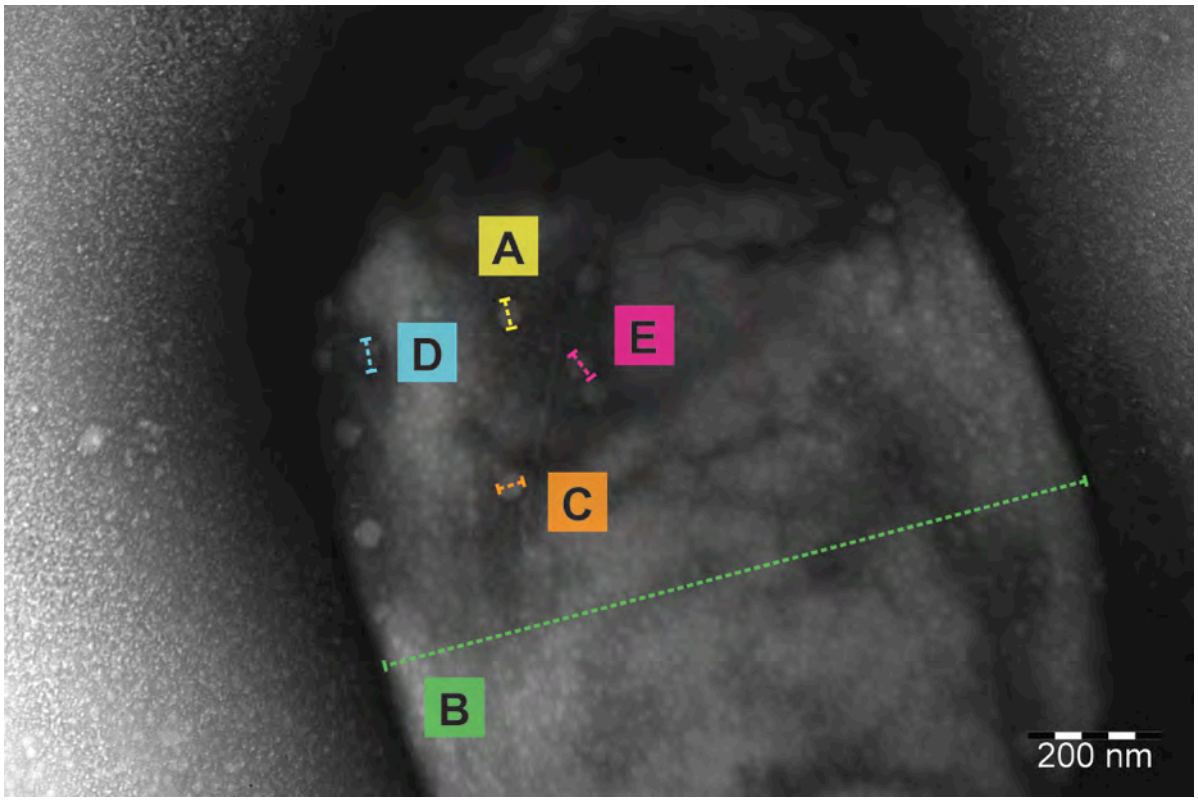


Figure 10i. Micrograph showing some vB_EcoX-U9 phages associated with a UEmw_9 cell. The envelope is no longer visible and the phage capsid sizes are shown: A – 44.0nm; C – 45.0nm; D – 48.8nm; E – 45.52nm. The width of the UEmw_9 cell pictured was measured at B – 1086.39nm.

3.6.2 Bacteriophage genetic characterization

Genetic characterization was carried out for vB_EcoP-U8, but was not achieved for vB_EcoX-U9 as the phage nucleic acid extraction was unsuccessful despite numerous attempts made using various protocols and methodologies.

3.6.2.1 Bacteriophage genome extraction

Extraction of the bacteriophage nucleic acid was successful for vB_EcoP-U8 using the Roche High Pure Viral Nucleic Acid Extraction Kit, and the manual extraction procedure adapted from McCarthy & Romanowski.(91) Neither of these methods, nor any of the other procedures attempted yielded any appreciable amounts of DNA or RNA from vB_EcoX-U9. Nanodrop analysis of the eluents from samples of both vB_EcoP-U8 and vB_EcoX-U9 in which viral nucleic acid was expected was inconclusive, consistently producing high (> 3.0) A260/280 and A260/230 readings for samples and negative controls (PCR grade dH_2O). When PCR grade water was tested it gave low negative absorbance readings but when the same water was run on the extraction kit columns the resulting eluent had high absorbance readings. The elution buffer and the glass wool of the columns are known to make spectrophotometry readings unreliable. Attempts at restriction enzyme digestion of the eluents yielded no results (no bands) and electrophoresis in a 0.8% (w/v) agarose gel showed presence of single nucleic acid band greater than 10 000 base pairs in size for both the manual extraction and the High Pure Kit extraction eluted phage vB_EcoP-U8.

3.6.2.2 Bacteriophage sequencing

The nucleic acids obtained from bacteriophage vB_EcoP-U8 extraction procedures were subjected to next generation sequencing. The NGS achieved 1 035 068 reads, 99,9% of those being of high quality. De novo assembly (without a reference guide) of the obtained reads revealed a contig representing a 40 294 base pair genome for vB_EcoP-U8, with a GC content of 50.14%. It was shown to code for 52 genes; of those 51 CDS and 1 tRNA gene. Figure 12 below provides a graphical representation of the derived coding DNA sequence with genes and their positions in the genome labeled. BLAST analysis indicated high genetic similarity with another podovirus coliphage, namely: Escherichia phage LM33 (Genbank accession LT594300.1). The results indicate definitively that a novel podovirus active against a clinically isolated *E. coli* strain has been isolated.

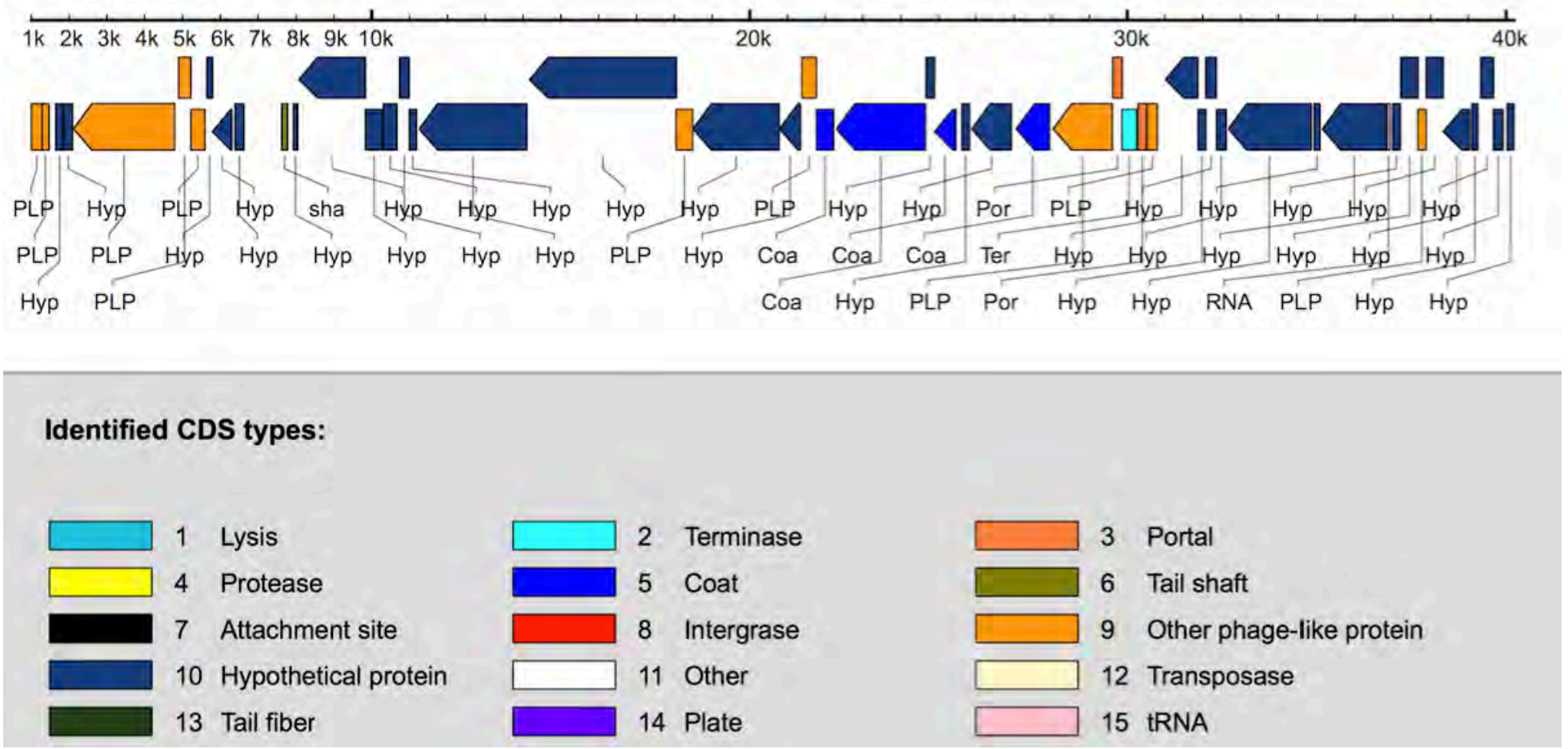


Figure 11. Annotated coding DNA sequence (CDS) of bacteriophage vB_EcoP-U8 generated from next generation sequencing data.
The genome of vB_EcoP-U8, coding for 1 tRNA and 51 bacteriophage proteins including tail shaft protein, coat protein and terminase and lysis enzymes.

3.7 Biofilm catheter assays

3.7.1 Biofilm degradation assay

The catheter segments in which UEmw_8 and UEmw_9 biofilms were grown, were treated either with "Treatment" ($1x10^7$ pfu/ml vB_EcoP-U8 or vB_EcoX-U9) or negative "Control" (Sterile AUM). The levels of cells and EPS remaining were measured by absorbance and plate count. The data collected are shown in Figures 13 and 14 below.

The absorbance data, which were collected as optical density measurements at a wavelength of 595nm (OD_{595}), are a measure of the turbidity of the collected biofilm suspensions. Such data are an indication of the biomass of the samples for each incubation time interval, i.e. the levels of biofilm EPS and cells present (including cell debris and non-viable cells).

The cell count data are a measure of the number of cells that are both viable and cultivable.

3.7.1.1 Degradation of UEmw_8 and UEmw_9 biofilms measured by absorbance

The absorbance measurements of UEmw_8 and UEmw_9 catheter-lumen derived samples obtained from the both the control and phage-treated catheter sections are graphed in Figure 12. For the UEmw_8 catheter derived biofilms, the biomass (as measured by absorbance) of the control samples differed markedly from the vB_EcoP-U8 treated samples. While both treatment and control absorbance decreased at the onset, a major distinction can be seen from 4 h onwards, where the control begins to level out and then increases and the treatment absorbance decreases. The overall difference (across all incubation times) between biofilm levels formed in the control and the phage-treated catheters was not significant ($F = 1.017$, $p = 0.316$), as assessed by changes in biomass. However, when considering the difference in biofilm levels between the treatment and control by the end of the 24 h incubation time, the treatment absorbance is significantly lower ($F = 5.858$, $p = 0.024$) than the control absorbance. This difference can be seen in Figure 12 in comparing the distance between the curves of the treatment and control at 24 h.

For UEmw_9 catheter derived biofilms the control samples showed a recovery in biomass from 2 h onward (as opposed to 4 h as was seen for UEmw_8) but the vB_EcoX-U9 treated samples followed an almost identical trend to that seen for UEmw_8 treated with vB_EcoP-U8. Once again the treatment absorbance was seen to be significantly lower ($F = 4.513$, $p = 0.045$) than the control absorbance by the end of the 24 h incubation. The overall difference in levels of biofilm formed were not significant ($F = 1.493$, $p = 0.224$) when considering all incubation times.

For both the assayed bacterial strains it was seen that the biomass decreased below the levels recorded for the controls at some time point between the 4 and 8 h incubation period.

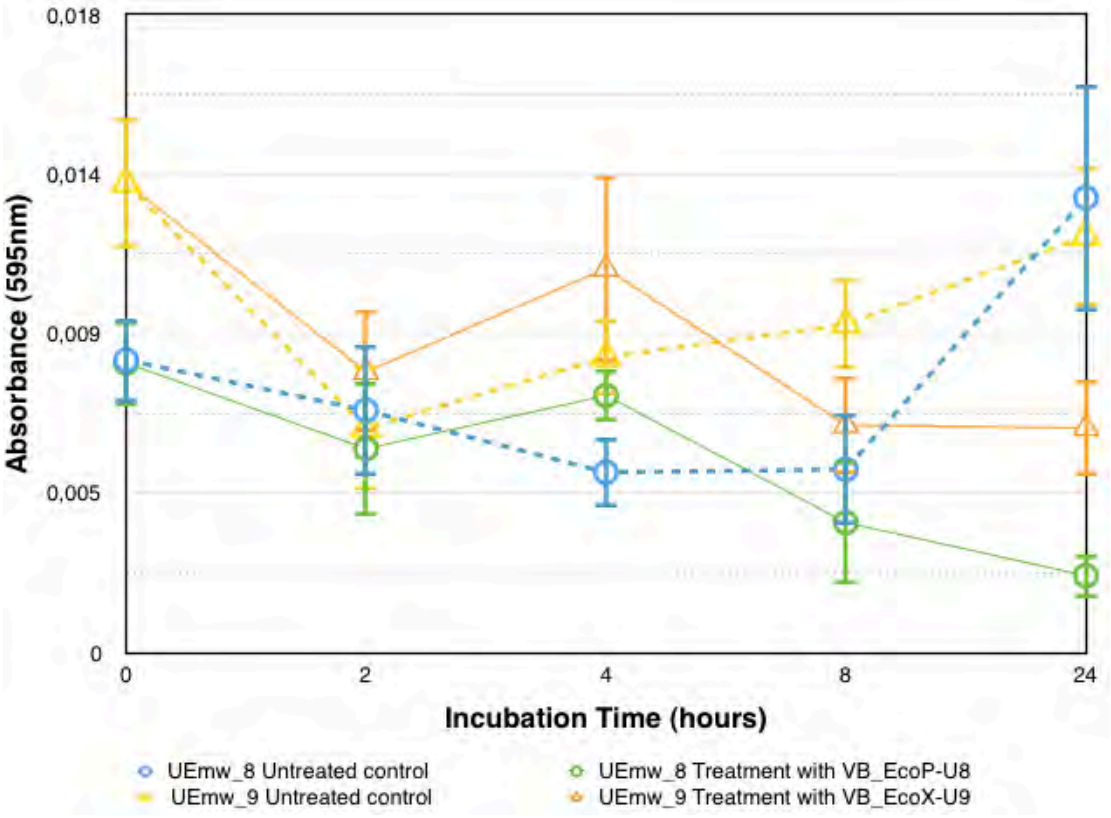


Figure 12. Changes in absorbance (OD_{595}) at standardised time intervals of UEmw_8 and UEmw_9 culture catheter segments treated with vB_EcoP-U8 and vB_EcoX-U9.
For UEmw_8 a decrease in the absorbance is seen between 0 and 2 h for both treatment and control. A lag phase is seen between 2 and 4 h where treatment has no effect, and absorbance increases in treatment compared with the control. A sharp increase in absorbance was seen in the control between 8 and 24 h which is not seen in the treatment. For UEmw_9 a decrease in the absorbance is seen between 0 and 2 h. A lag phase is seen between 2 and 4 h where treatment has no visible effect. A steady increase in absorbance was seen in the control between 4 and 24 h in contrast with a sharp decrease in absorbance for the treatment which stabilises between 8 and 24 h.

3.7.1.2 Degradation of UEmw_8 and UEmw_9 biofilms measured by plate count

The degradation assay plate count data are shown in Figure 13 for both UEmw_8 and UEmw_9 catheter-lumen derived samples obtained from both the control and phage-treated catheter sections of each. The data are graphed as the log of the number of colony forming units for each sample group. For UEmw_8, the vB_EcoP-U8 treated samples had lower cell numbers than did the corresponding untreated control samples. A major divergence in the treatment and control trends can be seen from 8 hours onwards where the treatment samples' viable cell counts drop and the control viable cell counts increase markedly. By 24 h of incubation with vB_EcoP-U8 there is a significant difference ($F= 87.921$; $p = 0.000$) in the number of viable cells as compared to the untreated control. Across all incubation periods the

decrease in viable cells measured for phage treated compared to untreated controls was significant (F = 43.827; p =0.000).

In the UEmw_9 samples the phage-treated cultures present lower cell counts than the untreated controls as was seen with the UEmw_8. After dropping distinctly from 4 h onward, the phage-treated samples showed a recovery in cell numbers from 8 h until 24 h, a trend that was not seen in the control.

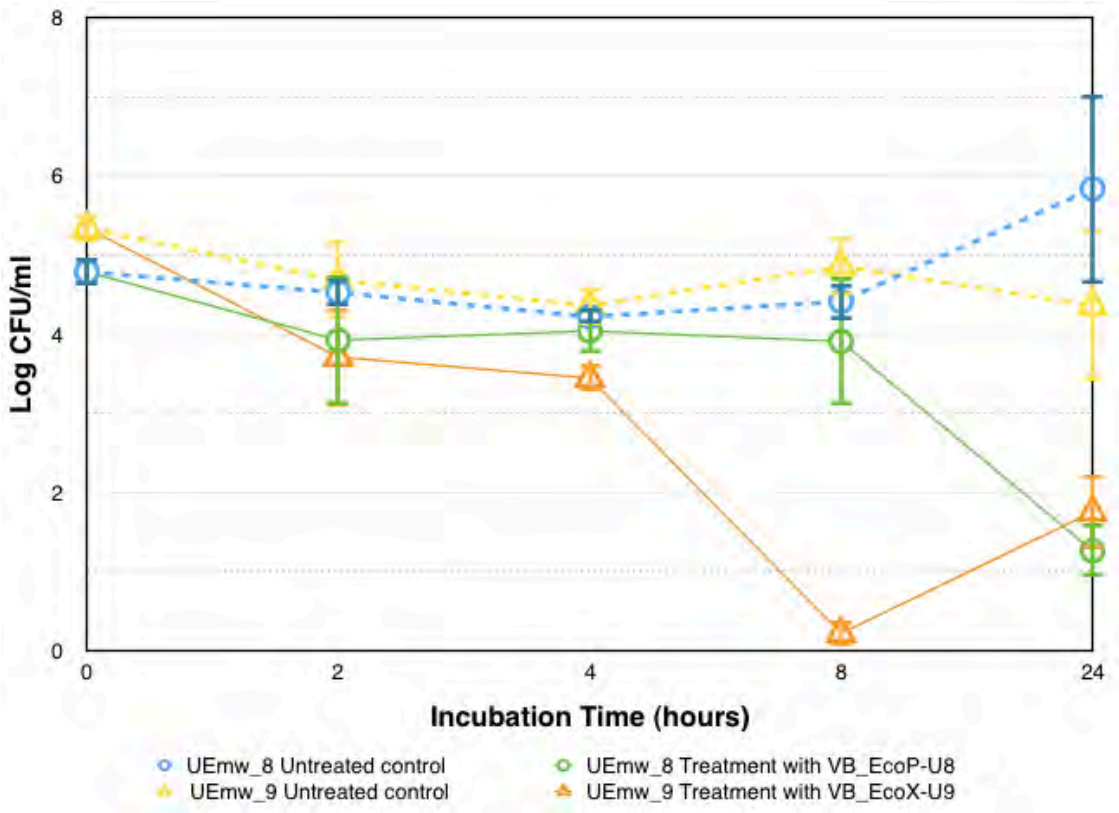


Figure 13. Changes in viable cell count (recorded as log CFU/ml) of UEmw_8 and UEmw_9 following treatment with vB_EcoP-U8 and vB_EcoX-U9.
For UEmw_8 a decrease in plate count is seen between 0 and 2 h in both control and treatment. A lag phase is seen between 2 and 4 h where treatment has no effect. From 4 h onward a slight divergence is seen in treatment and control with the number of colonies dropping in the treatment and rising in the control. For UEmw_9 decrease in plate count is seen between 0 and 2 h in both control and treatment, although slightly greater for the treatment. A lag phase is seen between 2 and 4 h where treatment has no effect. From 4 to 8 h a sharp divergence is seen between treatment and control with the number of colonies dropping in the treatment and rising in the control. From 8 to 24 h a convergence is seen with treatment cells counts increasing and control counts decreasing slightly.

By 24 h of treatment there are significantly less viable, cultivable cells present in the UEmw_9 samples in comparison to the controls. (F = 41.186; p = 0.000). Across all incubation times the difference between cell counts of the treatment samples and control samples was significant (F = 69.479; p = 0.00).

It is noted that overall, for the degradation assays the shape of the curves for the treatment is largely consistent when comparing effects measured by absorbance (Figures 12) and those measured by cell count data (Figure 13) in each sample. However, the UEmw_9 control curves do not correlate well when comparing biomass measurement to cell viability.

When comparing the two different bacterial strains in terms of response to phage treatment, the absorbance data showed similar effects. For the application of vB_EcoX-U9 to biofilms of UEmw_9, the decrease in viable cell count occurs from 4 h onward, whereas for UEmw_8 treated with vB_EcoP-U8 the rate of cell killing is lower and a major difference between the treatment and controls is only seen somewhere between 8 and 24 h. Overall, in all cases there is a significant decrease in biofilm parameters in the treatments as compared to the controls by 24 h of incubation with phage.

3.7.2 Biofilm prevention assay

In the prevention assays, prior to the incubation with bacteria for biofilm formation, pre-treatment with bacteriophages of the treatment group catheters was needed and “no pre-treatment” of the controls was needed. Thus, control and treatment samples necessarily had to be run on different catheter segments from the beginning of the assay. This was unlike the procedure for the degradation assays, where the same catheter segment was split into treatment and control sections after initial growth of the biofilms, a process which yielded identical starting values for treatment and control catheters. Unsurprisingly in the prevention assays, variations in the initial values for both absorbance measurements and cell counts (and thus the subsequent values as well) were seen. This in general would be the case for measurement of phenomena in which the initial values differ markedly between the different conditions or sample groups. Such variation was indeed expected and was taken into consideration in interpreting and comparing the control and treatment data.

3.7.2.1 Prevention of UEmw_8 and UEmw_9 biofilms by pre-treatment measured by absorbance

For UEmw_8 the absorbance measurements (Figure 14) showed an initial decrease in biomass for the untreated control between 2 and 4 h, followed by a steady increase from 4 h onward. The treatment samples showed an initial increase in biomass between 2 and 4 h, followed by a sharp decrease between 4 and 8 h. Following this drop in biomass however, a gradual recovery between 8 and 24 h was seen with the rate of decrease slowing distinctly. The overall difference between treatment and control was significant across all incubation times ($F = 8.891$; $p = 0.004$). By the end of the 24 h biofilm growth period post phage pre-treatment, there was significantly less biofilm in the pre-treated catheters than in the controls ($F = 5.532$; $p = 0.028$). It is noted however that the initial levels of biofilm measured (at 2 h catheter culture incubation) were different.

The UEmw_9 phage pre-treated sample absorbance data show an initial decrease in biomass followed by stabilization between 4 and 8 h and then a gradual decrease in biomass between 8 and 24 h. The untreated control samples yielded absorbance data showing a very slight initial increase between 2 and 4 h, followed by a decrease in biomass between 4 and 8 h and then an increase from 8 h onwards. The data indicate that there is no significant difference in the levels of biofilm measured between the control and the pretreated catheters after 24 h of catheter irrigation following pre-treatment. From the graph it can clearly be seen that levels of biofilm are higher in the treatment catheters than in the control catheters except at 4 h, and it is clear that phage pre-treatment did not significantly decrease levels of biofilm as compared to the controls. It is noted however that the decrease in the level of biofilm from

2 h to 24 h of incubation in the pre-treated catheters was far greater than the decrease in biofilm levels seen in the control catheters.

The measured absorbances of the untreated controls for UEmw_8 and UEmw_9 catheter derived cultures showed decreases in biomass over the course of the incubation from the initial sampling to final sampling. Such decreases were also noted in the degradation assay controls (Figure 12). What is noteworthy in the prevention assays however is that the degree of decrease seen for the controls is lower than what is seen in the pre-treated catheters. The fact that the initial levels of measured biofilm in the treatments and controls were so discrepant indicates a difficulty in comparison without transformation of the data to allow for comparison of proportional change in biofilm in response to pre-treatment or lack thereof.

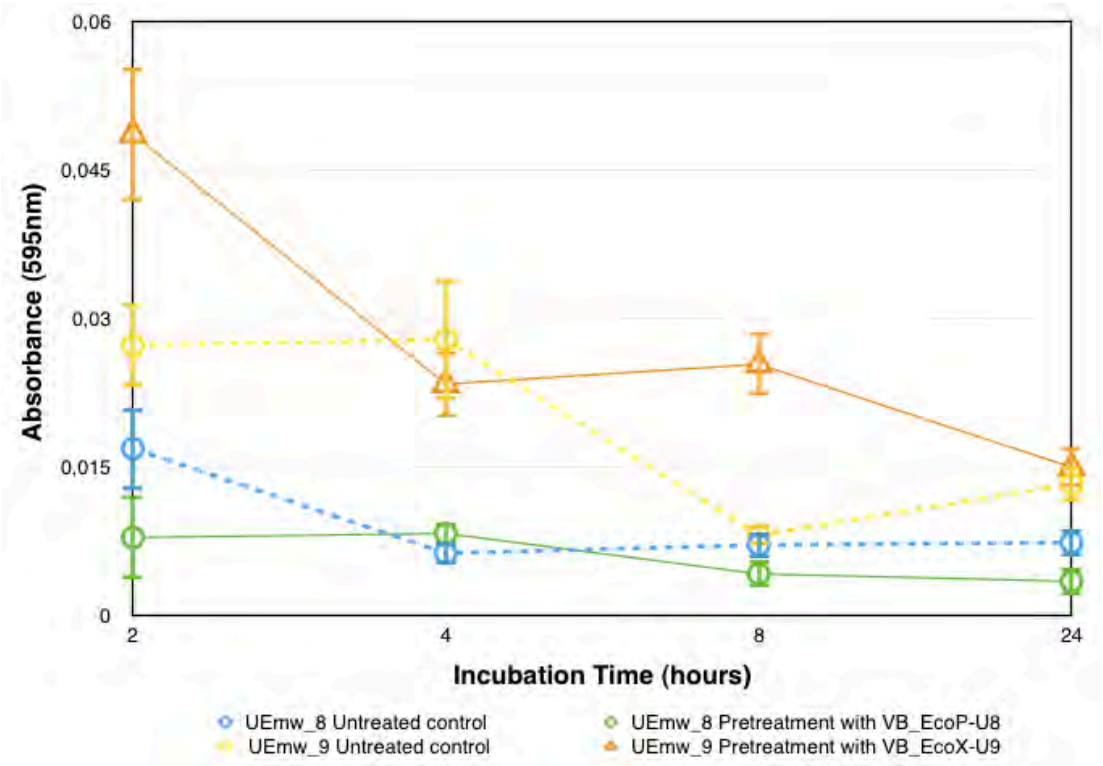


Figure 14. Absorbance (OD_{595}) of UEmw_8 and UEmw_9 following pre-treatment with vB_EcoP-U8 and VB_EcoX-U9.
For UEmw_8 lag phase is seen between 2 and 4 h where treatment has no effect, and absorbance increases in slightly treatment. An increase in absorbance is seen in the control between 4 and 24 h which is not seen in the treatment. Between 8 and 24 h there is a slowing of the rate of decrease of biofilm compared to that seen between 4 and 8 h. For UEmw_9 a decrease in the absorbance is seen between 0 and 2 h. A lag phase is seen between 2 and 4 h where treatment has no visible effect. A steady increase in absorbance was seen in the control between 8 and 24 h in contrast with a decrease in absorbance for the treatment over the same period.

3.7.2.1 Prevention of UEmw_8 and UEmw_9 biofilms by pre-treatment measured by plate count

Figure 15 shows the changes in the plate count data (as the logarithm of the colony forming units) for each condition (pre-treatment or untreated control) for UEmw_8 and UEmw_9 cultures. The pre-treatment samples of strain UEmw_8 showed a decrease in viable cultivable cells by 24 h, as compared with initial 2 h measured values (see start- and end-points of the green solid line in Figure 15) and in comparison with the control ($F = 13.638$; $p = 0.004$). The overall decrease in cell counts across all incubation periods compared with the control was significant ($F = 31.565$; $p = 0.000$).

For UEmw_9 catheter cultures pre-treated with vB_EcoX-U9 at every successive incubation period higher numbers of viable cells were counted. This data shows that while pre-treatment lessened the ability of the bacteria to form biofilms (compared to the control), it could not prevent biofilms from developing. By the end of the 24 h incubation period cell counts for UEmw_9 pre-treated samples were lower than the untreated control, but not significantly so. ($F=0.130$ $p = 0.726$)

The effect of pre-treatment with phage (vB_EcoP-U8) was greater in UEmw_8 cultures than it was for UEmw_9 (pre-treated with vB_EcoX-U9). Both strains' plate counts were initially higher than their respective control groups', but by 24 h of incubation following phage pre-treatment viable cell numbers had dropped below their corresponding control counts.

Overall, for phage pre-treatment of catheters in which biofilms of UEmw_8 and UEmw_9 were grown, comparisons between the absorbance and plate count data seem to show entirely different effects on biomass and on cell viability. The plate count data indicates a similarity in effects of pre-treatment on the two bacterial strains assayed, but the absorbance data is far more mutable.

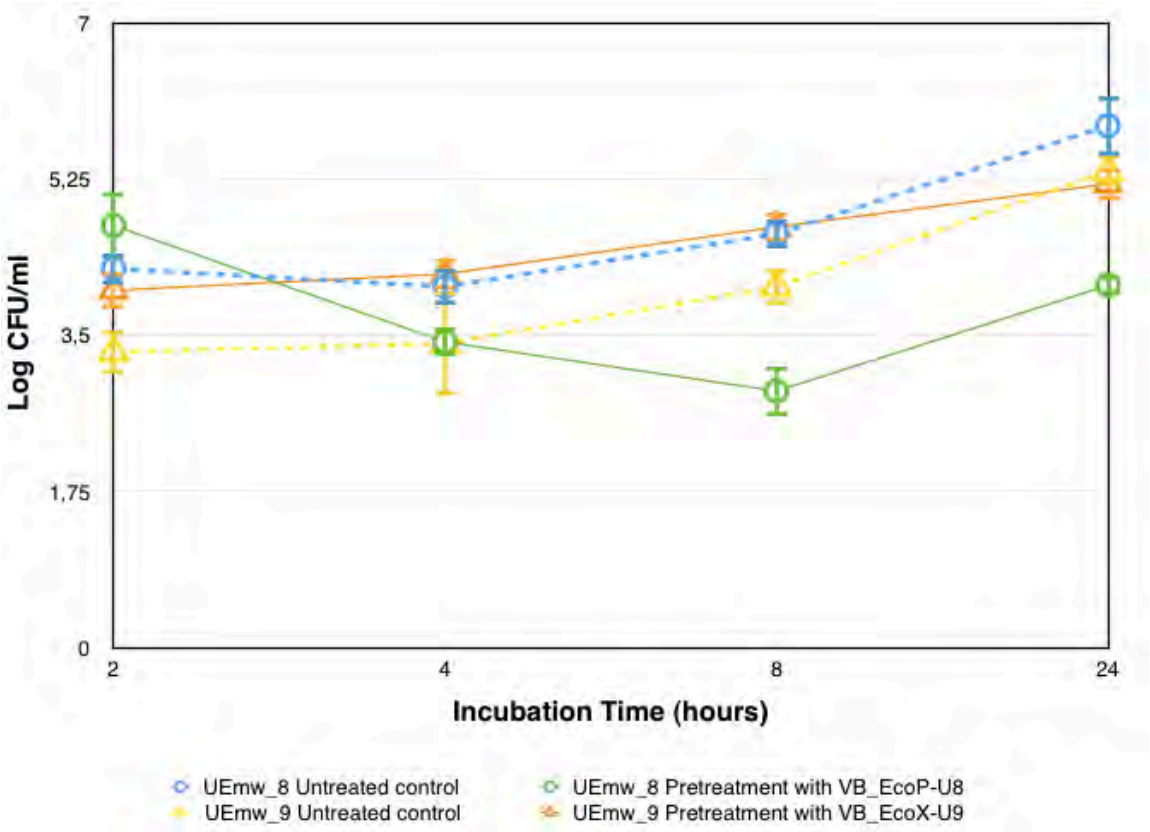


Figure 15. Changes in viable cell counts (measured as log CFU/ml) over time of UEmw_8 and UEmw_9 following pre-treatment with vB_EcoP-U8 and vB_EcoX-U9.
For UEmw_8 a decrease in plate count is seen between 2 and 4 h in both control and treatment representing lag phase, where treatment has no effect. From 4 h onward a divergence is seen in treatment and control with the number of colonies dropping in the treatment and rising in the control. Recovery of the biofilm is seen in the treatment from 8 h onwards. For UEmw_9 a continued increase in plate count is seen between 0 and 24 h in both control and treatment, although the treatment cells increase less than the controls with the difference in trend increasing over time.

3.8 Flow cytometry

The suspensions from the biofilm degradation and prevention assays were additionally analyzed using nucleic acid double staining flow cytometry (as described in section 2.9) that allowed for discrimination between populations of live and dead cells for each incubation period as a function of membrane integrity (as opposed to turbidity and cultivability). The flow cytograms below indicate these cell populations by the distribution of dots representing stained *E. coli* cells of different fluorescence intensities. The fluorescence was measured at two wavelengths corresponding to the emission maxima of the two dyes used, and thus the dots on the plots are divided between four quadrants. In this type of assay cells that have intact cell membranes are considered viable/ “live” and are stained only by membrane permeant SYBR Green (SG); such cells are visible in the upper left quadrant (ULQ) on the resulting cytograms/plots. Cells that are non-viable/ “dead” have lost membrane integrity and are permeable to both propidium iodide (PI) and SG. The SG fluorescence is however quenched by the PI molecules in the process of fluorescence resonance energy transfer (FRET), and in addition the PI has a higher binding affinity for DNA so physically displaces the bound SG in these cells, and they fluoresce red.(99,100) These dead cells are visualized in the lower right quadrant (LRQ). Cells that are in an intermediate state or have only slight membrane disruption allowing low levels of PI entry, have both high intensity PI and SG emission and are seen in the upper right quadrant (URQ). Such cells are considered to be non-viable as well.

The placement of the quadrant vertices is based on the cell population distribution data obtained from the controls, and the quadrants thus serve as a visual aid in conceptualizing the distribution of the cells’ populations. In the statistical analyses and graphing of the data the cells were gated as either live or dead on each plot using compensation based on the specific controls run for each data acquisition.

Unstained cells and cellular debris would ideally not be detected by the cytometer, as the assay was set up with thresholds based on size discrimination of the cells of interest. However, flow cytometric “noise” can be visualized in the lower left quadrant (LLQ). Emission data is represented on a log scale with the FL1 channel data representing the green emission of SG and FL3 measuring the red emission of PI. For optimal configuration of the cytometric assay both live cells and cells with compromised membrane integrity were used as controls.

The measurement of fluorescence of live UEmw_8 cells differentially stained with SG (plot A) and PI (plot B) are shown below in Figure 16.

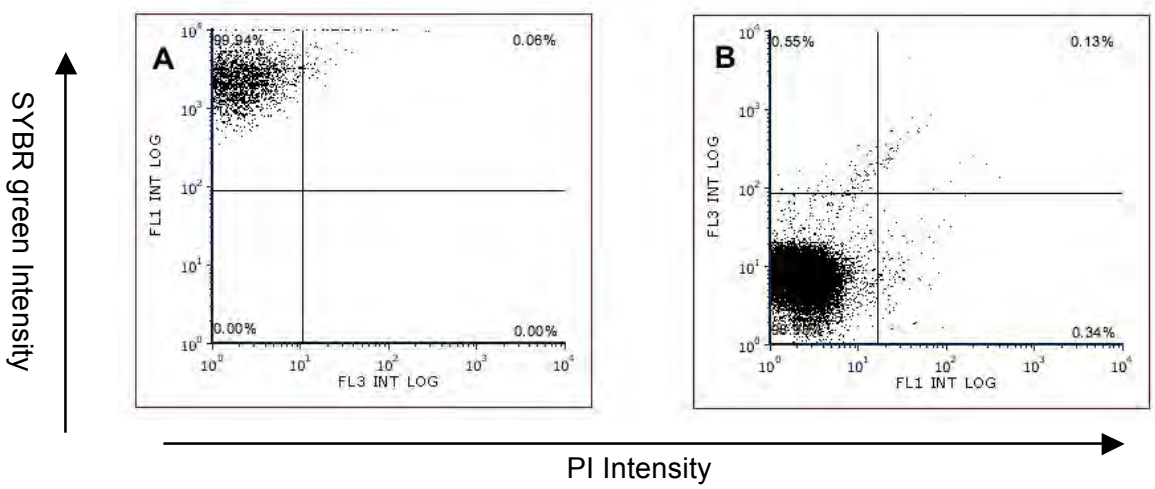


Figure 16. Flow cytogram of live cells individually stained with SYBR green and propidium iodide.
Plot A shows live cells stained only with SG. Plot B shows live cells stained only with PI - here all events measured are a result of flow cytometer noise and do not represent cell populations to be measured in analyses. Arrows showing direction for dye fluorescence intensity apply to all plots in panel.

Figure 17 shows the plots of heat-treated (membrane disrupted) UEmw_8 and UEmw_9 cells that were; stained with only PI (plots A and E), only SG (plots B and F), double stained i.e. stained with both dyes (plots C and G), or unstained (plots D and H). For these controls it is noted that some fluorescence noise is visible in the unstained samples that cannot be overcome by compensation, the vast majority of which is visualized in the LLQ.

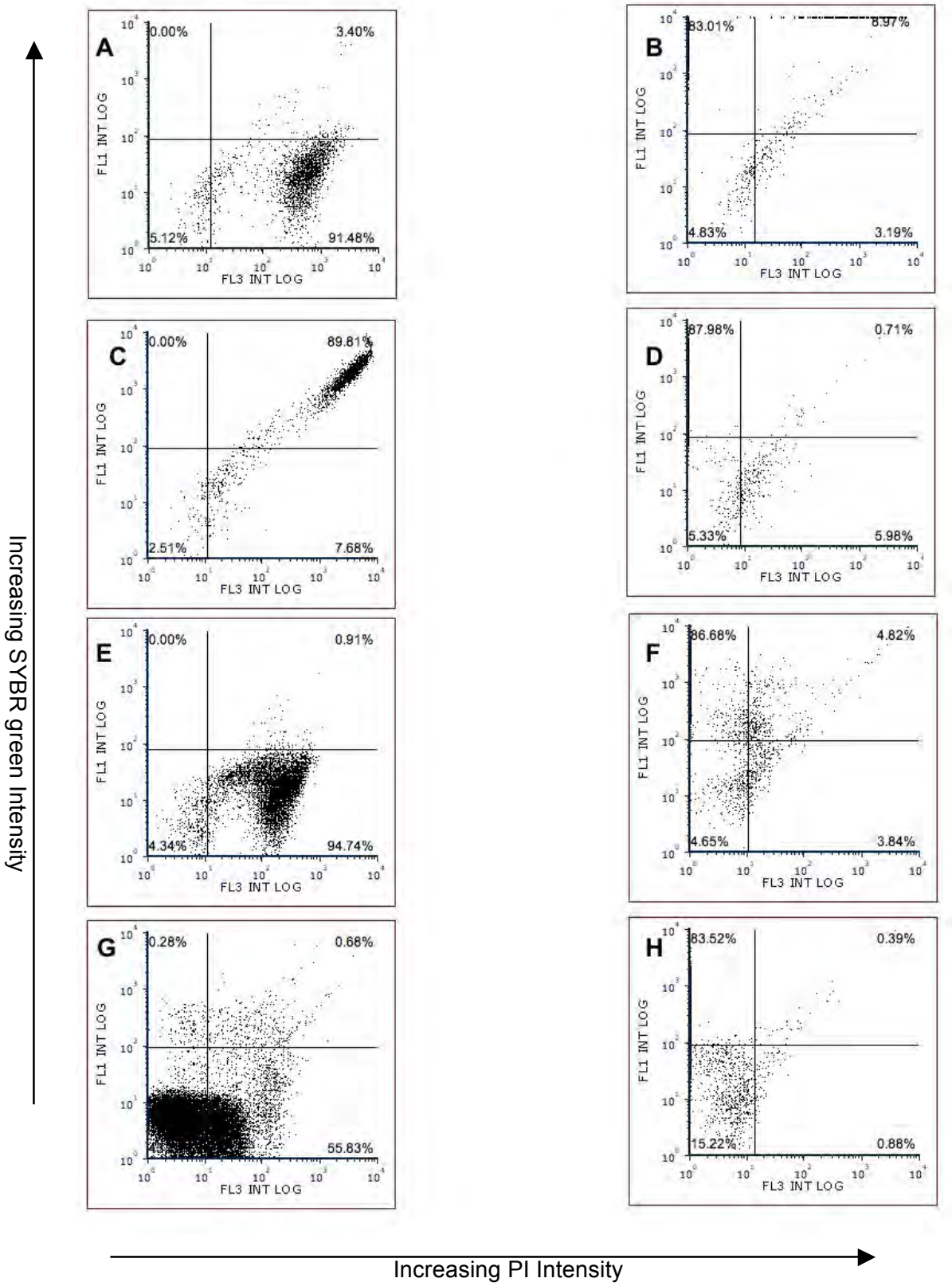


Figure 17. UEmw_8 and UEmw_9 dead cell controls variably stained with PI and SG.
Plots: A to D (top 4 plots) indicate UEmw_9 controls and plots E to H (bottom 4 plots) represent UEmw_8 controls. A & E: PI only. B & F: SYBR Green only. C & G: PI and SG double stained. D & H: Unstained cells. Arrows showing direction for dye fluorescence intensity apply to all plots in panel.

3.8.1 Biofilm degradation flow cytometric analysis

Following bacteriophage treatment, a change in the distribution of both live and dead cells is seen as compared to the controls. Figures 19 and 21 are representative sets of cytograms from one replicate measurement for each strain showing the changes as the live cells diminish and dead cells increase. As cells are killed by phage treatment a distinction in the pattern of cell distribution between the control and the treatment at each incubation time is apparent, with cells moving downward and to the right away from the upper left quadrant. The number of events seen in the “noise” quadrant (LLQ) also increases as the incubation time increases on the treatment plots. This is an indication that cells are being killed by the infecting phages but upon complete lysis the resulting cell fragments no longer fall within the correct size range for detection. The debris however still harbour bound stain and thus still emit light at the wavelengths of interest, which is tracked by the detectors as signal noise.
The obtained flow cytometric data describe relative amounts of cells falling in each of the four quadrants.

3.8.1.1 Degradation of UEmw_8 biofilms

Figure 18 shows the changes in live and dead cells at the various sampling times (2 h, 4 h, 8 h and 24 h) over the course of the 24 h incubation period. The percentage of live cells decreased compared to both the untreated control counts and compared to the measured initial values for the treatment group. The number of dead cells counted dropped at 4 h but then increased throughout the rest of the incubation. The number of dead cells as a proportion of the total cell count is higher for the controls than for the treatment group.

By 24 h of incubation with phage vB_EcoP-U8 a significant ($F = 5.858$; $p = 0.036$) increase in dead cells was seen compared to the initial (2 h) counts. The ~20% decrease in live cells at 24 h of phage incubation between the control and the treatment was not significant ($F = 1.536$ $p = 0.244$). For the treatment catheters a 34.2% decrease in live cells was seen over the 24 h incubation compared to a decrease of 14.5% in the live cells control over the same period, this difference was not statistically significant ($F = 2.050$; $p = 0.159$). An increase of 9.47% dead cells was seen between 2 h and 24 h of incubation with phage, whereas dead cells increased by 6.24% over the same period in the untreated controls. This difference in the increase in dead cells is not significant ($F = 6.553$; $p = 0.14$).

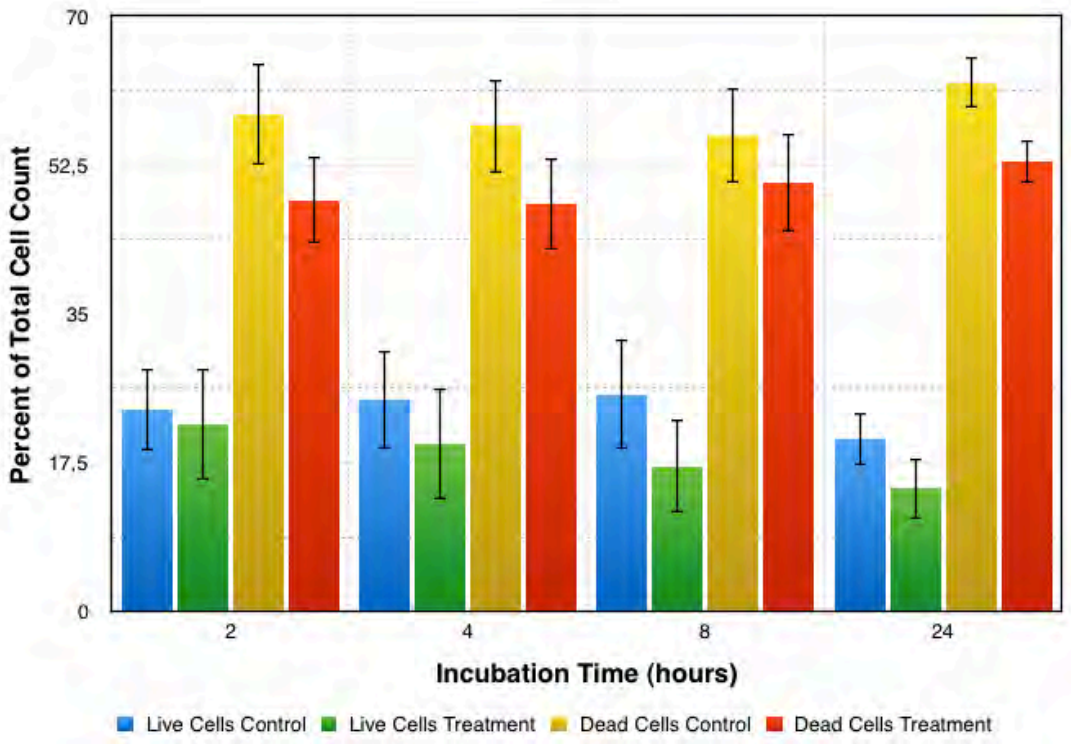


Figure 18. Percentage of live and dead UEmw_8 cells following bacteriophage treatment.
A decrease in the number of live cell is seen over time with treatment as compared to the control, and the number of dead cells measured increases with time.

Figure 19 shows a representative set of cytograms from one of the replicate measurements showing representative changes in dead and live cells for a control and corresponding treatment of UEmw_8 biofilm derived cells in the biofilm degradation assay.

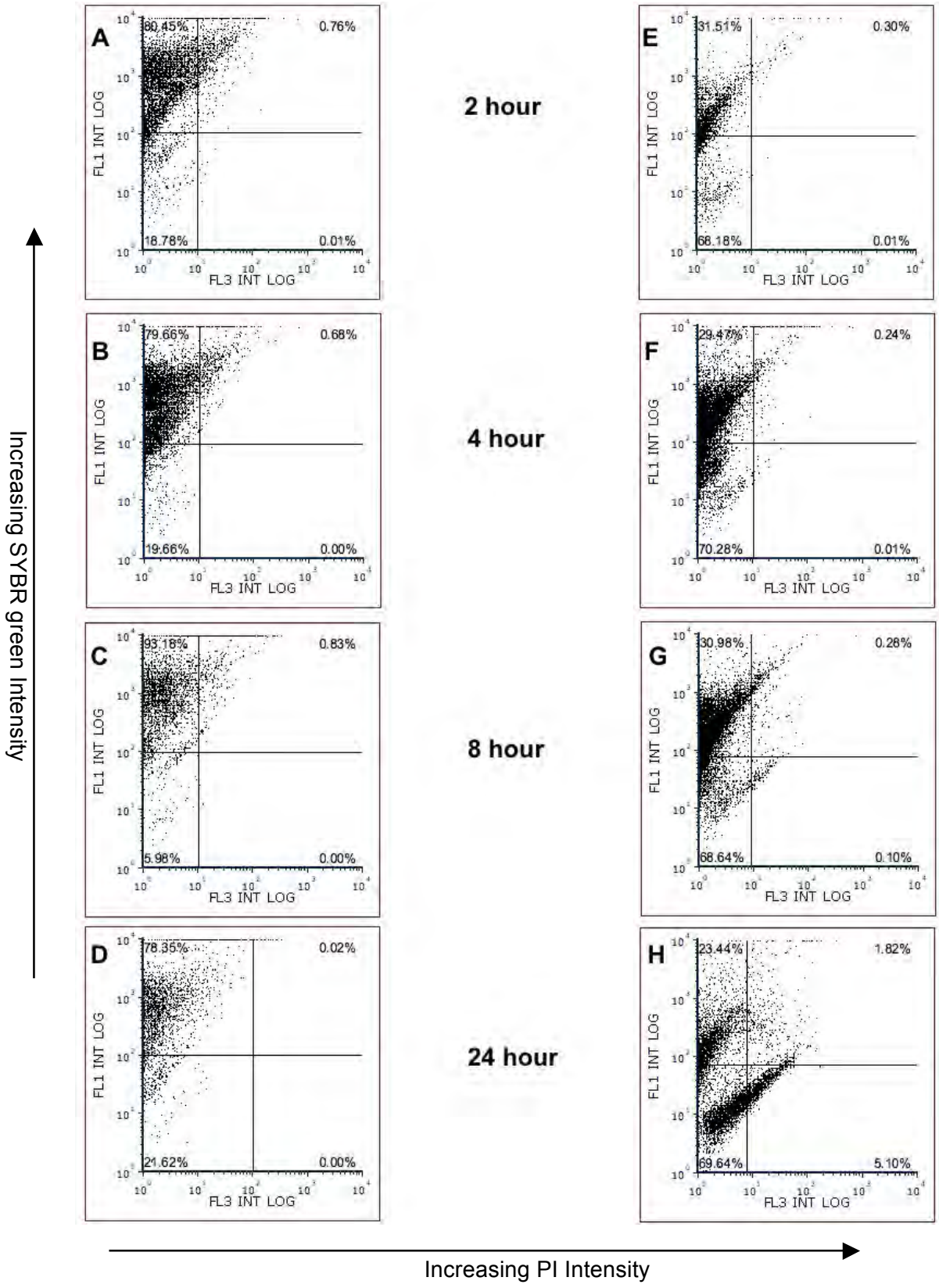


Figure 19. Flow cytograms of UEmw_8 degradation assay control vs. treatment.
All plots indicate double stained samples. Plots: A to D represent control (sterile AUM treated) cells. Plots E to H indicate vB_EcoP-U8 treated cells. A progression is seen from the single live population to divergence into two distinct populations with longer exposure to bacteriophage. Arrows showing direction for dye fluorescence intensity apply to all plots in panel.

3.8.1.2 Degradation of UEmw_9 biofilms

Figure 20 shows the changes in live and dead UEmw_9 cells over time after treatment of catheter derived biofilms with phage vB_EcoX-U9. As was seen with UEmw_8, overall the live cells decrease and dead cells increase with longer phage incubation. Once again dead cell proportions were higher in the controls than in the treated biofilm. Live cell counts were measured as a lower proportion of total cell counts in the controls for UEmw_9 biofilms, but over time the control live cells increased whereas the phage treated live cell counts dropped.

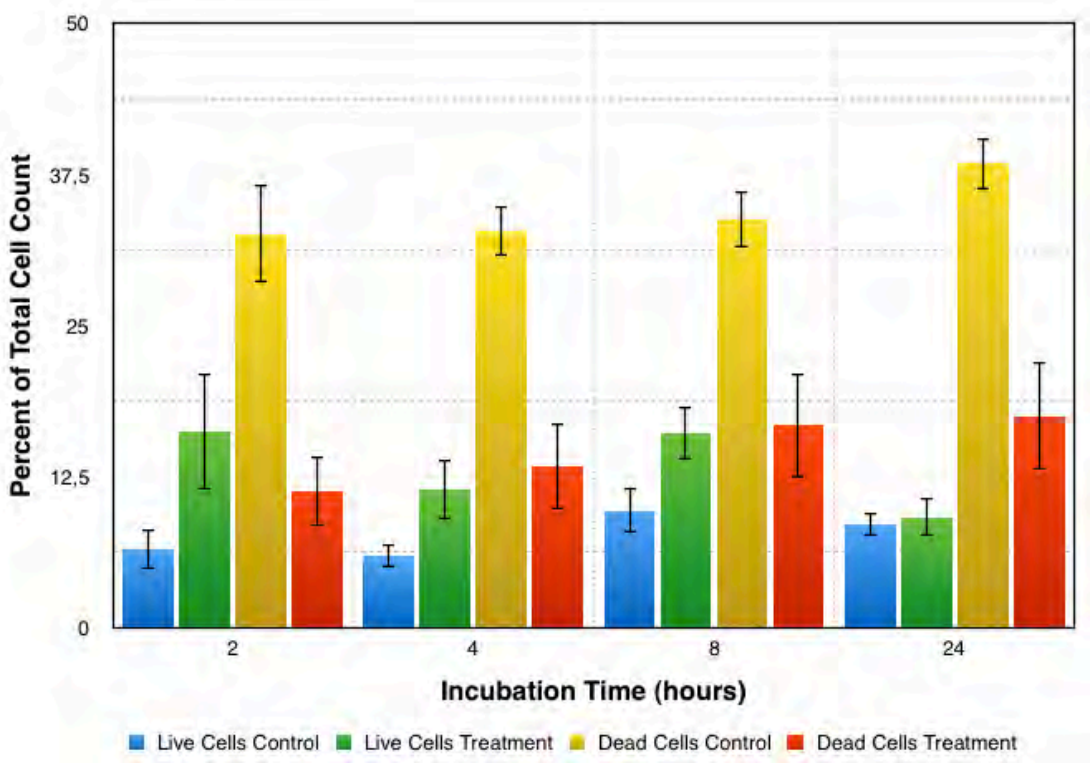


Figure 20. Percentage of live and dead UEmw_9 cells following bacteriophage treatment.
A decrease in the percentage of live cells is seen over time with treatment as compared to the control, and the percentage of dead cells measured increases with time compared to the control.

By 24 h of incubation with phage vB_EcoX-U9 neither the decrease in live cells nor the increase in dead cells was significant for the treatment samples. There was no significant difference in the live cell counts for the treatment vs. the control over the course of the incubation with phage. For the treatment a 43.91% decrease in live cells whereas a 31.39% decrease in the live cell population was seen for the control. An increase in dead cells of 55% was seen in the phage treated biofilm cultures compared to only 18% increase in dead cells over the same period for the controls.

As seen in the UEmw_9 degradation cytograms (Figure 21), treatment with phage vB_EcoX-U9 results in a divergence over time in the pattern of cell distribution between the control and the treatment.

Comparison of the effects of bacteriophage treatment on the two bacterial strains by flow cytometric analysis showed a similar effect of treatment on established biofilm cells for both of the bacterial strains. It was seen that for both strains of isolated uropathogenic *E. coli,* the lytic phages were able to access cells associated and/or within the EPS matrix and kill some of the viable cells.

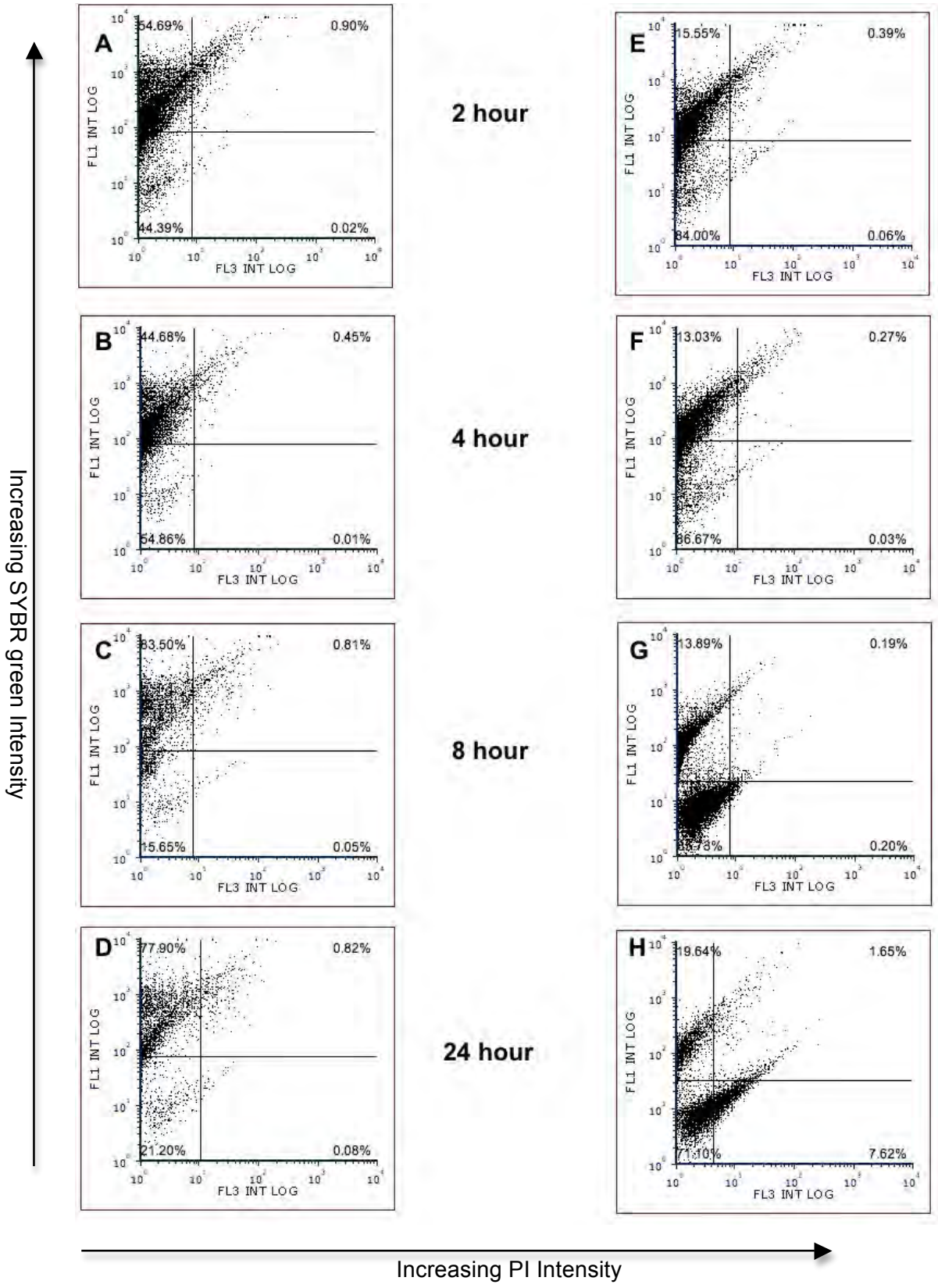


Figure 21. Flow cytograms of UEmw_9 degradation assay control vs. treatment.
All plots indicate double stained samples. Plots: A to D represent control (sterile AUM treated) cells. Plots E to H indicate vB_EcoX-U9 treated cell suspensions. A progression is seen from the single live population to divergence into a live and a dead population increasing with longer exposure to bacteriophage in the treatment plots. Arrows showing direction for dye fluorescence intensity apply to all plots in panel.

3.8.2 Biofilm prevention flow cytometric analysis

The data obtained from the flow cytometric analysis of biofilm cell suspensions from the catheters pre-treated with phage showed, as in the absorbance measurements of the same suspensions in section 3.7.2, a complex response to pre-treatment as compared to the controls.

3.8.2.1 Prevention of UEmw_8 biofilms

Figure 22 indicates the effect of pre-treatment of catheter segments before growth of biofilms for UEmw_8 compared to untreated controls. The live cell counts recorded are lower in the pre-treated catheters than those measured in the controls but they increase over time, indicating a possible negative effect on biofilm production but not an ability to prevent it. The dead cell counts over time showed that as incubation time increases the number of dead cells increase, which was an effect not seen in the controls. Once again measured dead cell counts were higher in untreated controls.

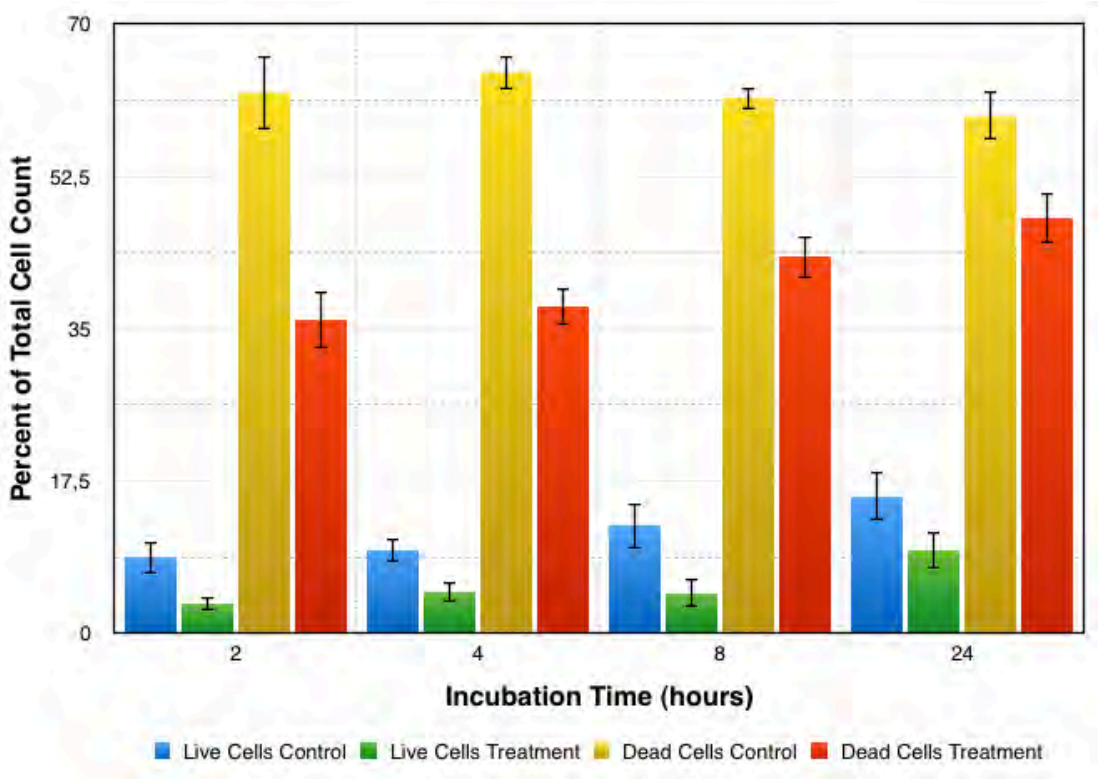


Figure 22. Percentage of live and dead UEmw_8 cells from bacteriophage prevention assay catheter segments.
Live cells are seen to increase over time with treatment as compared to the control and in comparison with initial values. The number of dead cells measured increases with time compared to the control and also compared to initial dead cell numbers.

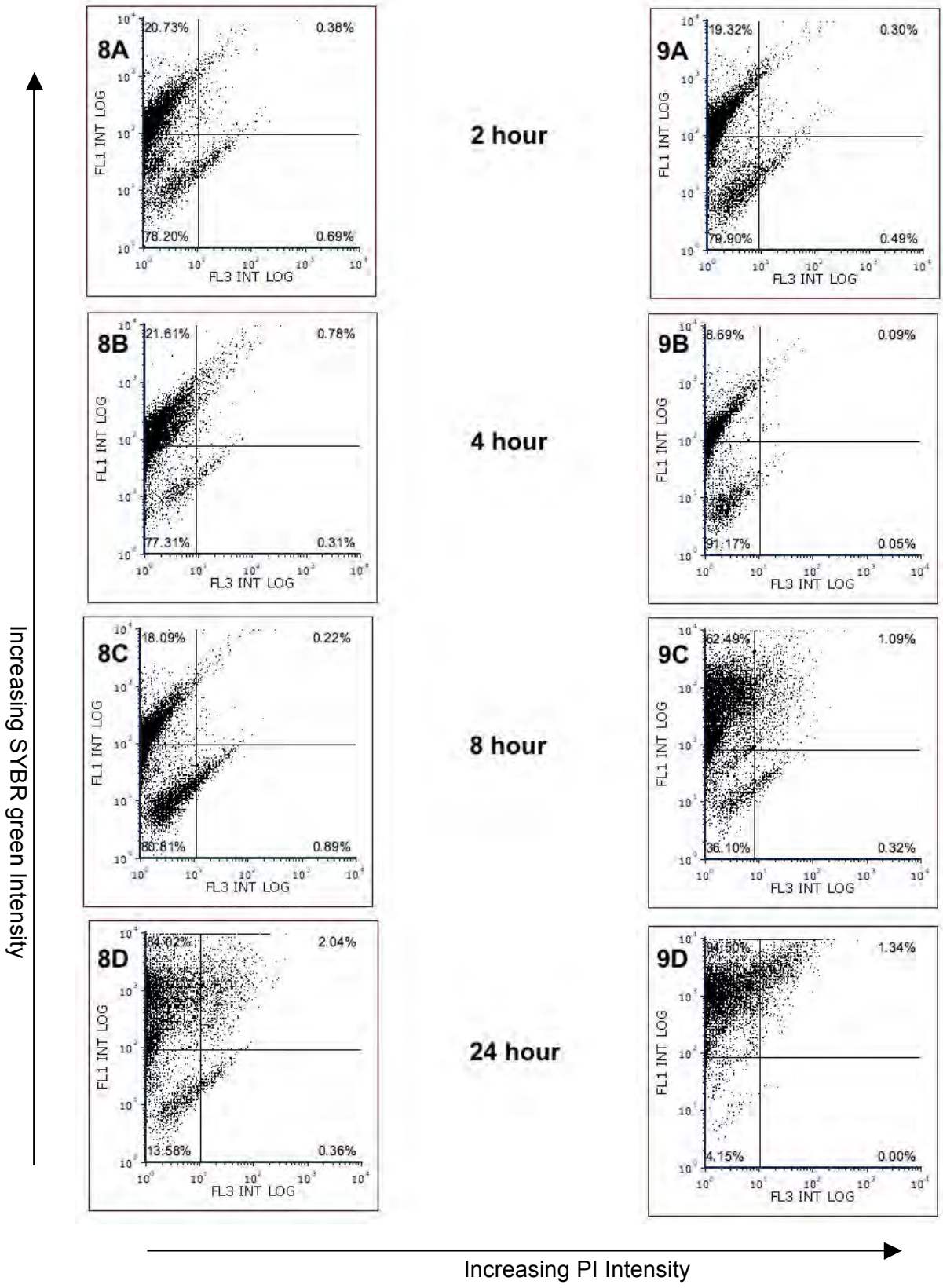


Figure 23. Flow cytograms of UEmw_8 and UEmw_9 prevention assay.
All plots indicate double stained samples. Plots: 8A to 8D represent control UEmw_8 cells following pre-treatment with vB_EcoP-U8. Plots 9A to 9D indicate vB_EcoX-U9 pre-treated treated UEmw_9 cells. The progression is markedly different for each strain but recovery is seen with a large number of live cells in both groups by 24 h. Arrows showing direction for dye fluorescence intensity apply to all plots in panel.

The left column of the flow cytograms in Figure 23 (plots 8A-D) indicate these anomalous patterns in live and dead cell distributions as measured for UEmw_8. An increase of 32.33% dead cells was seen in the treatment samples between 2 and 24 h (red bars in Figure 22) post phage pre-treatment where a decrease in dead cells of 4.13% is seen in the control (yellow bar in Figure 22).

3.8.2.2 Prevention of UEmw_9 biofilms

As with UEmw_8, the effects of pre-treatment with bacteriophage seem to be rather complex have little consistency of effect over time as measured by flow cytometry. This can be seen in the right column of cytograms in Figure 23 (plots 9A-9D) and in the graph below (Figure 24). Once again, live cell counts measure higher than those seen in the control. The changes in numbers of dead cells over time indicate that as incubation period increases the number of dead cells initially decrease and then begin to increase but they never reach the levels initially measured. It is noted that the proportion of dead cells in the control is significantly lower in the controls than in the pre-treated catheters, an effect not mirrored in the degradation assays or the prevention assay for UEmw_8.

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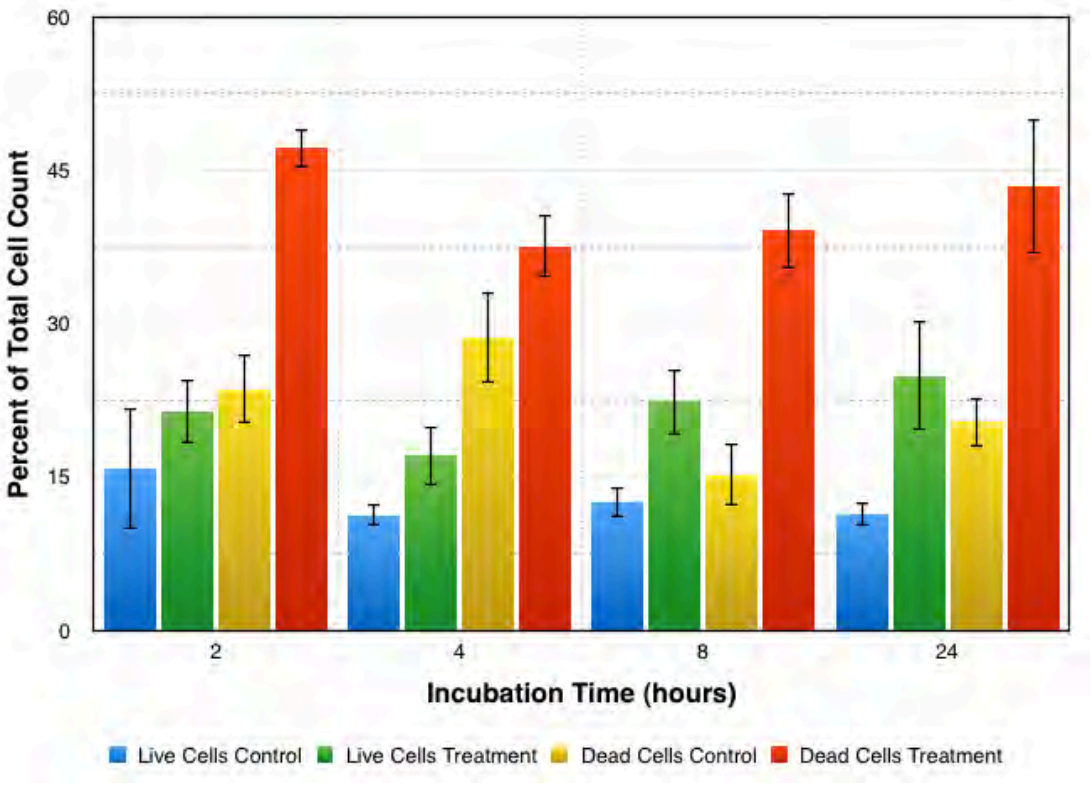


Figure 24. Percentage of live and dead UEmw_9 cells from bacteriophage prevention assay catheter segments.
Live cells are seen to initially decrease and then increase over time from 4 h with pre-treatment as compared to the control and in comparison to initial values. Live cell percentages are higher in the treatment compared with the control. The number of dead cells measured initially decreases and then increases with time from 4 h onward but remain below the mean of the cells measured at 2 h.

CHAPTER 4 – DISCUSSION

4.1 Bacterial isolation and culture

Given the goal of finding bacteriophages capable of infecting clinically derived uropathogenic *E. coli* as outlined above, the first step was to isolate the UPEC strains from urine samples of hospitalized patients. The study design focused on sampling that would yield at the very least two UPEC strains that were capable of forming significant biofilms that could be used for downstream assays. The literature indicated that, any UTI has anywhere from 30% to over 80% chance of being *E. coli* associated.(18,20,36,101–103)

It is also evident from the literature that any isolated *E. coli* would be more likely than not to produce biofilms under the right conditions. In their 2008 study, Rijavic et al. found more than half (53%) of UTI derived UPECs to be biofilm forming.(104) Indeed, biofilm formation is understood to be a phenotypic "strategy" rather than a strain specific function,(4,48,104) being inducible by nutrient and environmental changes. According to Costerton et al. "biofilm bacteria predominate, numerically and metabolically, in virtually all nutrient-sufficient ecosystems".(4)

In consideration of these data, sampling was structured to facilitate continuous and successive screening of UTI-urine samples until 15 *E. coli* strains had been isolated, to ensure several biofilm forming isolates could be attained, with a high degree of certainty (taking even the most modest estimates of *E. coli* responsibility for UTI) that sampling would not require the processing of more than 50 urine samples. As indicated in the results, and in line with the projected numbers, actual sampling from 38 suspected UTI urines was required to yield the desired 15 clinical UPEC strains. The preliminary species identification was achieved by direct culture of urine derived bacterial populations on Uriselect™ 4 Chromogenic Medium plates, with *E. coli* cells growing on these plates as small pink colonies. A mix of multispecies and single species cultures was observed in the preliminary screening (See figure C2 in Appendix C). Single pink colonies were picked, whether from single- or multi-organism cultures, from each of the 15 chromogenic medium plates that indicated presence of *E. coli* in the corresponding urine sample. These strains were subcultured on MacConkey agar plates to attain a completely pure culture of each isolate for Microscan™ analysis. (Microscan™ identity analysis results can be found in Appendix E).

At this point sample number 28 was excluded from the study as the analysis indicated that it was a sorbitol non-fermenter, and thus a possible strain of *E. coli* O157:H7.(105) It is well established that pathogenicity factors may be transferred by transduction (106) into phage genomes and subsequently thus contribute to "the creation of new pathogenic variants".(107)

Since the overall rationale for this narrow field of study is to find possible routes to novel therapeutic tools and practices, the decision was taken to avoid carrying out downstream phage infection of bacteria which harbor toxin encoding genes. The remaining 14 samples were all confirmed as uropathogenic *E. coli* with identification confidence of over 80% probability. At this point the sample strains were preliminarily designated simply UEmw_1 to UEmw_14. This naming scheme indicates preliminary identification as uropathogenic *E. coli* (UE), the researcher's initials (mw), and a numeral component of the designation indicating simply the chronological order of isolation as per the naming criteria outlined by Demerec et al. (Section III).(108)

The prevalence of *E. coli* in the urine samples tested was 39.5%. Thus 60.5% of the infections were caused by a variety of other microbes but with no other single species making up as large a proportion of the total number of infections as *E. coli*. This result is in line with those reported in the literature in that it supports the assertion that *E. coli* is the most common causative agent of UTI in hospitalized patients. Indeed, Habte et al. having sampled 2203 hospital associated UTI isolates in South Africa over a period of 1 year found 39.0% to be *E. coli,* a result differing from the sampling in this study by only 0.5%.(18) The result is higher than that found by Abdallah et al. who reported 31.7% of tested isolates to be *E. coli* when 72 patients with UTI in Egypt were screened.(20) It is lower than the numbers indicated by Jones et al. who reported 48.6% of tested isolates to be *E. coli* from a pool of 1698 pathogens from patients in the USA (1308 patients) and Canada (390 patients).(36) It is also a lower proportion than indicated by the data from Gales et al., who found that 60.4% of all hospital associated UTI infections in their study on patients in Latin America, were caused by *E. coli.*(101)

So although in various cases and studies with differing sample sizes, the percentage of *E. coli* contributing to UTI differs fairly markedly, across the globe it has been clearly demonstrated that *E. coli* strains are the most prevalent causative agent of UTI in hospitalized patients. The current study made no attempt to confirm or refute this assertion but consideration of it was imperative in designing the sampling procedure. The result however has reaffirmed the practicality and applicability of using clinically isolated *E. coli* strains as model organisms for general enquiry into the use of phages for combating common, biofilm forming pathogens.

4.2 Preliminary biofilm assay

The preliminary biofilm assay was carried out in order to screen for and relatively quantify biofilm formation and thereby establish a hierarchy, across all the isolated UPEC strains. The outcome of this preliminary procedure was then used to select the two bacterial strains that would be most likely to form biofilms under similar conditions, provided that they were

susceptible to phage infection and lysis, so that they could be used for the primary, downstream biofilm degradation and prevention assays.

The protocol for this procedure was described by O'Toole et al.(61) building on previous work by those authors and Christensen et al.(109) and has been used as a preliminary screen for capacity to form biofilms in many subsequent studies.(17,59,110) The technique is most powerful when there is a large sample with many distinct strains, and there is a need for a rapid method of establishing which strains are biofilm formers and which are not. In this study it was utilized less categorically and more for relative ordering of isolated strains, although the same cut-off criteria for "significant" biofilm formation was used, i.e. at least 3 x the value obtained from the negative control of bacteria free AUM.(17) The results (Figure 3) indicate that 8 of the 14 tested strains (57,1%) formed biofilms at a significant level. These numbers correlate well with those reported by Rijavic et al. (104), who found 53% of their isolates to be significant biofilm formers. The important result from the assay is that two of the 6 biofilm formers (Uemw_8 and UEmw_9) were best at producing biofilms under the conditions used and thus were preliminarily selected as the strains to be used for all further assays.

Although the method is often used and cited in the literature, and its efficacy as a preliminary screen has been well established, it was found that the manual washing steps (due to the nature of the procedure itself) could not be repeated with absolute precision (this notwithstanding numerous attempts at maintaining consistent protocol). This notion is considered and explored by the original authors in describing the procedure who state that: "…the incubation and rinsing conditions themselves determine the properties of the biofilm".(61) Thus, although repeated assays supply sufficient qualitative data about biofilm formation per strain in relation to the other strains assayed in the same sample batch, they failed to provide useful quantitative data. In light of the fact that it is just a preliminary assay and not intended to quantify absolutely the specific strains' biofilm forming capacities anyway, this was not considered as a meaningful shortcoming. It did however prompt the decision to repeat the assay several times and take the averages rather than using just duplicate data collection.

The assay can be carried out with or without shaking during the incubation period. Although Moreira et al. found no statistically significant difference in capacity of *E. coli* to form biofilms in microtitre plates under shaking or static conditions, they did find phenotypic differences in cells of biofilm populations grown under each condition.(110) In addition O'Toole et al. describe typical conditions to not include agitation.(61) Thus, no shaking was used in this assay in an attempt to keep adherence and growth conditions as close to those that would be used in downstream phage treatment assays where the bacteria would be cultured on the catheter segments.

Because of the complexity of biofilm formation and the abovementioned established understanding that biofilms of the same strain form to varying degrees on different surfaces,(111) a parallel test for combined biofilm formation and cell count was carried out on all 14 isolated strains on the surface of catheters of the same make and composition as those to be used in the downstream assays. This was a quick secondary test used as a check for possible large discrepancies. Surprisingly, the results of this procedure indicated very little difference in the ordering of the bacterial strains, with UEmw_8 and UEmw_9 being the best biofilm formers on the catheter segments, as in the microtitre plate assay. However, some small differences in some of the strains were seen and this does lend support to the idea that inducibility of biofilm formation is to a degree dependent on the surface material. This consideration was noted but the lack of detection of any large discrepancies and the fact that the microtitre plate assay is standard procedure in biofilm screening studies, prompted the continued use of this standard methodology as the primary screening procedure in this study. The confirmation afforded however by running this secondary check; that UEmw_8 and UEmw_9 were the strains that formed the highest levels of biofilm, was a valuable insight and a graph of the data from this modified catheter section biofilm screen assay is included (Figure B1) in Appendix B.

4.3 Bacterial genetic characterization

The rationale behind using phenotypic, biochemical, and genetic evaluations to screen for and characterize the specific bacteria involved in the infections of interest was to increase certainty of the identity of those microbes. This is a seemingly obvious endeavour, but one that can and is sometimes overlooked. Indeed, Janda and Abbott state that: “Reliance on a single identification system, phenotypic or genotypic, to identify an organism provides more opportunity for misidentifying bacterial species, and if the trend described above continues, the risk will become even greater in the future.”(64) Thus bacterial genetic characterization was carried out, a process that began with genomic DNA extraction. In analyzing the success of extraction of the bacterial genomic DNA as a preliminary step to enable amplification of the 16S rRNA gene, it was noted that the published desired figures for purity and yield are ‘rules of thumb’ and dependent on nucleotide composition (NanoDrop Technical Bulletin) and pH.(95) Thus comparisons of the results of this study with those in the literature indicated that the total nucleic acid extracted for each strain was indeed acceptable for the downstream processing to be carried out i.e. 16S rDNA amplification using PCR.

The final step in the characterization of the two bacterial strains (UEmw_8 and UEmw_9) used for the biofilm prevention and degradation assays was 16S rRNA gene analysis. The universal primers used for the amplification of the gene from the isolated genomic DNA for each strain were the forward primer 4F, which binds from base position 4 to 25 on the 16S rDNA, and the reverse primer BAKR which binds from position 1540 to 1521. In line with the

current taxonomic characterization methodologies,(112) the universal primers were used, rather than opting for the design of novel primers for the published *E. coli* 16S rDNA sequences. This is because the use of universal primers for amplification of such conserved housekeeping genes is a well-established standard procedure. The alignment of the primers with the published 1541bp sequence of the full *E. coli* 16S rDNA sequence indicated two primer mismatches between the 16S rDNA and primer 4F (at positions 7 and 19 on the target), and 1 mismatch with primer BAKR (at position 1426 on the target). Such mismatches, when evaluated in the primer design/analysis software tools at "Primer Blast" available at https://www.ncbi.nlm.nih.gov/tools/primer-blast/ and "Primer Map" available at http://www.bioinformatics.org/sms2/primer_map.html resulted in limited binding and no amplification of the gene of interest *in silico.*

The reality of the situation is however quite different in practice in that strong amplification of the gene of interest was achieved despite the mismatches. A single band corresponding to a nucleotide fragment of around 1500bp was observed during electrophoresis of the amplicons for both propagation strains and the control strain (CFT 073). The observed outcome was as expected based on the understanding that, as explained by Sipos et al.: "Primer mismatch is an inherent characteristic of PCR with 'universal' primers, while, owing to single nucleotide variability even in the evolutionarily highly conserved regions of the rRNA genes, the designation of a perfectly matching 'universal' primer is simply not possible".(113) Indeed, it is noted that Stern and Holland assert that identity as low as 70% of universal primers with the target sequence is adequate for the amplification of genes from pure cultures.(114) Baker et al. suggest that the choice of universal primers should be based on both identity with the target, and a combination of the various characteristics of the primers.(112) These include consideration of primer length, reaction temperature optimums, GC content, and self-complementarity in addition to the target sequence specificity (identity). The caveat for allowance of mismatches in universal primers and the target sequence is that no mismatch should be tolerated if it occurs at or within ~5 bases of the 3' end of the primer sequence.(112,115,116) The forward primer 4F, although bearing one mismatch in the 22 bp sequence still sustains 95.5% identity with the proposed target. For the 20bp reverse primer 90% identity with the target was seen and it was noted that more than 3 G or C residues are found near the 3' end. Considering all these parameters together and seeing that both primers have been used successfully in the literature and are in use in combination at the NHLS, preliminary optimization runs were carried out. It was found that together the primers yielded good amplification of the genes of interest.

The spin column clean-up of the DNA from the PCR yielded different levels of pure 16S rDNA amplicon from each sample, and from the control, as can be expected for amplification of homologous genes from different strains, using the same primers. The DNA clean-up was considered successful as no large or unexpected discrepancies were seen.

Bacteriophages are characterized in terms of the specific bacterial host species/strains, which they infect. Thus accurate and robust characterization of the UPEC species in this study is important in its own right but even more so because upon it rests the characterization of the isolated bacteriophages. In the process of categorizing the UPEC strains however, it was found that, as with any measurement, the degree of certainty of results is intrinsically linked to the precision of the methods used. That is, in this case, if only phenotypic methods were used, the criteria for inclusion into the group "uropathogenic *E. coli*" would have been met by all samples after the initial chromogenic medium based isolation, before microscan analysis and before 16S rDNA analysis. The drift toward ever more refined and stringent taxonomic classification schemes fuelled by increasingly precise data collection and analysis tools is a necessary and somewhat desired phenomenon – but an obvious consequence is the need for standard cut-offs of levels to which classification is carried out in varying situations.(64) This topic has been covered fairly extensively by Janda and Abbott who assert that the acceptable degree of certainty of species identification should be determined by the purpose of the identification, and should be a compromise between the feasibility (cost and complexity) of the methods and the time necessary for characterization.(64,118)

With these general taxonomic considerations applied to the context of this study it is noted that the literature highlights clearly the genetic similarity between some genera within the Enterobacteriaceae family,(119) with which this study is concerned. When considering *Shigella* and *E. coli*, Johnson sums up the common biochemical differences as follows: "whereas most *E. coli* are motile, ferment lactose, are lysine decarboxylase- and indole-positive, and are mostly avirulent, *shigellae* are non-motile, are typically lactose-, lysine decarboxylase- and indole-negative, and are obligate pathogens".(120) The difference however in sequence identity for *Shigella* spp. and *E. coli* 16S ribosomal RNA genes is far smaller than what is considered necessary for species differentiation i.e. 1.3%.(121) In fact the literature indicates that in terms of currently practiced and accepted phylogenetic characterization methodologies (by the comparison of housekeeping genes), *E. coli* and the four *Shigella* species (which themselves are only distinguishable in terms of antigenic expression), should be considered one species.(122–124) Such extreme relatedness between these two genera begs the question; why distinguish between them? The answer to that lies in the distinct functional pathogenic capabilities of the two groups, which from an epidemiological and infection control perspective is exceptionally important. A whole genome based "feature frequency profile" approach has been used to successfully group evolutionary relatedness of these species into pathogenesis aligned clades.(125) More recently Zuo et al. have shown that through another whole genome based, alignment free comparison methodology (CVTree), the two genera are distinct "sister" species.(126) The ongoing debate and functional need for rapid discrimination between these closely related pathogens brings

to the forefront the abovementioned quandary of the methods of investigation providing new criteria for distinction.

By nucleotide BLAST analysis both UEmw_8 and UEmw_9 showed 99% identity with the *E. coli, Escherichia fergusonii, and Shigella flexneri* sequences. Analyses using the Geneious 16S Biodiversity Tool confirmed that UEmw_8 and UEmw_9 have identity with 100% confidence up to the family level, grouping both strains as members of *Enterobacteriaceae.* Distinction, however, by sequence analysis could not be made further than the genus level because, as has already been discussed, *Shigella* spp. and *E. coli* 16S rRNA genes are phylogenetically identical.

Considering the above and noting that the isolated strains were seen to be motile gram-negative bacilli by morphology, and that the chromogenic media preliminarily indicated species as being *E. coli*, and that the automated biochemical analyses confirmed identification with 99% confidence, it is suggested that the strains UEmw_8 and UEmw_9 are in fact *E. coli* strains. It is accepted that the combination of phenotypic, biochemical, and 16S rRNA gene analyses has successfully identified the two strains of interest; UEmw_8 and UEmw_9, as biofilm forming uropathogenic *E. coli* species as previously defined.

4.4 Bacteriophage isolation

The preliminary protocol for the isolation of a range of bacteriophages from sewage, centered on removing as much debris, bacteria, and other contaminants as possible from the samples while limiting the loss of concentration and variety of bacteria-infecting viruses. Several options were available, all with their own distinct pros and cons. Filtration would remove the debris and bacteria but leave all other chemical and molecular waste, including toxins, LPS, and bacterial metabolites like bacteriocins, which can mask the infection efficacy or mimic infectivity of the phages.(74) Ultracentrifugation purifies phages well but can lead to loss of phage titre, may discriminate between viruses of different size,(77) and cause aggregation or clumping of tailed phages.(76) The PEG procedure is inexpensive and was found to be effective when the cold chain was maintained, functioning well as a preliminary step before culture and propagation of specific phages in host strains.

The 14 UPEC strains were affected differently when treated with the initial phage lysate. The lysate, which in essence was a cocktail of many different bacteriophages, had a range of different bacterial killing capacity, with some strains' bacterial lawns being highly susceptible to lysis and some not being susceptible at all. Of the 14 strains only two (UEmw_2 and UEmw_14) showed no plaques/visible cell death. This result does not rule out phage infection for these two strains, only phage mediated lysis.

From this vast difference in reaction to treatment with the lysate, and consideration of the procedure used to obtain the phage lysate it can be confidently inferred that 1) there was no cytotoxic chemical or enzyme present in the preparation able to kill all bacterial strains, and 2) that it was highly probable that the plaques formed were a result of phage infection and lysis. The extreme host specificity of bacteriophages could at this preliminary juncture already be observed in action. An ordinal listing of the bacterial isolates was generated from the qualitative evaluation of the plaque assay results. This list was compared with the results from the biofilm-forming hierarchy obtained from the preliminary biofilm assay. While bacterial strains UEmw – 1, 5, 6, 8, 9, 10 and 14 were the best biofilm formers, strains 10 and 1 were hardly affected by the crude phage lysate. And while strains 3 and 4 were most susceptible to lysis, they were poor biofilm formers. UEmw_8 and UEmw_9 were selected as propagation strains for bacteriophage isolation and for further analyses, as they were shown to be both good biofilm formers and susceptible to infection by lytic phages within the sewage derived crude lysate.

4.5 Bacteriophage purification

The crude lysate, having been shown to be containing various bacteriophages by the ability to form plaques on some *E. coli* isolate-grown bacterial lawns while having no effect on others (indicating a specific activity against some strains rather than a general cytotoxicity), was inoculated into a liquid culture of each of the propagation strains. Only phages capable of infecting each specific bacterial strain would have been propagated in these cultures. The repeated isolation and infection of phage in the propagation strains yielded a distinct, single-lineage phage, designated preliminarily as vB_Eco-U8 and vB_Eco-U9 respectively, effective at inducing lysis in each of their hosts.

Rigorous analyses of the methods employed, coupled with continuous optimization thereof, were required during the repeated attempts at adequately concentrating the phages from the crude sewage-derived lysate, the results of which could only be seen after the process was complete. Throughout the process it was found that any disruption in the cold chain led to loss of titre. Bacteriophage vB_Eco-U8 did not grow to appreciable levels in liquid culture even with optimization of liquid volumes and shaking conditions. Conversely vB_Eco-U9 did grow to high titre in liquid culture of its host. The difference in capacity to grow in liquid culture is striking in that both viral strains, though at this point obviously distinct, are coliphages infecting uropathogenic *E. coli*. Besides, their specific affinity for related hosts there seems to be little similarity in the two bacteriophages. After the incubation period of the phages in each of their host cultures, it was noted that a thick sediment of presumably dead cells had formed at the bottom of the UEmw_9 - vB_Eco-U9 vessel, with considerable clearing of the media. For UEmw_8 - vB_Eco-U8 culture however, the media remained turbid and very little settlement of cells/debris was seen. It is thus postulated that some trigger for conversion from lytic to lysogenic modality was seen in the movement of vB_Eco-U8 from host infection on

plates to liquid culture, perhaps relating to the differential composition of AUM and cation supplemented LB agar plates. This notion is supported by the fact the plate enrichment procedure for vB_Eco-U8 was successful at producing high titre phage.

The isolated bacteriophages designated vB_Eco-U8 and vB_Eco-U9 were found to have strain-level specificity in their capacity to kill UTI associated bacteria. That is vB_Eco-U8 was tested against all 14 strains and CFT073 and was found to be active only against UEmw_8. Likewise, in treating all 14 UPEC isolates and the control strain with vB_Eco-U9, only UEmw_9 showed any sign of susceptibility to infection and lysis. Although the specificity of bacteriophages has been documented extensively in the literature,(11,13,127) the result that not one of the other UPEC strains were affected by these phages was surprising, given that Brüssow describes the capacity of some coliphages to alter their host ranges by various mechanisms (128) and that Chibani-Chennoufi found that four lytic coliphages isolated from stool and wastewater were able to infect nineteen of the pathogenic *E. coli* strains assayed in their study.(129) The specificity of a bacteriophage for its host is dependent on that phage's unique tail fibre association with host cell phage receptors,(130) so it is a combination of the *E. coli* strain membrane characteristics and the viruses themselves that impart this level of specificity. The result lends support to the theory that any viable therapeutic agent based on bacteriophage will need to incorporate a phage cocktail rather than any single viral species.

4.6 Bacteriophage characterization

In the preparation for virus viewing via TEM, the staining and rinsing procedures, sample and stain volumes, staining and drying times and treatment of the grids prior to microscopic visualization required multiple adjustments and refinements to get to the point where imaging was acceptable for morphological analysis. It was found that these aspects had as much or more effect as the most critical aspects described in the literature, those being pH and the choice of stain used.(131,132) In addition, it was found that the beam intensity had marked effects on the stability of the perforated formvar/carbon films on the grids, and it is known that organic material often diffuses to the edges of these holes on the grids.(132). Single staining with potassium phosphotungstate and uranyl acetate as well as dual staining with UA and lead citrate were attempted before the recorded methodology was decided upon, giving the best results and sample/grid stability.

The bacteriophages infecting UEmw_8 were seen to have icosahedral heads averaging around ~44nm in diameter. A short non-contractile tail and the base plate from which short spikes protrude can be seen in the transmission electron micrographs of the virus. Because of these distinctive morphological features the bacteriophage was designated fully as vB_EcoP-U8 the "P" indicating that it is a member of the family *Podoviridae*, one of the three families of the order Caudovirales, the tailed bacteriophages.

For vB_EcoP-U8, the DNA extraction process was carried out many times with all methods that were attempted for vB_EcoX-U9 as described in the Materials and Methods. It was found that spectrophotometric analysis of the nucleic acids was not in any instance indicative of true yield or quality, and even in cases where it may have been, the discrepancies seen in the results from the different extraction methods made it impossible to know which were accurate prior to sequencing. Two different Nanodrop spectrophotometers (Thermo Scientific NanoDrop 2000 and NanoDrop 1000) were used to double check samples. The results remained unclear when using the High Pure Kit (which gave the best extraction results based on electrophoresis), and it is thought that the composition of the specific kit's elution buffer and/or residual glass fibres from the elution tube may have been involved in the difficulty experienced in gaining clear results by the spectrophotometric analysis. Despite the problems with the spectrophotometry, following genomic DNA extraction, electrophoresis was carried out and indicated the presence of a large band of DNA, greater than 10 000 bp. This indicated that the extraction of DNA was successful to some extent, but attempts at restriction digestion for sizing proved fruitless. Because DNA was found to be present when the eluent was run on an electrophoresis gel, the samples were sent to the NICD for sequencing. Sequencing was successful and subsequent genetic analysis indicated that the virus has a linear genome of 40294 base pairs in length. The phage genome codes for 51 CDS genes including tail fibre, coat, tail shaft, plate protein, and other phage protein genes and enzymes including protease, terminase and integrase. The genome also codes for one tRNA gene. The genome size, GC content, number of structural proteins and tail and head size and morphology of vB_EcoP-U8 indicate that it is a member of the T7-like viruses according to the criteria as outlined by the International Committee on Taxonomy of Viruses (ICTV), 9th report.(134)

The phages infecting UEmw_9 were morphologically distinct from any known bacteriophages, and have been designated vB_EcoX-U9 to indicate that the family is yet to be definitively determined. The viruses are enveloped, having circular lipid membranes surrounding capsids of around ~40nm (over 60nm when encapsulated). These phages are most closely related to the Cystoviruses but are on average around 20% smaller.(98) Difficulty in extracting phage nucleic acids prevented genetic characterization of vB_EcoX-U9 and further work must be done to accurately characterize this unique virus.

Isolation and description of two distinct and highly specific bacteriophages that are capable of infecting clinically isolated UPEC strains has thus been achieved.

4.7 The ability of bacteriophages to degrade and prevent formation of biofilms

This study involved finding and isolating lytic phages active against patient derived *E. coli* strains and then determining if the isolated phages could alter biofilm formation in urinary

catheters *in vitro.* Having isolated two lytic bacteriophages capable of specifically infecting two biofilm-forming UPEC strains, the primary objectives lay in determining how effective the phages were at either reducing or preventing biofilm growth. Three methods were used to detect changes in biofilm cell numbers and biofilm biomass in an attempt to get a holistic view of the effects of application of phages to the bacterial biofilms, and to close the gaps that are left by using fewer or less broad quantification assays.

Use of the direct cell viability assay (flow cytometric NADS method) was chosen as an adjunct to biomass comparisons and plate counts described above to circumvent the errors in results that inherently arise from the limitations of cell culturing and the possible metabolic effects of disruption of the biofilm on specific subpopulations of biofilm cells. Together the assays aimed to measure lytic effects of the phage treatment directly on the cells from the treated biofilms rather than making attempts at indirectly assessing their viability based on the assumed capacity for replication following phage treatment. This is in light of the established understanding that phenotypic differences and thus possible differences in cultivability of biofilm cells underlie biofilm formation.

In considering the three methods used to track changes in biofilms, the following aspects are found to be important. The plate count method is a standard viability assay but does not measure viable but not culturable cells,(79) and it under-represents slower growing cells that may actually be the major cellular components of the biofilms.(19) Plate counts also have no capacity to determine changes in EPS levels or extent of penetration of antimicrobial agents into EPS. The absorbance measurements detect cellular debris and EPS as well as live cells, so it gives an indication of total biofilm present. The changes it tracks therefore occur in biofilms at rates different to those detectable by the indirect (because it relies on culture) plate count assay, as even though dead cells don't produce EPS, the EPS already produced remains detectable by spectrophotometry. In addition, rapidly replicating viruses within a high number of bacterial cells may increase or have no effect on absorbance when infection is highest, but at such a point flow cytometric analysis (where viability is based upon membrane integrity) will indicate the highest number of dead cells. The plate counts of such highly infected cells would also undoubtedly indicate very low numbers of viable cells following culture. Lastly it is noted that when bacterial cells have been completely lysed by the activity of rapidly replicating phages, they are excluded from the analysis by the flow cytometer detectors as they no longer have the characteristic shape and size of cells being measured, so the assay more realistically measures "live and dying cells", rather than "live and dead cells" when cell death is associated with cellular lysis. So, because the NADS flow cytometry procedure cannot measure fully-lysed bacterial cells, the dead cell count would be highest and the live cell count the lowest when the highest numbers of bacterial cells have viruses replicating within them and have not yet fully lysed, which would seem at odds with spectrophotometric measurements of the same samples.

For some of the above reasons it seems that in this specific case, the addition of quantification methods may in fact complicate rather than simplify the elucidation of the effects measured by the generation of a large set of data that may seem to be, at times, at odds with each other. This effect may be exacerbated by shortcomings in the execution of the experimental procedures, or because of the obvious time lag between cell killing and changes in EPS production. In addition, the shortcomings in each of the techniques may overlap more so than do the benefits. In previous studies often only one or two of these methods were used (16,44,50) and the data is seemingly clear and definitive. In this study however, oftentimes different measured aspects of the effects on biofilms seemed to indicate different effects of application of the lytic bacteriophages.

In the degradation assays the changes mapped by the three techniques were somewhat congruent. In the biofilm prevention assays however, determining the effects of pre-treatment of catheters on biofilm growth was complicated by the necessity of having controls and treatment catheters showing variation in initial measured values. At first the results seem both anomalous for each technique in isolation and disjunct when scrutinized in concert. However, upon closer inspection it seems that the observed effects may simply be an indication that the pre-treatment of a surface with lytic phages causes a complex phage-bacteria interaction in biofilm-producing bacteria cultivated on that surface.

The interplay between the abovementioned assay-specific subtleties may be confounding factors in elucidating the effects of phage on biofilms, but they seem to have less of an impact in the measurement of the extent of degradation of established biofilms than in the prevention of biofilms by pre-treatment.

So, focusing firstly on the ability of phages to degrade existing biofilms, the data from the absorbance and plate count assays for UEmw_8 demonstrated a decrease in cells and biofilm mass from 4 hours onward. A lag phase was seen between 2 and 4 hours where no significant effect of phage was seen. This effect was also noted in the flow cytometric dead cell count data, but not in the live cell counts. The observed lag phase is mirrored by those recorded by Curtin and Donlan for phage activity of bacteriophage *456* on *S. epidermidis* biofilms grown on catheters,(16) in that they found no decrease in biofilm cells during the first two hours of treatment with phage. Chibeu et al. however saw no such latency in their phage treatment of *E. coli* biofilms formed in microtitre plates.(17) This delayed onset of phage activity in the catheter segments could just be an effect of the time necessary for specific phage to establish infection in biofilm cells under the conditions in effect, as is suggested by Curtin and Donlan.(16) This suggestion seems to be likely given that such activity dynamics are seen in flow and drip reactors along lengths of catheters but not in the stationary conditions and small surface areas associated with microtitre plates. The fact that the lag was

not seen in the live cell counts by flow cytometric analysis but is seen in all other measurement hints at a difference in the direct and indirect methods of investigating cell viability.

The results were similar for application of vB_EcoX_P9 to established biofilms of UEmw_9 where a lag phase is detected between 2 and 4 hours. Estimation of the extent to which biofilms are able to grow during this period following phage application is different depending on the method used to measure it. Plate counts show a very slight decrease in viable cells but absorbance measurements show an increase in biomass. The lag is not seen in the flow cytometric data. Again, these results may at first glance seem discrepant but in consideration of the nature of biofilm formation, they indicate that cells at the early stages of phage infection may still be producing EPS and establishing biofilms. The greatest decreases in biofilm cells and biomass occurred between 4 and 8 hours according to absorbance measurements, plate count data, and differences in dead cells in the flow cytometric analyses. Live cells decreased most from 8 hours onwards according to the flow cytometry data, but a recovery or decrease in biofilm reduction was seen in all other measurements between 8 and 24 hours.

Overall, the effect of phage treatment on established biofilms was similar for both UEmw_8 and UEmw_9. The statistical analysis of the effect of treatment compared to no treatment for each strain showed that treatment with vB_EcoP-U8 and vB_EcoX-U9 significantly decreased the number of biofilm cells and the biomass of UEmw_8 and UEmw_9 respectively by 24 hours when measuring turbidity of samples and cell counts. Flow cytometric analysis also showed that the number of dead cells within the treated samples is significantly higher after a 24 hour period, and that live cells are diminished with respect to controls for UEmw_8 and with respect to initial values for UEmw_8 and UEmw_9. It was seen that for the most part, the live cell counts and dead cell counts from the flow cytometric analysis were congruent with the absorbance data in terms of trends following phage application.

In the flow cytometric analysis it was seen that for both UEmw_8 and UEmw_9 dead cells measured as a percentage of total cell count were higher in the control than in the treatments. This effect was also seen in the pre-treatment data from the biofilm prevention assay for UEmw_8. Initially the data seem anomalous, but in consideration of the outcome of effective lytic phage infection (i.e. cell lysis) it becomes an almost obvious and expected result. The observed phenomenon results from the fact that cells that are fully lysed are not counted as dead or live, but rather are detected and recorded as noise by the flow cytometer, triggering count events but not adding to proportion of cells gated as members of dead cell population.

Neither the percentages of decrease in biofilm levels following phage application nor the significance of the changes are equivalent for the three measurement techniques at each time interval. This result is to be expected as each method measures a different aspect of the

biofilm's viability and each has different levels of variability between replicates. As discussed above, the timeframe of detection of changes must differ for different aspects of biofilm growth parameters as well.

In assessing the ability of bacteriophages to degrade existing biofilms, two main criteria were distinguished, namely the effects of treatment overall all as compared to no treatment (control), and secondly the effects of treatment over time. Firstly, the effect of phage treatment was examined overall by comparisons between the change in live cell numbers between the control and the treatment over the complete incubation time, and by comparing only the final values (means of the measured values at the final time point; at 24 hours). Then to determine the effects of treatment for increasing durations independent of the controls, the number of live cells at 2 hours' incubation was compared to the number after 24 hours of incubation within the treatment group. For UEmw_8 the decrease in live cells between the control and treatment after 24 hours as measured by plate count was a 78.3% decrease, by absorbance measurement an 83.1% decrease, and by flow cytometric analysis a 19.7% decrease was seen.

The change in live biofilm cells in the treatment catheters from 2 to 24 hours was measured as a 67.8% reduction by plate count, 62.3% reduction by absorbance measurement and 34.2% decrease by flow cytometry.

The point of highest reduction for vB_EcoX-U9 application to UEmw_9 biofilms was at 8 hours, before a recovery of biofilm was seen, and this data provided (in brackets below) is considered to be indicative of phage activity.

For UEmw_9 the change in live cells between the control and treatment samples after 24 hours as measured by plate count was a 59.7% (95.4% after 8 hours) decrease, by absorbance measurement a 46.1% decrease, and by flow cytometric analysis a 75.3% decrease.

The change in live UEmw_9 biofilm cells in the treatment catheters from 2 to 24 hours was measured as being a 52.8% (94.0% after 8 hours) reduction by plate count, a 20.0% reduction by absorbance measurement and 43.9% decrease by flow cytometry.

These data for biofilm cell reductions are slightly below those presented by Sillankorva for the use of bacteriophage ϕS1 to reduce *P. fluourescens* by ~85%,(135) and are far lower than the figures presented by *Carson* et al. for reduction of *E. coli* biofilms.(45) As mentioned however, the amount of biofilm formed initially was higher for the treatment than it was for the control in both samples, an indication of variability of the formation of biofilms under similar conditions. The 99.99% reduction in biofilm mass reported by Carson et al. was achieved in a

microtitre plate peg device and not under the flowing conditions of catheter lumina.(45) For the catheter grown biofilms a 90% reduction in biofilm was seen in that study (by pre-treatment of catheters with phage). In that specific study however, it is noted that measurement of biofilm was by CV staining and software based analysis and thus was not presented as an outright indication of biofilm-associated cell killing capacity, but of actual reduction in EPS compared to controls.

It has thus been observed that both the isolated bacteriophages vB_EcoP-U8 and vB_EcoX-U9 are capable of killing biofilm associated host bacteria and to some extent degrading the host cell biofilms grown in catheters *in vitro*.

The graphs indicating effects of pre-treatment of catheters by vB_EcoP-U8 and vB_EcoX-U9 by absorbance and plate counts and by flow cytometric analysis indicate that the capacity for prevention of biofilms in these phages' specific hosts vary markedly.

For vB_EcoP-U8, the flow cytometry data agrees with the data obtained from the absorbance measurements, but the plate count data varies from these. Whereas in both the biomass determination and the live cell count data from the flow cytometry a decrease in biofilm is seen compared to the control, the plate count data shows an initial decrease in cells following which a recovery emerges after 8 hours. Flow cytometry and absorbance measures indicate that the biofilms began to become established on the catheter surface for the first 4 hours after having been treated with phage. Again, this latency in infection/lysis has been recorded by Curtin & Donlan.(16) Doolittle et al. found that temperature and nutrient concentration both effect time-to-lysis in *E. coli* biofilms by coliphages.(41) Profound degradation of the biofilm was then seen over the next 4 hours in the absorbance measurements mirrored by a decrease in live cells in flow cytometry. Following these decreases in biomass and cell numbers, the biofilm again began to establish itself (also recorderd by plate count data) in what can be seen as either a movement towards an equilibrium between bacteria and host, or the beginning of emergence of some phage resistant cells. Like in many similar studies in which a single phage species is used, recovery of bacterial populations following phage attack is seen after initial prevention of biofilm formation.(44,130) However, the specific activity seen between host and phage in this instance, where capacity for lysis and then a possible conversion to lysogeny under certain conditions (vB_EcoP-U8 could not be concentrated in liquid culture, see section 3.5) have already been established, is obviously a complex interplay between infection, host killing and nutrient dynamics, all of which have been discussed in detail by Abedon.(136) Consideration of these points leads to the notion that perhaps the situation is not as simple as "the bacteria were susceptible, and now are resistant" but rather, hinges on evolutionary phage mechanisms to remain viable when host numbers drop beyond a certain level. This having been said, the results show that pre-treatment of the catheter segments with vB_EcoP-U8 did result in a decrease in the levels of

biofilm formed although UEmw_8 populations recovered quickly. For plate count, absorbance data, and flow cytometric measurement of live cells, the final biofilm quantities are lower than the control values. For the flow cytometric analysis the percentages of live cells are higher than the initial recorded values, but increased less than the control live cells did. The percentages of dead cells measured in the flow cytometry assay once again (as in the degradation assays) correlates with the outcomes of the plate count data. The control dead cell counts are higher than the treatment dead cell counts again reinforcing the idea that as biofilm cells become fully lysed they no longer are counted as dead cells by the cytometer detectors.

For vB_EcoX-U9, once again (as with vB_EcoP-U8) the trend lines of the percent live cells from the flow cytometric data show a high similarity with the seemingly erratic/anomalous data seen in the absorbance measurements. Once again the plate count data does not show similarity in trend to either of these but is similar in implication to the dead cell numbers measured in the flow cytometry. Overall, between 2 and 4 hours, the level of biofilm and cells decrease. Following this, between 4 and 8 hours the live cell numbers as measured by flow cytometry, and the biomass begin to increase. Then between 8 and 24 hours, the rate of increase of cells decreases, as does the biomass. The observed overall effect of pre-treatment of catheters for the specific strain was difficult to gauge with decrease in biofilm biomass and live cells counted by flow cytometry compared to initial values. Across the full incubation compared to the untreated control the absorbance and plate count data showed higher biofilm levels than were seen in the controls. The overall changes are so varied between the techniques that comparison of the numbers are not meaningful. It is noted however that comparison of the trends of all data with their corresponding control data shows an effect of lowered capacity for biofilm formation in pre-treated catheters, but an inability to prevent biofilm growth.

Thus while both viral strains were shown to be able to slightly diminish the amount of growth of biofilms of the uropathogens over the tested incubation periods, neither was able to do so without an initial establishment of bacterial colonies/biofilms in which the phages could then propagate. In addition, the pre-treatment of the catheter surfaces with phages inevitably leads to a transient decrease in biofilms followed by recovery of the host cells within the 24 hour incubation period. Such activity may show that the phages are largely incapable of affecting biofilm formation and the transient decreases seen before biofilm recovery are an artefact of biofilm growth kinetics, or perhaps that resistance to phage induced lysis by some mechanism is at play. Such a phenomenon could be indicative of the existence of and skewed proliferation of phage resistant bacterial cells within the biofilm mass or of an interaction leading to lysogenic maintenance of the phage genomes in the biofilm associated hosts. Another more likely option is that the interaction between biofilm-forming bacterial cultures growing from their inception with infecting viral pathogens is extremely complex with

a fluctuation in levels of both the bacterial host and the bacteriophage populations. Similar conclusions have been drawn by Tait et al. who found that complete eradication of biofilms by phage was impossible with less than three distinct phages active against the same host.(137) The exact mechanism of the observed biofilm recovery is not purported to be known here and further study into the cause of this outcome is needed. The result does however lend support to the idea and practice of bacteriophage cocktail usage.

It has been shown that pre-treatment of catheters with lytic phages did not significantly reduce biofilms in either of the two UPEC strains compared to the untreated controls, after 24 hours of incubation but that pre-treatment of catheters was able to decrease the rate of increase in biofilms formed by UEmw_8 and UEmw_9.

CHAPTER 5 – CONCLUSION

5.1 Conclusion

The three objectives of the study were met. A range of various bacteria was isolated from UTI patient urine samples and all isolates were subjected to phenotypic characterization using chromogenic selective media and automated biochemical analyses. Fifteen of these strains were found to be *E. coli* isolates and were characterized as UPEC strains as described. One isolate was rejected from the study. All 14 remaining UPEC strains were tested for capacity to form biofilms. The two isolated UPEC strains that were most effective at producing biofilms and that demonstrated susceptibility to phage lysis were characterized genetically, using 16S rRNA gene sequencing and biodiversity analysis, in fulfillment of a polyphasic identification methodology recommended in the literature. These strains were designated UEmw_8 and UEmw_9. Two wastewater-derived bacteriophages, each capable of infecting one of the two UPEC strains, were isolated, purified, cultured and morphologically characterized using TEM. The two described bacteriophages were designated vB_EcoP-U8 and vB_EcoX-U9. The phages were tested for the capacity to i) degrade established UPEC biofilms and ii) prevent the formation of UPEC biofilms in urinary catheters *in vitro*. Both bacteriophages were found to be capable of degrading their respective hosts' biofilms grown in catheter lumina. Neither of bacteriophages were reliably able to decrease the amount of biofilm formed compared to controls when used to pre-treat catheters as a preventative measure against biofilm formation. In both the biofilm degradation and prevention assays it was noted that the capacity of the phages to combat biofilms would be insufficient for complete elimination of bacterial biofilms and would need to be supplemented in therapeutic applications by other phages in the form of phage cocktails, or by adjunctive antimicrobial application.

The outcomes are further valuable in that they have led to characterization and to storage of phages with the capacity to degrade biofilms formed by UPEC species in catheters, which could later be used in combination with other such phages as cocktails or combination therapies.

5.2 Proposed further research

The described bacteriophage stock cultures and their hosts should be maintained as a starting point for the creation of a bacteriophage repository for further enquiry and work towards possible therapeutic applications. Such a repository would be indispensable in investigations into the use of phage cocktails rather than single phage species/strains.

Following the description and morphological characterization of the phages achieved during this study, and the genetic characterization of vB_EcoP-U8, further attempts at full genetic

characterization of vB_EcoX-U9 should be carried out. Understanding the genetic makeup of the phages will greatly enhance the understanding of their specific interactions with host cells and biofilms. In addition, the mechanisms of the phage lytic activity against the UPEC strains, and particularly their capacity to disrupt host-produced biofilms, should further be investigated. In the very likely event that biofilm disruption is in part due to phage-encoded lysins, these proteins should be characterized and tested for capacity to disrupt biofilms without the presence of the phages themselves.

5.3 Limitations

Even though bacterial genome extraction and phage vB_EcoP-U8 DNA extraction proved successful, the viral nucleic acid extraction remained unachieved for vB_EcoX-U9 despite the exceedingly similar techniques and principles underlying both. Extraction protocols were repeated, optimised, and altered while constantly checking that the phages still maintained infectivity. After several months however, the working sample of vB_EcoX-U9 lost lytic capacity and a new high titre lysate had to be cultured from the frozen stock. Collaboration with scientists at the University of Pretoria, with a postdoctoral candidate in another department at Wits, and consultations with extraction kit company representatives all proved fruitless. Five viral genome extraction kits were used and an automated nucleic acid protocol was followed and then rerun by scientists and the NHLS, none of which yielded any appreciable amounts of viral nucleic acid. Under consideration of advice received from various members of staff, the genetic characterization of phage vB_EcoX-U9 was abandoned as part of this project. In addition, despite numerous attempts at restriction enzyme digest of the extracted vB_EcoP-U8 DNA, the procedure was always unsuccessful so preliminary sizing of extracted fragment was also unsuccessful. All that could be done was confirmation of presence of DNA by electrophoresis alongside an inappropriately sized (10 000 bp) DNA ladder (gels not shown). Further attempts were made at sizing the fragment accurately by running electrophoresis gels with a DNA ladder of appropriate size (+48 000 bp) but, both molecular weight markers that were available were degraded, and exceptionally long lead times for receiving the ladder from suppliers made it impossible within the time frame needed for completion of the project. The sizing was achieved via the NGS procedures but electrophoretic evidence would have strengthened the quality of the work.

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Additional Resources

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- The Sequence Manipulation Suite:

  Stothard P (2000) The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques 28:1102-1104

- Geneious and Geneious 16S Biodiversity Tool:

  Geneious version 10.0.9 created by Biomatters. Available from http://www.geneious.com/

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APPENDICES

Appendix A – Buffer and Media Compositions

AUM was sterile filtered in a Class II Biological Safety Cabinet (BSC), using Sterivex filter units (Millipore), as composition does not allow for sterilization by autoclaving, which induces precipitation of some constituents. The media contained per 1L:

Table A1. Composition of Artificial Urine Medium (AUM)

Reagent	Mass (grams)	Volume (litres)	Supplier
Iron (II) Sulphate	0.001		Merck (Darmstadt, Germany)
Yeast Extract Powder	0.005		Merck (Darmstadt, Germany)
Uric Acid	0.07		Sigma Life Sciences
Calcium Chloride	0.37		Merck (Darmstadt, Germany)
Citric Acid	0.40		Merck (Darmstadt, Germany)
Magnesium Sulphate Heptahydrate	0.49		Merck (Darmstadt, Germany)
Creatinine	0.80		Sigma-Aldrich
Potassium Dihydrogen Phosphate	0.95		Merck (Darmstadt, Germany)
Peptone	1.00		Merck (Darmstadt, Germany)
Di-Potassium Hydrogen Phosphate	1.20		Merck (Darmstadt, Germany)
Ammonium Chloride	1.30		Merck (Darmstadt, Germany)
Sodium Sulphate	1.42		Merck (Darmstadt, Germany)
Sodium Hydrogen Carbonate	2.10		BDH (Midrand,South Africa)
Sodium Chloride	5.20		Merck (Darmstadt, Germany)
Urea	10.0		Merck (Darmstadt, Germany)
Lactic Acid		8.26×10^{-6}	Merck (Darmstadt, Germany)
dH_2O		1.00	Millipore Filtration Device

Luria bertani (LB) broth:

10g Bacto-tryptone
5g Yeast extract
10g NaCl

Add to 800mL dH2O
Adjust pH to 7.5 with NaOH
Adjust volume to 1 L with dH2O
Sterilize by autoclaving.

Phage buffer (SM buffer):

5.8 g NaCl
1.2 g MgSo4
50 mL 1M Tris-HCl, pH 7.5
0.1 g Gelatin

Add to 800mL dH2O
Adjust volume to 1 L with dH_2O
Sterilize by autoclaving

Nutrient Broth (ATCC nutrient broth #3):

3.0g Beef Extract
5.0g Peptone
Final pH 6.8 +/- 0.2

Add to 800mL dH2O
Sterilize by autoclaving.

Phosphate buffered saline (PBS):

8.0g NaCl
0.2g KCl
1.44g Na_2HPO_4
0.24g KH_2PO_4

Add to 800mL dH_2O
Adjust pH to 7.4 with HCL
Adjust volume to 1 L with dH_2O
Sterilize by autoclaving

10 X TBE buffer:

107.69 g Tris (0.89 M)
7.44g EDTA (0.02 M)
55g Boric Acid (0.89 M)

Add 980ml DEPC dH_2O
Adjust to pH7.2 using Acetic Acid
Bring to 1L using DEPC dH_2O

0.1M Ammonium acetate solution (phage genome wash solution)

0.77g Ammonium Acetate
add 80ml dH_2O
adjust pH to 7.5
Bring to a volume

Appendix B – Results of catheter-grown biofilm screening assay

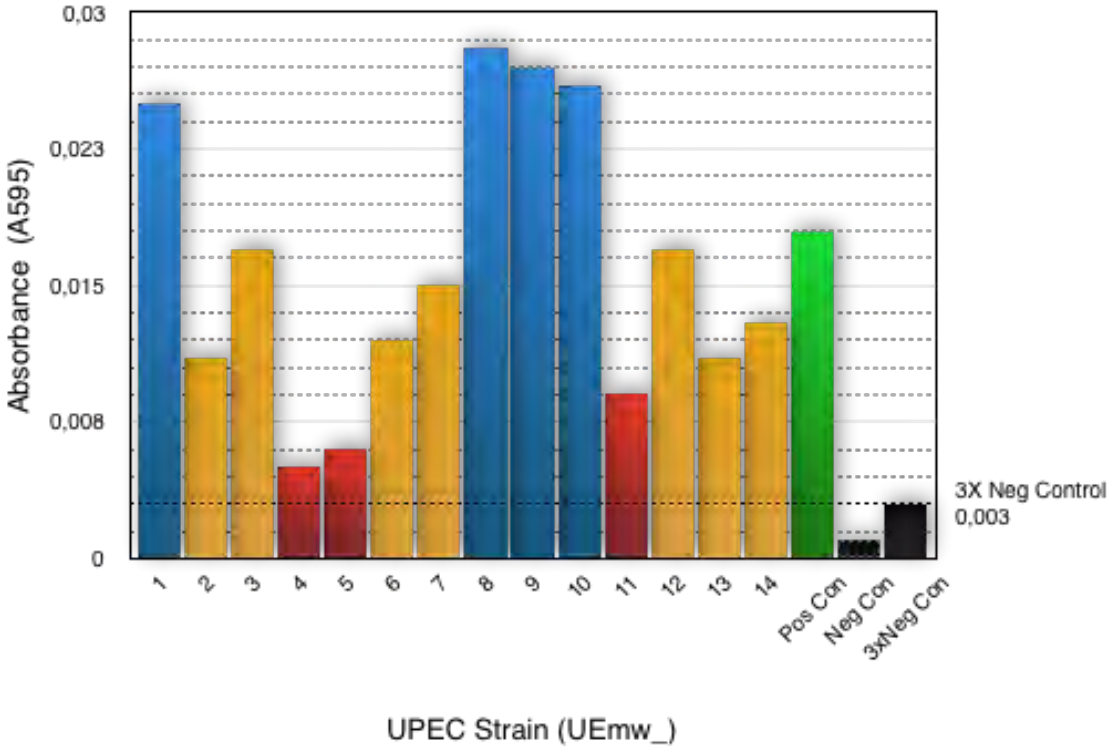


Figure B1. Graph of average absorbance of UEmw1-14 in catheter biofilm screen assay. UEmw_8 and UEmw_9 showed the highest capacity for biofilm formation on catheter segments. The relative negative control value is much lower than in the microtitre plate assay indicating a higher number of significant biofilm formers when screening on catheters rather than microtitre plates. Blue bars show high level-biofilm forming strains and red bars indicate low biofilm formers. The strains represented by orange bars have an average capacity for biofilm formation on catheters *in vitro*.

The results of the additional procedure (described in section 2.3) to check the propensity of the isolated strains to form biofilms confirmed that UEmw_8 and UEmw_9 would form significant levels of biofilms in catheter lumina as indicated in the microtitre plate biofilm assay. It indicated however that all tested strains from significant biofilms which is contrary to the result obtained when the test was run in microtitre plates.

Appendix C – Images of plaque assay plates, bacterial culture and catheter sections from degradation assays.

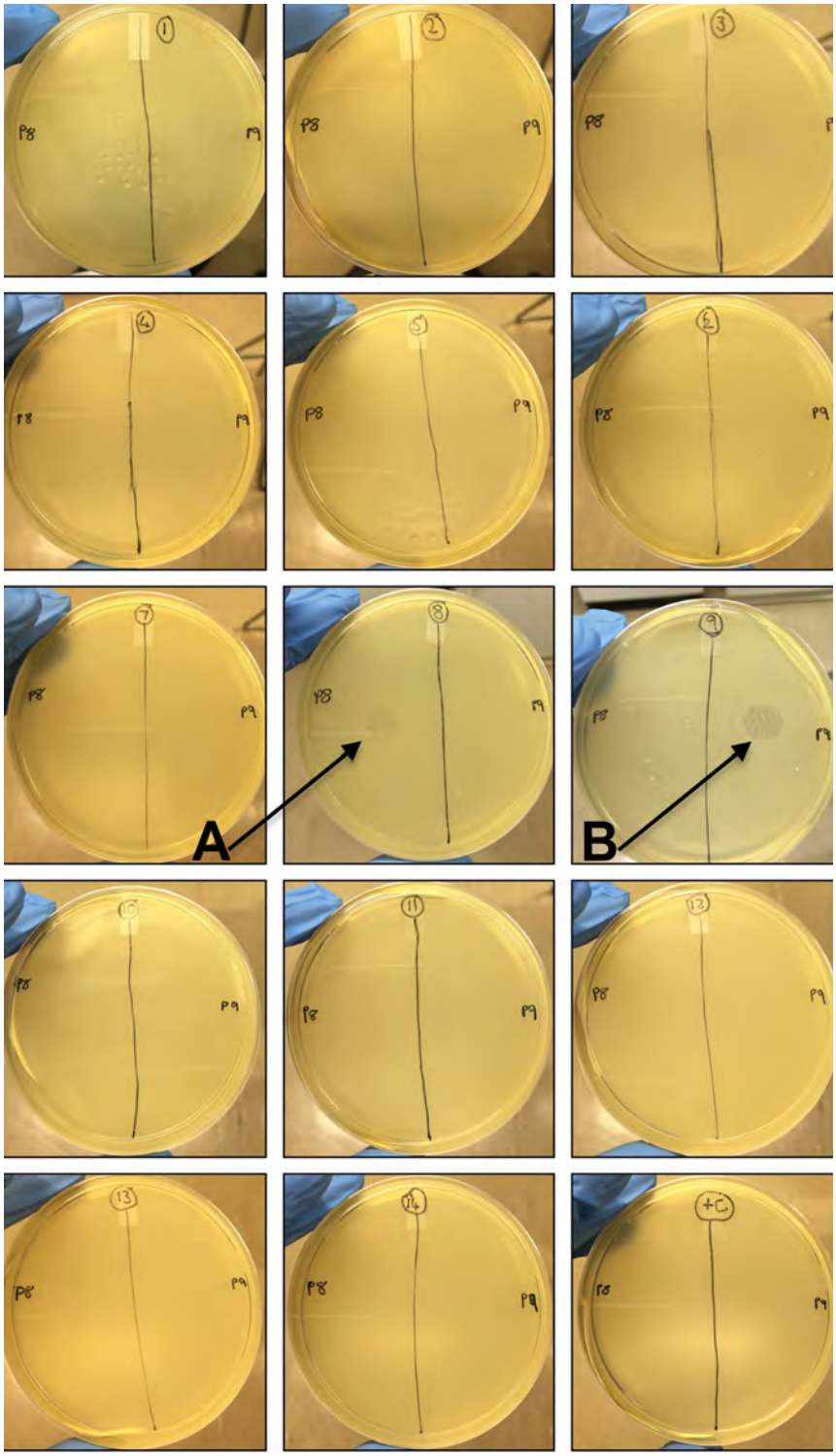


Figure C1. Phage host range determination plates.
vB_EcoP-U8 (left side of plates), and vB_EcoX-U9 (right side of plates) were inoculated onto each of the 14 isolated *E. coli* strains and control strain *E. coli CFT073*. Strains are pictured sequentially from UEmw_1 at top left (1) to UEmw14 (middle in the bottom row), and control (+C). vB_EcoP-U8 was exclusively active against UEmw_8 (Arrow A) and vB_EcoX-U9 was exclusively active against UEmw_9 (Arrow B). In all 13 of the other bacterial strains, no plaques or signs of phage activity were observed.

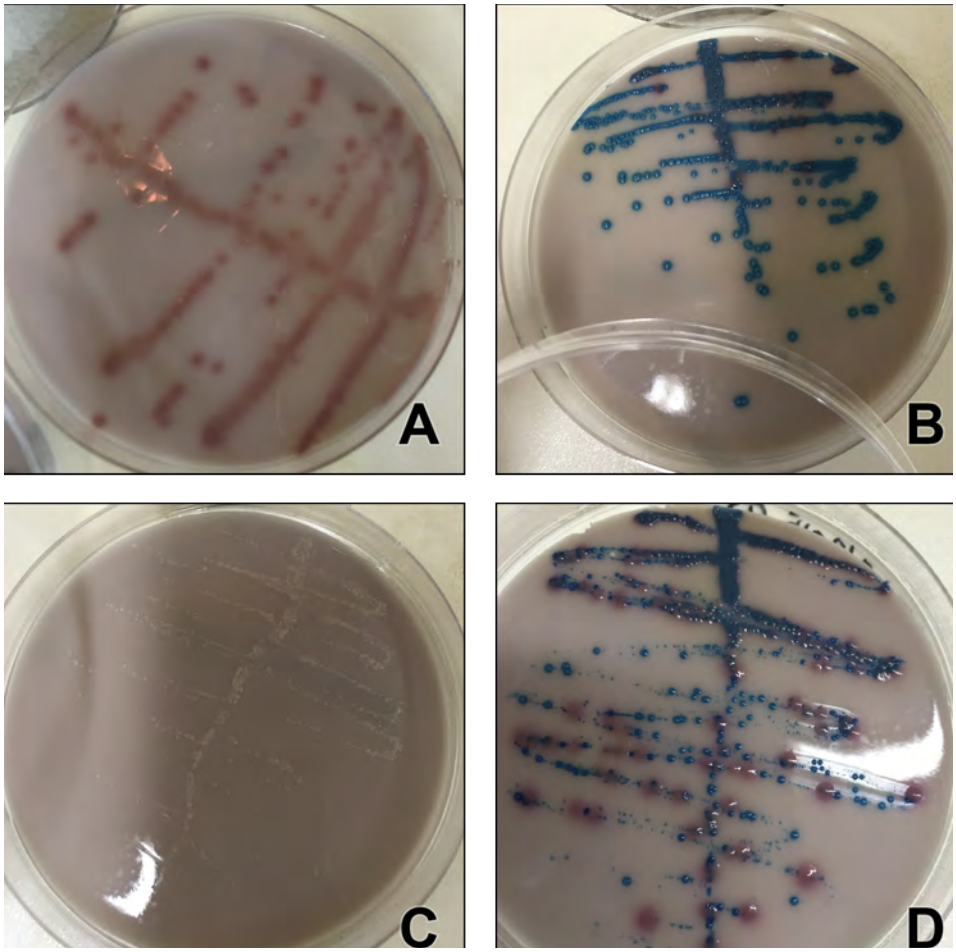


Figure C2. Bacterial colonies growing on some of the Uriselect 4 chromogenic medium plates for preliminary bacterial screening.
The initial urine streaking procedure revealed various bacterial populations in the urines including multi-species and single-species cultures. *E.coli* cells grow as pink colonies on the chromogenic medium plates. Image A (top left) - pure *E. coli* culture; Image B (top right) - *C. Koseri* and *E. coli* multi-species culture; image C (bottom left) – *S. epidermidis* pure culture; Image D (bottom right) – *E. coli* and *E. faecium* multi-species culture.

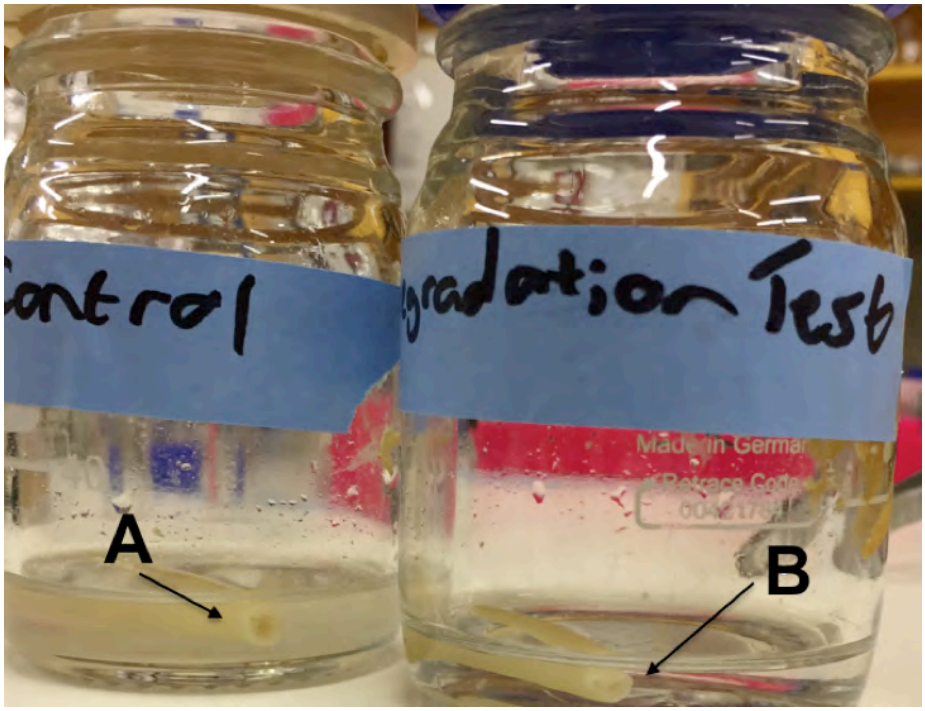


Figure C3. UEmw_8 degradation assay catheter pieces' treatment vs. control after 24 hours.
Control (A) and bacteriophage treated catheter segment (B) during the degradation assay. The treatment catheter segment for UEmw_8 is seen on the right, having been treated with bacteriophage vB_EcoP-U8. The clear liquid containing the phage indicates no bacterial growth. The untreated control on the left, treated only with AUM, has turbid liquid media full of bacterial.

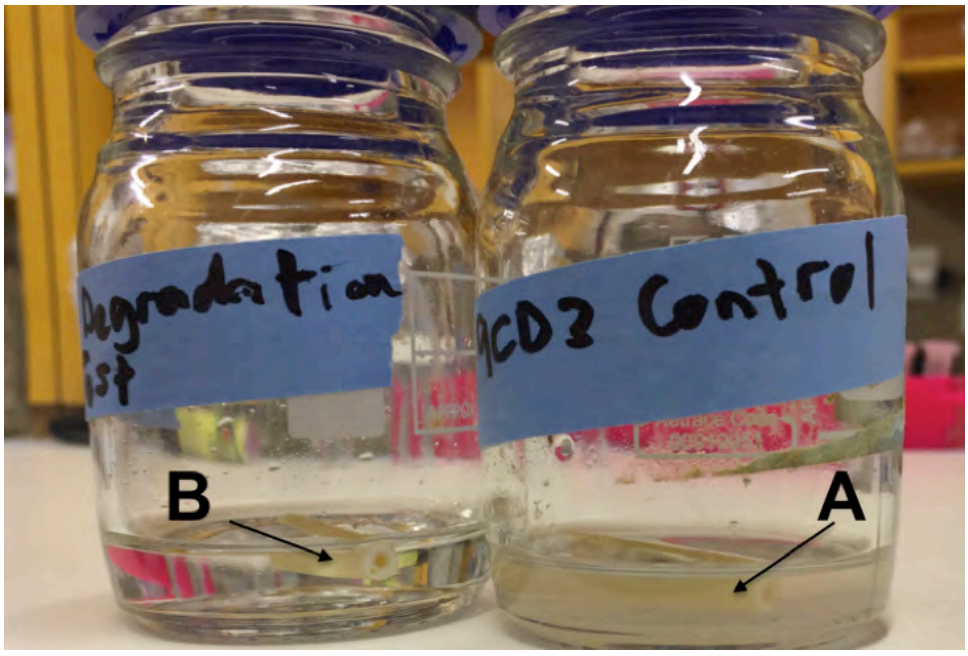


Figure C4. UEmw_9 degradation assay catheter pieces' treatment vs. control after 24 hours.
Control (A) and bacteriophage treated catheter segment (B) during the UEmw_9 degradation assay. The treatment catheter segment for UEmw_9 is seen on the left, having been treated with bacteriophage vB_EcoX-U9. The clear liquid containing the phage indicates no bacterial growth. The untreated control on the left, treated only with AUM, has turbid liquid media full of bacteria.

Appendix D – pGEM 3Zf (+) Plasmid consensus aligned to published sequence

```
>X65307 - realigned Alignment of 2 sequences: PLASMID.ab1 - bases 19 - 968, X65
307

Score = 4732.0, Identities = 946/950 (99%),
Positives = 950/950 (100%), Gaps = 0/950 (0%)

PLASMID.ab1 - bases 19 - 968   19 TCGAGCTCGGTACCCGGGGATCCTCTAGAGYCGACCTGCAGGCATGCAAGCTTGAGTATT  78
                                  TCGAGCTCGGTACCCGGGGATCCTCTAGAG+CGACCTGCAGGCATGCAAGCTTGAGTATT
X65307                          9 TCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGAGTATT  68

PLASMID.ab1 - bases 19 - 968   79 CWATAGTGTCAGCTAAATAGCTTGGCGTAATCATGKYCATAGCTGTTTCCTGTGTGAAAT 138
                                  C+ATAGTGTCAGCTAAATAGCTTGGCGTAATCATG++CATAGCTGTTTCCTGTGTGAAAT
X65307                         69 CTATAGTGTCAGCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAAT 128

PLASMID.ab1 - bases 19 - 968  139 TGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGG 198
                                  TGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGG
X65307                        129 TGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGG 188

PLASMID.ab1 - bases 19 - 968  199 GGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAG 258
                                  GGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAG
X65307                        189 GGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAG 248

PLASMID.ab1 - bases 19 - 968  259 TCGCGAAACTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT 318
                                  TCGCGAAACTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT
X65307                        249 TCGCGAAACTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT 308

PLASMID.ab1 - bases 19 - 968  319 TTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGG 378
                                  TTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGG
X65307                        309 TTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGG 368

PLASMID.ab1 - bases 19 - 968  379 CTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGG 438
                                  CTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGG
X65307                        369 CTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGG 428

PLASMID.ab1 - bases 19 - 968  439 GATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAG 498
```

```
X65307                          429 GATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAG 488

PLASMID.ab1 - bases 19 - 968    499 GCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGA 558
                                    GCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGA
X65307                          489 GCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGA 548

PLASMID.ab1 - bases 19 - 968    559 CGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCT 618
                                    CGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCT
X65307                          549 CGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCT 608

PLASMID.ab1 - bases 19 - 968    619 GGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCC 678
                                    GGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCC
X65307                          609 GGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCC 668

PLASMID.ab1 - bases 19 - 968    679 TTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCG 738
                                    TTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCG
X65307                          669 TTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCG 728

PLASMID.ab1 - bases 19 - 968    739 GTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGC 798
                                    GTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGC
X65307                          729 GTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGC 788

PLASMID.ab1 - bases 19 - 968    799 TGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCA 858
                                    TGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCA
X65307                          789 TGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCA 848

PLASMID.ab1 - bases 19 - 968    859 CTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG 918
                                    CTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG
X65307                          849 CTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG 908

PLASMID.ab1 - bases 19 - 968    919 TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGG 968
                                    TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGG
X65307                          909 TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGG 958
```

Appendix E – Microscan bacterial ID analysis results

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 8579

Patient Name:	, MARK		
Patient ID:	1	Collect Date:	7/1/2016
Specimen/Isolate:	M1-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:07:52 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	8
Panel Status:	In WalkAway, Complete		

Exceptions:

Data obtained directly from the WalkAway

Biotype: 520260700365474

Organism Identification:

	Organism	% Probability	Footnotes	Special Characteristics
	E. coli	95.56		
2	Cit bra/freu/sed	4.44		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL	+	BGL		BMAN		AGLT		GGLT	+	PYG		PHO1	+	**DCB**		ORN		ADN		GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		BGLR	+	BXYL		**ARG**	+	GLT2	+	TYR		PH1A	+	DCB2		ONB		ADN2	+	GLU	+	MAN2		RAF		**SUC**	+	URE	
BGAL	+	CHB	-	NAG	-	GGA	-	PRO	-	TYR2		PHO2	+	LYS	-	ABT	-	ARA	+	INO		MEL	+	SAL		IND	+	INI	

Data stored in the LabPro Database

Biotype: 520260700365474

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL	+	BGL		BMAN		AGLT		GGLT	+	PYG		PHO1	+	**DCB**		ORN		ADN		GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		BGLR	+	BXYL		**ARG**	+	GLT2	+	TYR		PH1A	+	DCB2		ONB		ADN2	+	GLU	+	MAN2		RAF		**SUC**	+	URE	
BGAL	+	CHB	-	NAG		GGA		PRO		TYR2	-	PHO2	+	LYS	-	ABT		ARA	+	INO		MEL	+	SAL		IND	+	INI	

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 8579

Patient Name:	, MARK		
Patient ID:	2	Collect Date:	7/1/2016
Specimen/Isolate:	M2-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:08:29 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	9
Panel Status:	In WalkAway, Complete		

Exceptions:

Data obtained directly from the WalkAway

Biotype: 520240556365454

Organism Identification:

Organism	Probability	Footnotes	Special Characteristics
E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL	+	BGL	BMAN	AGLT	GGLT	+	PYG	PHO1	+	DCB	+	ORN	+	ADN	GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		BGLR	+ BXYL	ARG	+	GLT2	TYR	PH1A		**DCB2**		ONB	+	ADN2	+ GLU	+	MAN2		RAF		SUC		URE	
BGAL	+	CHB	NAG	- GGA	-	PRO	- TYR2	PHO2	+	LYS	+	ABT	-	ARA	+ INO		MEL	+	SAL		IND	+	INI	

Data stored in the LabPro Database

Biotype: 520240556365454

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL	+	BGL	BMAN	AGLT	GGLT	+	PYG	PHO1	+	DCB	+	ORN	+	ADN	GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		BGLR	+ BXYL	ARG	+	GLT2	TYR	PH1A		**DCB2**		ONB	+	ADN2	+ GLU	+	MAN2		RAF		SUC		URE	
BGAL	+	CHB	- NAG	- GGA	-	PRO	- TYR2	- PHO2	+	LYS	+	ABT	-	ARA	+ INO		MEL	+	SAL		IND	+	INI	

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 857

Patient Name:	, MARK		
Patient ID:	3	Collect Date:	7/1/2016
Specimen/Isolate:	M3-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:08:50 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	10
Panel Status:	In WalkAway, Complete		

Exceptions:

Data obtained directly from the WalkAway

Biotype: 500040572365454

Organism Identification:

	Organism	% Probability	Footnotes	Special Characteristics
1	E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL +	BGL	BMAN	AGLT	GGLT +	PYG	PHO1 +	DCB	+ ORN	ADN	GLR	+ MAN	+ MNS	+ SOR	+ ARA2 +
AGL	**BGLR**	BXYL	ARG	GLT2	TYR	PH1A	DCB2	+ ONB	+ ADN2	+ GLU	+ MAN2	RAF	SUC	URE
BGAL +	CHB -	NAG -	GGA -	PRO -	TYR2 -	PHO2 +	LYS	+ ABT	- ARA	+ INO	MEL	+ SAL	IND	+ INI

Data stored in the LabPro Database

Biotype: 500040572365454

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL +	BGL	BMAN	AGLT	GGLT +	PYG	PHO1 +	DCB	+ ORN	ADN	GLR	+ MAN	+ MNS	+ SOR	+ ARA2 +
AGL	**BGLR** -	BXYL	ARG	GLT2	TYR	PH1A	DCB2	+ ONB	+ ADN2	+ GLU	+ MAN2	RAF	SUC	URE
BGAL +	CHB -	NAG -	GGA -	PRO -	TYR2 -	PHO2 +	LYS	+ ABT	ARA	+ INO	MEL	+ SAL	IND	+ INI

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 8579

Patient Name:	, MARK		
Patient ID:	4	Collect Date:	7/1/2016
Specimen/Isolate:	M4-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:09:44 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	11
Panel Status:	In WalkAway, Complete		
Exceptions:			

Data obtained directly from the WalkAway

Biotype: 120060556264470

Organism Identification:

	Organism	% Probability	Footnotes	Special Characteristics
1	E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL	BGL	BMAN	AGLT	GGLT +	PYG	PHO1 +	DCB	+ ORN	+ ADN	GLR	+ MAN	+ MNS	+ SOR	+ ARA2
AGL	BGLR +	BXYL	ARG	GLT2 +	TYR	PH1A	**DCB2**	ONB	+ ADN2	+ GLU	+ MAN2	RAF	SUC	+ URE
BGAL +	CHB	NAG	GGA	PRO	TYR2	PHO2 +	LYS	+ ABT	ARA	INO	MEL	SAL	IND	+ INI

Data stored in the LabPro Database

Biotype: 120060556264470

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL	BGL	BMAN	AGLT	GGLT +	PYG	PHO1 +	DCB	+ ORN	+ ADN	GLR	+ MAN	+ MNS	+ SOR	+ ARA2
AGL	BGLR +	BXYL	ARG	GLT2 +	TYR	PH1A	**DCB2**	ONB	+ ADN2	+ GLU	+ MAN2	RAF	SUC	+ URE
BGAL +	CHB -	NAG -	GGA -	PRO -	TYR2	PHO2 +	LYS	+ ABT	ARA	INO	MEL	SAL	IND	+ INI

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 8579

Patient Name:	, mark		
Patient ID:	5	Collect Date:	7/1/2016
Specimen/Isolate:	M5-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:10:37 PM
Ward of Isolation:		Next Read	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	12
Panel Status:	In WalkAway, Complete		
Exceptions:			

Data obtained directly from the WalkAway

Biotype: 500241572365454

Organism Identification:

Organism	% Probability	Footnotes	Special Characteristics
E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL	+	BGL	BMAN	AGLT		GGLT	+	PYG	PHO1	+	DCB	+	ORN	ADN	GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		**BGLR**	BXYL	ARG	+	GLT2		TYR	PH1A		DCB2	+	ONB	+ ADN2	+ GLU	+	MAN2		RAF		SUC		URE	
BGAL	+	CHB	- NAG	- GGA	-	PRO	-	**TYR2**	+ PHO2	+	LYS	+	ABT	- ARA	+ INO	-	MEL	+	SAL		IND	+	INI	

Data stored in the LabPro Database

Biotype: 500241572365454

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL	+	BGL	BMAN	AGLT		GGLT	+	PYG	PHO1	+	DCB	+	ORN	ADN	GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		**BGLR**	BXYL	ARG	+	GLT2		TYR	PH1A		DCB2	+	ONB	+ ADN2	+ GLU	+	MAN2		RAF		SUC		URE	
BGAL	+	CHB	- NAG	- GGA	-	PRO	-	**TYR2**	+ PHO2	+	LYS	+	ABT	ARA	+ INO		MEL	+	SAL		IND	+	INI	

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 8579

Patient Name:	, MARK		
Patient ID:	6	Collect Date:	7/1/2016
Specimen/Isolate:	M6-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:11:02 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	5
WalkAway:	WalkAway 965925	Slot:	
Panel Status:	In WalkAway, Complete		
Exceptions:			

Data obtained directly from the WalkAway

Biotype: 500040576365474

Organism Identification:

	Organism	% Probability	Footnotes	Special Characteristics
1	E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL +	BGL	BMAN	AGLT	GGLT +	PYG	PHO1 +	DCB	+ ORN	+ ADN	GLR	+ MAN	+ MNS	+ SOR	+ ARA2 +		
AGL	**BGLR** -	BXYL	ARG	GLT2	TYR	PH1A	DCB2	+ ONB	- ADN2	+ GLU	+ MAN2	RAF	**SUC**	+ URE		
BGAL +	CHB -	NAG -	GGA -	PRO	TYR2 -	PHO2 +	LYS	+ ABT	ARA	+ INO	- MEL	+ SAL	IND	+ INI		

Data stored in the LabPro Database

Biotype: 500040576365474
Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL +	BGL	BMAN	AGLT	GGLT +	PYG	PHO1 +	DCB	+ ORN	+ ADN	GLR	+ MAN	+ MNS	+ SOR	+ ARA2 +		
AGL	**BGLR** -	BXYL	ARG	GLT2	TYR	PH1A	DCB2	+ ONB	- ADN2	+ GLU	+ MAN2	RAF	**SUC**	+ URE		
BGAL +	CHB -	NAG	GGA	PRO	TYR2	PHO2 +	LYS	+ ABT	ARA	+ INO	MEL	+ SAL	IND	+ INI		

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 8579

Patient Name:	MARK		
Patient ID:	7	Collect Date	7/1/2016
Specimen/Isolate:	M7-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:11:57 PM
Ward of Isolation:		Next Read	
Panel Type:	Rapid Neg ID 3	Tower:	5
WalkAway:	WalkAway 965925	Slot:	2
Panel Status:	In WalkAway, Complete		
Exceptions:			

Data obtained directly from the WalkAway

Biotype: 520060576364454

Organism Identification:

Organism	% Probability	Footnotes	Special Characteristics
E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL	+	BGL		BMAN		AGLT		GGLT	+	PYG		PHO1	+	DCB	+	ORN	+	ADN		GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		BGLR	+	BXYL		ARG		GLT2	+	TYR		PH1A		DCB2	+	ONB	+	ADN2	+	GLU	+	MAN2		RAF		SUC		URE	
BGAL	+	CHB	-	NAG	-	GGA	-	PRO	-	TYR2	-	PHO2	+	LYS	+	ABT		ARA	+	INO	-	MEL		SAL		IND	+	INI	

Data stored in the LabPro Database

Biotype: 520060576364454

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL	+	BGL		BMAN		AGLT		GGLT	+	PYG		PHO1	+	DCB	+	ORN	+	ADN		GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		BGLR	+	BXYL		ARG		GLT2	+	TYR		PH1A		DCB2	+	ONB	+	ADN2	+	GLU	+	MAN2		RAF		SUC		URE	
BGAL	+	CHB	-	NAG	-	GGA	-	PRO	-	TYR2	-	PHO2	+	LYS	+	ABT		ARA	+	INO	-	MEL		SAL		IND	+	INI	

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 8579

Patient Name:	MARK		
Patient ID:	8	Collect Date:	7/1/2016
Specimen/Isolate:	M8-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:07:31 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	7
Panel Status:	In WalkAway, Complete		
Exceptions:			

Data obtained directly from the WalkAway

Biotype: 120060576364474

Organism Identification:

Organism	% Probability	Footnotes	Special Characteristics
E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL	BGL	BMAN	AGLT	GGLT +	PYG	PHO1 +	DCB	+ ORN	+ ADN	GLR	+ MAN	+ MNS	+ SOR	+ ARA2	+	
AGL	BGLR +	BXYL	ARG	GLT2 +	TYR	PH1A	DCB2	+ ONB	+ ADN2	+ GLU	+ MAN2	RAF	SUC	+ URE		
BGAL +	CHB -	NAG -	GGA -	PRO -	TYR2 -	PHO2 +	LYS	+ ABT	ARA	+ INO	MEL	SAL	IND	+ INI		

Data stored in the LabPro Database

Biotype: 120060576364474

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL	BGL	BMAN	AGLT	GGLT +	PYG	PHO1 +	DCB	+ ORN	+ ADN	GLR	+ MAN	+ MNS	+ SOR	+ ARA2	+	
AGL	BGLR +	BXYL	ARG	GLT2 +	TYR	PH1A	DCB2	+ ONB	+ ADN2	+ GLU	+ MAN2	RAF	SUC	+ URE		
BGAL +	CHB -	NAG -	GGA -	PRO -	TYR2	PHO2 +	LYS	+ ABT	ARA	+ INO	MEL	SAL	IND	+ INI		

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 857

Patient Name:	, mark		
Patient ID:	9	Collect Date:	7/1/2016
Specimen/Isolate:	M9-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:07:06 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	6
Panel Status:	In WalkAway, Complete		
Exceptions:			

Data obtained directly from the WalkAway

Biotype: 520040156365454

Organism Identification:

Organism	% Probability	Footnotes	Special Characteristics
E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL +	BGL	BMAN	AGLT	**GGLT** +	PYG	PHO1	DCB	+ ORN	+ ADN	GLR	+ MAN	+ MNS	+ SOR	- ARA2	+
AGL	BGLR +	BXYL	ARG	GLT2	TYR	PH1A	**DCB2**	ONB	+ ADN2	+ GLU	+ MAN2	RAF	SUC	URE	
BGAL +	CHB -	NAG -	GGA -	PRO -	TYR2 -	PHO2 +	LYS	+ ABT	- ARA	+ INO	MEL	+ SAL	IND	+ INI	

Data stored in the LabPro Database

Biotype: 520040156365454

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL +	BGL	BMAN	AGLT	**GGLT** +	PYG	PHO1	DCB	+ ORN	+ ADN	GLR	+ MAN	+ MNS	+ SOR	- ARA2	+
AGL	BGLR +	BXYL	ARG	GLT2	TYR	PH1A	**DCB2**	ONB	+ ADN2	+ GLU	+ MAN2	RAF	SUC	URE	
BGAL +	CHB -	NAG -	GGA -	PRO -	TYR2 -	PHO2 +	LYS	+ ABT	- ARA	+ INO	MEL	+ SAL	IND	+ INI	

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 857

Patient Name:	mark		
Patient ID:	10	Collect Date:	7/1/2016
Specimen/Isolate:	M10-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:06:45 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	5

Panel Status: In WalkAway, Complete

Exceptions:

Data obtained directly from the WalkAway

Biotype: 520240576365670

Organism Identification:

Organism	% Probability	Footnotes	Special Characteristics
E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL	+	BGL		BMAN		AGLT		**GGLT**	+	PYG		PHO1	+	DCB	+	ORN	+	ADN		GLR	+	MAN	+	MNS	+	SOR	+	**ARA2**
AGL		BGLR	+	BXYL		ARG	+	GLT2		TYR		PH1A		DCB2	+	ONB	+	ADN2	+	GLU	+	MAN2		RAF	+	SUC	+	URE
BGAL	+	CHB	-	NAG	-	GGA	-	PRO	-	TYR2	-	PHO2	+	LYS	+	ABT	-	ARA	+	INO	-	MEL	+	SAL		IND	+	INI

Data stored in the LabPro Database

Biotype: 520240576365670

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL	+	BGL		BMAN		AGLT		**GGLT**	+	PYG		PHO1	+	DCB	+	ORN	+	ADN		GLR	+	MAN	+	MNS	+	SOR	+	**ARA2**
AGL		BGLR	+	BXYL	-	ARG	+	GLT2		TYR		PH1A		DCB2	+	ONB	+	ADN2	+	GLU	+	MAN2		RAF	+	SUC	+	URE
BGAL	+	CHB		NAG	-	GGA	-	PRO	-	TYR2	-	PHO2	+	LYS	+	ABT		ARA	+	INO		MEL	+	SAL		IND	+	INI

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 857

Patient Name:	, MARK		
Patient ID:	11	Collect Date:	7/1/2016
Specimen/Isolate:	M11-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:05:58 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	4
Panel Status:	In WalkAway, Complete		
Exceptions:			

Data obtained directly from the WalkAway

Biotype: 120240572365454

Organism Identification:

Organism	% Probability	Footnotes	Special Characteristics
E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL	BGL	BMAN	AGLT	GGLT +	PYG	PHO1 +	DCB +	ORN	ADN	GLR +	MAN +	MNS +	SOR +	ARA2 +		
AGL	BGLR +	BXYL	ARG +	GLT2	TYR	PH1A	DCB2 +	ONB +	ADN2 +	GLU +	MAN2	RAF	SUC	URE		
BGAL +	CHB -	NAG -	GGA -	PRO	TYR2 -	PHO2 +	LYS +	ABT -	ARA +	INO	MEL +	SAL	IND +	INI		

Data stored in the LabPro Database

Biotype: 120240572365454
Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL	BGL	BMAN -	AGLT	GGLT +	PYG	PHO1 +	DCB +	ORN	ADN	GLR +	MAN +	MNS +	SOR +	ARA2 +		
AGL	BGLR +	BXYL	ARG +	GLT2	TYR	PH1A	DCB2 +	ONB +	ADN2 +	GLU +	MAN2	RAF	SUC	URE		
BGAL +	CHB	NAG -	GGA -	PRO -	TYR2 -	PHO2 +	LYS +	ABT -	ARA +	INO	MEL +	SAL	IND +	INI		

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 857

Patient Name:	, mark		
Patient ID:	12	Collect Date:	7/1/2016
Specimen/Isolate:	M12-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:04:54 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	3

Panel Status: In WalkAway, Complete

Exceptions:

Data obtained directly from the WalkAway

Biotype: 520240572365454

Organism Identification:

Organism	% Probability	Footnotes	Special Characteristics
E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL	+	BGL		BMAN		AGLT		GGLT	+	PYG		PHO1	+	DCB	+	ORN		ADN		GLR	+	MAN	+	MNS	-	SOR	+	ARA2	+
AGL		BGLR	+	BXYL		ARG	+	GLT2		TYR		PH1A		DCB2	+	ONB	+	ADN2	+	GLU	+	MAN2		RAF		SUC		URE	
BGAL	+	CHB	-	NAG	-	GGA	-	PRO	-	TYR2	-	PHO2	+	LYS	+	ABT		ARA	+	INO	-	MEL	+	SAL		IND	+	INI	

Data stored in the LabPro Database

Biotype: 520240572365454

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL	+	BGL		BMAN		AGLT		GGLT	+	PYG		PHO1	+	DCB	+	ORN		ADN		GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		BGLR	+	BXYL		ARG	+	GLT2		TYR		PH1A		DCB2	+	ONB	+	ADN2	+	GLU	+	MAN2		RAF		SUC		URE	
BGAL	+	CHB	-	NAG	-	GGA	-	PRO	-	TYR2	-	PHO2	+	LYS	+	ABT	-	ARA	+	INO		MEL	+	SAL		IND	+	INI	

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 8579

Patient Name:	mark		
Patient ID:	13	Collect Date:	7/1/2016
Specimen/Isolate:	M13-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:04:32 PM
Ward of Isolation:		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	2

Panel Status: In WalkAway, Complete

Exceptions:

Data obtained directly from the WalkAway

Biotype: 120240572365454

Organism Identification:

	Organism	% Probability	Footnotes	Special Characteristics
1	E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL		BGL		BMAN		AGLT		GGLT	+	PYG		PHO1	+	DCB	+	ORN		ADN		GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		BGLR	+	BXYL		ARG	+	GLT2		TYR		PH1A		DCB2	+	ONB	+	ADN2	+	GLU	+	MAN2		RAF		SUC		URE	
BGAL	+	CHB	-	NAG		GGA		PRO	-	TYR2	-	PHO2	+	LYS	+	ABT	-	ARA	+	INO	-	MEL	+	SAL		IND	+	INI	

Data stored in the LabPro Database

Biotype: 120240572365454

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL		BGL		BMAN		AGLT		GGLT	+	PYG		PHO1	+	DCB	+	ORN		ADN		GLR	+	MAN	+	MNS	+	SOR	+	ARA2	+
AGL		BGLR	+	BXYL		ARG	+	GLT2		TYR		PH1A		DCB2	+	ONB	+	ADN2	+	GLU	+	MAN2		RAF		SUC		URE	
BGAL	+	CHB	-	NAG	-	GGA	-	PRO		TYR2	-	PHO2	+	LYS	+	ABT	-	ARA	+	INO	-	MEL	+	SAL		IND	+	INI	

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 8579

Patient Name:	UPEC 14, MARK		
Patient ID:	MARJK	Collect Date:	8/27/2015
Specimen/Isolate:	UPEC 14-LF	Test Date:	8/28/2015
Source:		Last Read:	8/28/2015 10:40:26 AM
Ward of Isolation:		Next Read:	
Panel Type:	Neg Breakpoint Combo 43	Tower:	1
WalkAway:	WalkAway 965925	Slot:	8
Panel Status:	In WalkAway, Complete		
Exceptions:			

Data obtained directly from the WalkAway

Biotype: 53114000

Organism Identification:

Organism	% Probability	Footnotes	Special Characteristics
E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

GLU	+	RAF		INO		URE		LYS	+	TDA		CIT		CL4		ACE		K4		P4	+			
SUC	-	RHA	+	ADO	-	H2S	-	ARG	-	ESC	-	MAL	-	CF8	-	CET	-	NIT	+	TAR				
SOR	+	ARA	+	MEL	+	IND	+	ORN	-	VP	-	ONPG	-	OXI	-	FD64	-	OF/G	+	TO4				

MIC Results: (Antimicrobics marked with "Ø" are suppressed from Long and Short Format Patient Reports)

AM	AUG	AZT	P/T	CFZ	CFX	CRM	CFT	CAZ	CPE	ETP	IMP	MER	GM	AK
>16	<=8/4	<=8	<=16	<=8	<=8	<=4	<=1	<=1	<=8	<=1	<=4	<=1	<=4	<=16

TO	T/S	CP	LVX	TE	TGC	FOS	FD	CFT/CA	CAZ/CA	NXN	PI
<=4	>2/38	<=1	<=2	>8	<=1	<=32	<=32	<=0.5	<=0.25	<=4	>64

Extra Tests: ESBL -

Data stored in the LabPro Database

Biotype: 53114000

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

GLU	+	RAF		INO		URE		LYS	+	TDA		CIT		CL4		ACE		K4		P4	+			
SUC	-	RHA	+	ADO	-	H2S	-	ARG	-	ESC	-	MAL	-	CF8	-	CET	-	NIT	+	TAR				
SOR	+	ARA	+	MEL	+	IND	+	ORN	-	VP	-	ONPG	-	OXI	-	FD64	-	OF/G	+	TO4				

MIC Results: (Antimicrobics marked with "Ø" are suppressed from Long and Short Format Patient Reports)

AM	AUG	AZT	P/T	CFZ	CFX	CRM	CFT	CAZ	CPE	ETP	IMP	MER	GM	AK
>16	<=8/4	<=8	<=16	<=8	<=8	<=4	<=1	<=1	<=8	<=1	<=4	<=1	<=4	<=16
R	S	N/R	S	S	S	S	S	S	S	S*	S*	S*	S	S

TO	T/S	CP	LVX	TE	TGC	FOS	FD	CFT/CA	CAZ/CA	NXN	PI
<=4	>2/38	<=1	<=2	>8	<=1	<=32	<=32	<=0.5	<=0.25	<=4	>64
S	R	S	S	R	S	S					R

Extra Tests: ESBL -

Comprehensive Panel Status Report

Wits Medical School - Infection Control NHLS

Microbiology Department 011 489 8579

Patient Name:	, MARK		
Patient ID:	+C	Collect Date:	7/1/2016
Specimen/Isolate:	+C-GN	Test Date:	7/1/2016
Source:		Last Read:	7/1/2016 3:04:11 PM
Ward of Isolation		Next Read:	
Panel Type:	Rapid Neg ID 3	Tower:	4
WalkAway:	WalkAway 965925	Slot:	
Panel Status:	In WalkAway, Complete		
Exceptions:			

Data obtained directly from the WalkAway

Biotype: 500200576365454

Organism Identification:

	Organism	% Probability	Footnotes	Special Characteristics
1	E. coli	99.99		

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the first choice organism)

AGAL +	BGL	BMAN	AGLT	GGLT	PYG	PHO1 +	DCB +	ORN +	ADN	GLR +	MAN +	MNS +	SOR +	ARA2 +
AGL	**BGLR**	BXYL	ARG +	GLT2	TYR	PH1A	DCB2 +	ONB +	ADN2 +	GLU +	MAN2	RAF	SUC	URE
BGAL +	CHB	NAG	GGA -	PRO	TYR2	PHO2 +	LYS +	ABT	ARA +	INO	MEL +	SAL	IND +	INI

Data stored in the LabPro Database

Biotype: 500200576365454

Organism: E. coli

Biochemical Results: (Biochemicals that are bolded and underlined are atypical for the stored organism)

AGAL +	BGL	BMAN	AGLT	GGLT	PYG	PHO1 +	DCB +	ORN +	ADN	GLR +	MAN +	MNS +	SOR +	ARA2 +
AGL	**BGLR**	BXYL	ARG +	GLT2	TYR	PH1A	DCB2 +	ONB +	ADN2 +	GLU +	MAN2	RAF	SUC	URE
BGAL +	CHB	NAG	GGA	PRO	TYR2	PHO2 +	LYS +	ABT	ARA +	INO	MEL +	SAL	IND +	INI

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Tobias Health Sciences Building 2nd floor. Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-141008-1 08/10/2014

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Marc-Michel Wilson. (Student number 500914).

Project title: Comparison of treatment of uropathogenic bacterial species with bacteriophages and their lysins in both forming and established biofilms.

Reason: This is a laboratory study of biofilms formed *in vitro* on urinary catheters. Bacteriophages to be used on the bacteria in the biofilms will be isolated from sewage. No humans are participants in the study.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON - JONES
HREC (MEDICAL)
2014 -10- 03
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat : Anisa Keshav, Zanele Ndlovu.