Experimental Studies on the Microbiota associated with Urinary Tract Infections

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Abstract

Urinary tract infections (UTIs) are one of the most common healthcare associated infections (HCAIs) accounting for 17.2% of the total HCAI's in England. Some of the underlying issues associated with UTIs include recurrent infections, catheter associated UTIs and antibiotic resistance. These issues are responsible for prolonged hospital admissions, increased costs and significant morbidity. Another possible issue relates to the ubiquitous protozoa, *Acanthamoeba*. Although it is known to cause infections in humans, the amoeba has been isolated from apparently healthy people. Furthermore, *Acanthamoeba* is known to have an endosymbiotic relationship with bacteria. Therefore, it is reasonable to hypothesise that *Acanthamoeba* may possibly play an important role in UTIs.

Clinical isolates of *E. coli*, *K. pneumoniae* and *P. mirabilis* were used in the current study. All uropathogens exhibited the ability to form biofilms in a nutrient dependent manner and complete the biofilm cycle within 24h. They also displayed the ability to form intracellular bacterial communities in urothelial cells and induce significant cytotoxicity. Moreover, they were able to associate, invade and survive within *Acanthamoeba castellanii* (T4). Furthermore, 200 urine samples from patients suspected of UTIs were collected from Colchester University Hospital NHS Trust and analysed for the presence of *Acanthamoeba*. Nineteen samples were positive for *Acanthamoeba spp.* (unclassified) and two samples for *A. castellanii* supporting our hypothesis that the amoeba possibly plays a role in UTIs. This is the first study in the UK to have confirmed the presence of *Acanthamoeba* in urine. This study also

investigated the antimicrobial efficacy of cetylpyridinium chloride (CPC). CPC coated latex catheters were able to prevent biofilm formation at very low concentrations. This finding provides promising evidence for the potential application of CPC impregnated catheters in preventing CAUTIs.

In conclusion, the findings from this study can be used to develop targeted interventions aimed at the underlying issues associated with UTIs.

Dedicated to the memories of my nan T. Mary

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Proverbs 16:3 *'Commit thy works unto the LORD, and thy thoughts shall be established'*

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Abbreviations

A. castellanii ..... *Acanthamoeba castellanii*, I
AB .....Antibacterial, 54
ABR ..... Antibacterial resistance, 36
ABU .....Asymptomatic bacteriuria, 5
ACM .....*Acanthamoeba* conditioned medium, 178
AHLs .....*N*-acyl homoserine lactones, 33
AK .....*Acanthamoeba* keratitis, 67
AUM .....Artificial urine medium, 76
BBB .....Blood brain barrier, 68
Bcl-2 .....B-cell lymphoma 2, 146
CAUTIs .....Catheter associated UTIs, 4
CDC .....US Centers for Disease Control and Prevention, 36
CFUs .....Colony forming units, 18
Ch ..... Charrière, 22
CHUFT ..... Colchester Hospital University Foundation Trust, 77
CLED ..... Cysteine lactose electrolyte deficient, 77
CNF1 ..... Cytotoxic necrotising factor 1, 13
CNS .....Central nervous system, 68
CPC ..... Cetylypyridinium chloride, II
CTXM .....Cefotaximases-type, 9
CV .....Crystal violet, 84
DHFR ..... Dihydrofolate reductase, 40
DHPS ..... Dihydropteroic acid synthase, 40
DI ..... Disseminated infection, 67
ds .....Distilled sterilised, 79
DVLO ..... Derjaguin, Landau, Verwey and Overbeek, 29
E. coli ..... *Escherichia coli*, 9
EAU .....European Association of Urology, 4
ECDC .....European Centre for Disease Prevention and Control, 36
EF-G .....Elongation factor G, 39

EPS ..... Extra polymeric substances, 26
EPs .....Extracellular proteases, 66
ESBLs .....Extended-spectrum beta-lactamases, 9
ESIU .....European Section of Infection in Urology, 4
EUCAST .....European Committee on Antimicrobial Susceptibility Testing, 83
FA ..... Fusidic acid, 38
FLA .....Free-living amoeba, 58
FQ ..... Fluoroquinolone, 40
GAE .....Granulomatous amoebic encephalitis, 67
GBM ..... Glomerular basement membrane, 236
GC-MS ..... Gas chromatography–mass spectrometry, 285
GES .....Guiana-extended-spectrum-type, 11
GFB .....Glomerular filtration barrier, 237
GND-UC .....Gendine coated urinary catheter, 50
HCAIs ..... Healthcare associated infections, 6
HlyA .....α-haemolysin, 13
HMBEC .....Human brain microvascular endothelial cells, 219
HMP .....Human Microbiome Project, 7
HPA .....Health Protection Agency, 6
HPLC ..... High performance liquid chromatography, 248
HU .....Human urine, 83
JFC ..... Joint Formulary Committee, 18
K. pneumoniae ..... *Klebsiella pneumoniae*, 10
KGM-2 .....Keratinocyte growth medium 2, 149
LB .....Luria Bertani, 77
LDH .....Lactate dehydrogenase, 148
Lp-Gel ..... Liposome-in-gel system, 301
LPS ..... Lipopolysaccharide, 42
LTC ..... Long-term catheterisation, 21
LUTS ..... Lower urinary tract symptoms, 17
mAU .....Milli absorbance units, 272
MBP .....Mannose binding protein, 65
MH ..... Mueller Hinton, 83
MRSA ..... Methicillin-resistant *Staphylococcus aureus*, 39

NADH ..... Nicotinamide adenine dinucleotide, 148
NHS .....National Health Service, 6
NHUC .....Normal human urothelial cells, 2
NICE .....National Institute for Health and Care Excellence, 20
NIH .....National Institute for Health, 7
NNA ..... Non-nutrient agar, 223
OD .....Optical density, 77
OmpA .....Outer membrane protein A, 212
OXA .....Oxacillinase-type, 11
P. mirabilis .....*Proteus Mirabilis*, 10
PCR .....Polymerase chain reaction, 76
PER ..... *Pseudomonas* extended resistance-type, 11
PLs .....Phospholipases, 66
PS .....Phosphatidylserine, 147
PSA .....Phenol-Sulphuric Acid, 89
PTFE ..... Polytetrafluoroethylene, 22
PYG .....Peptone yeast extract glucose, 83
QACs .....Quaternary ammonium compounds, 52
QS .....Quorum sensing, 33
RBCs .....Red blood cells, 237
RIF ..... Rifampin, 40
RIP1 ..... Serine/threonine kinase receptor-interacting protein 1, 146
RIPA ..... Radio immuno precipitation assay, 180
ROS ..... Reactive oxygen species, 146
RpoE .....RNA polymerase, extracytoplasmic E, 170
RT ..... Room temperature, 78
rUTIs .....Recurrent UTIs, 4
SDS-PAGE ....Sodium dodecyl sulfate polyacrylamide gel electrophoresis, 90
SHV .....Sulphydryl variable, 9
STC .....Short-term catheterisation, 25
TEM ..... Temoneira, 9
TERT-NHUC .....Telomerase Reverse Transcriptase-immortalised Normal Human Urothelial Cell, 149
THP .....Tamm-Horsfall protein, 28

TMP-SMX ..... Trimethoprim-sulfamethoxazole, 40
UP ..... Uroplakins, 3
UPEC ..... Uropathogenic *E. coli*, 10
UTI .....Urinary tract infections, 2
WHO ..... World Health Organization, 36

Chapter 1

Literature review

1 Literature review

1.1 Urinary tract infections

The term, urinary tract infections (UTIs), refers to a broad range of infections associated with the urinary tract, and caused by microbial invasion. It ranges from a simple cystitis affecting the lower urinary tract to severe pyelonephritis affecting the upper tract (Gibson and Toscano, 2012; Solh *et al.*, 2017). A triad of dysuria, enhanced urgency and increased frequency are typical symptoms of a UTI (August and Rosa, 2012). Typical complaints, presence of leukocytes and nitrites in urine and a positive urine culture is usually sufficient to make a diagnosis (Hummers-Pradier and Kochen, 2002). Initial empirical antibiotic treatment further altered based on culture results is the normal strategy used for treating UTIs (Sheerin, 2011). UTIs are the second most common infections encountered in current clinical and community practice all around the world (Aboumarzouk, 2014).

1.1.1 Anatomy of the urinary tract

The human urinary tract is a 'contiguous hollow-organ system' (Hickling *et al.*, 2016). Anatomically, the urinary tract can be divided into the upper and lower tract. The former is composed of the kidneys and ureters whereas the latter comprises the bladder and urethra (Mahadevan, 2016). From the minor calyces (kidney) to the prostatic urethra, the luminal surface of the urinary tract is lined by transitional epithelial tissues known as the urothelium. Normal human urothelial cells (NHUC) are cells covering the mucosal layer of the urinary tract (Hickling *et al.*, 2016). NHUC covering the ureters, bladder and the urethra are

morphologically similar but have different embryological derivations (Al-Kurdi, 2017). However, data from recent studies indicate some degree of difference with regards to the morphological and biochemical features of NHUC lining different parts of the urinary tract (Hickling *et al.*, 2016). The urothelial lining is composed of three layers: superficial, intermediate and basal cells (Brandt *et al.*, 2009). The superficial layer is made up of mono or multinucleated large cells with mature tight junctions characterised by transmembrane glycoproteins known as uroplakins (UP) (Al-Kurdi, 2017) which act as a barrier to water, small non-electrolytes, and protons (Lee, 2011). There are four major UPs: Ia, Ib, II, and IIIa and one minor UP: IIIb (Hickling *et al.*, 2016). They also contain numerous aquaporins and urea transporters (Al-Kurdi, 2017).

The bladder urothelium is one of the most extensively studied urotheliums (Hickling *et al.*, 2016). It not only acts as a permeability barrier between the bladder and the urine content but also plays an important role in sensing the intravesical environment. The urothelium can form and release a number of signaling molecules including urothelial acetylcholine, ATP, adenosine, and nitric oxide (Winder *et al.*, 2014).

1.1.2 Classification

An appropriate classification of UTIs is essential as they can form a sound basis for clinical management (Tan and Chlebicki, 2016) research, quality measurement and teaching (Smelov *et al.*, 2016). Several criteria can be taken into consideration for classifying UTIs. These include clinical presentations, specificity and severity of symptoms, pattern of infection, possible risk factors,

aetiological agent, circumstances under which UTI was acquired and treatment options (Johansen *et al.*, 2011). Traditionally, UTIs are classified as complicated and uncomplicated based on clinical symptoms and laboratory data. However, classifications developed by the US Centers for Disease Control and Prevention (1988 and updated in 2008), the Infectious Diseases Society of America and the US Food and Drug Administration (1992), and the European Society of Clinical Microbiology and Infectious Diseases (1993) are most commonly used (Smelov *et al.*, 2016). UTIs can also be classified as community acquired or hospital acquired (Ahmed *et al.*, 2014).

The European Association of Urology (EAU) and European Section of Infection in Urology (ESIU) have proposed a new classification system based on clinical presentation, risk factors and severity scale (Johansen *et al.*, 2011). This system incorporates UTI severity groups and provides a clear and useful classification for patient assessment and clinical research (Johansen *et al.*, 2011; Smelov *et al.*, 2016). Figure 1.1 provides a summary of the EAU/ESIU UTI classification. Although not specifically included in UTI classification, recurrent UTIs (rUTIs) and catheter associated UTIs (CAUTIs) are clinically important types of UTIs. rUTIs are usually symptomatic and occur after the resolution of a previous UTI (Tan and Chlebicki, 2016). Greater than 2 episodes of UTIs in the last 6 months or greater than 3 episodes in the last 12 months is defined as a rUTI (Aydin *et al.*, 2015). On the other hand, symptomatic and bacteraemic cases of UTIs in patients with an indwelling catheter are known as CAUTIs (Press and Metlay, 2013).

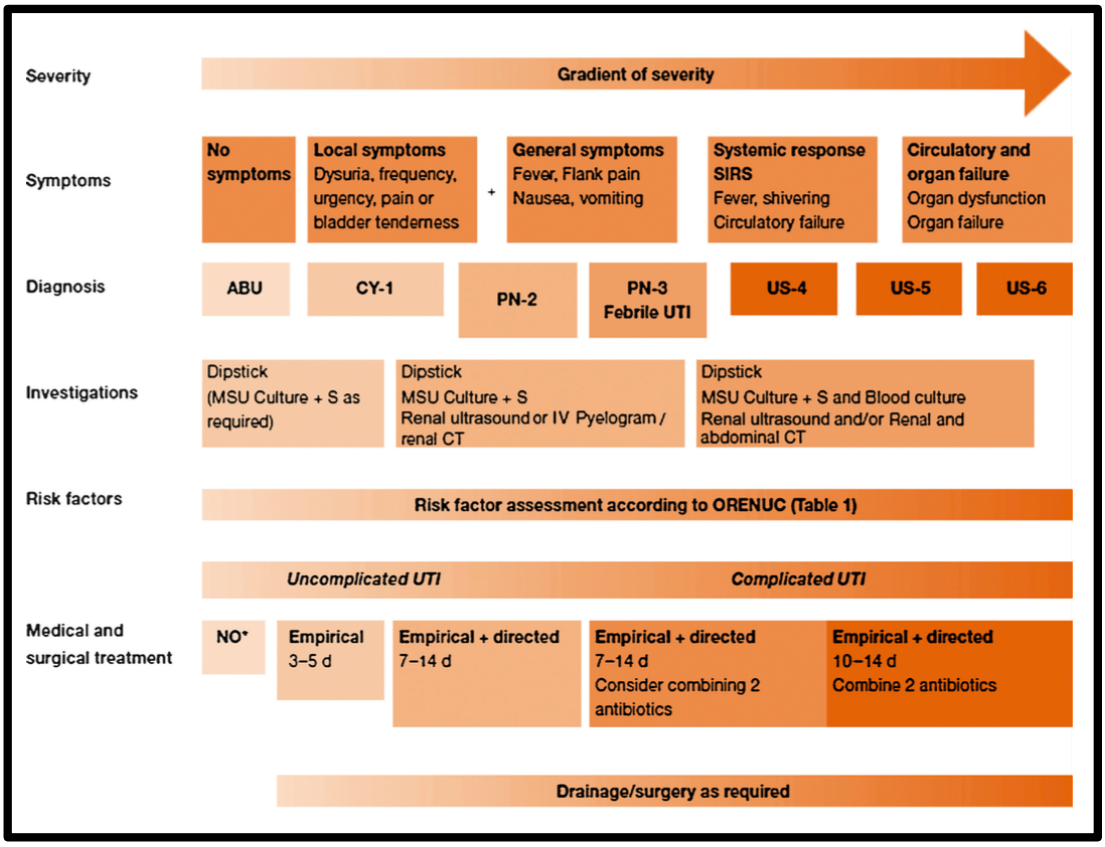

Figure 1.1: Classification of UTIs proposed by the EAU/ESIU.

Diagnosis: ABU – asymptomatic bacteriuria, CY-1 – cystitis, PN-2 – mild and moderate pyelonephritis, PN-3 – severe pyelonephritis, US-4 – simple urosepsis, US-5 – severe urosepsis, US-6 – uroseptic shock; CT - computed tomography; CY - cystitis; IV – intravenous; MSU -midstream sample of urine; ORENUC risk factor assessment: O – no known risk factors, R – risk factors for rUTIs, E – extra urogenital risk factor, N – nephropathic diseases, U – urological risk factors, C – urinary catheter (Smelov *et al.*, 2016). Reproduced with permission from Elsevier (license no: 4084491203843).

1.1.3 Incidence

UTIs are more common in women than in men due to anatomical differences in the genitourinary tract. In the UK, nearly one in two women develop the infection each year compared with only one in every 2000 men (NHS, 2012). In infants and children, UTIs are the most common bacterial infection and is generally difficult to diagnose because of non-specific signs and symptoms (NICE, 2007). rUTIs are more common in women (Kodner and Gupton, 2010) and CAUTIs are mostly associated with the use of an indwelling urinary catheter (Nicolle, 2014).

The healthcare associated infections (HCAIs) prevalence survey data released by the Health Protection Agency (HPA) shows that UTI's (n=605) are the second most common HCAI accounting for 17.2% of the total HCAIs (n=3506) in England, out of which 43% of infections were associated with the use of an indwelling catheter. This report also shows that UTI is the most frequent type of HCAI based on the onset of infection (HPA, 2011).

The National Health Service (NHS) spends approximately £1 billion a year to treat HCAIs in the UK. Fifty-six million of this is incurred after patients are discharged from hospitals (NICE 2012a). The high incidence of UTIs therefore has significant economic implications.

1.1.4 Aetiology

1.1.4.1 Urinary microbiota

In the early 1680s, Antonie van Leewenhoek was one of the first scientist to study the differences between the oral and faecal microbiota associated with states of health and disease. Since then, several studies have confirmed the diversity of the human microbiome (Ursell *et al*., 2013). The terms 'microbiota' and 'microbiome' are used in different contexts. 'Microbiota' refers to the taxonomic group of microorganisms associated with humans and consists of at least 10-100 trillion microbial cells primarily seen in the human gut. On the other hand, the term 'microbiome' refers to the inventory of these symbiotic microorganisms and their genes (Ursell *et al*., 2013; Blum, 2017).

The Human Microbiome Project (HMP) was established in 2007 by the National Institute for Health (NIH) (Proctor, 2011; Blum, 2017). The main aim of the project is to sequence all microbes associated with specific sites in the body including the urogenital system (Blum, 2017). The HMP focusses on creating a reference list of baseline microbiomes for healthy adults, gene sequences of these strains and the evaluation of microbiome properties associated with specific diseases (Proctor, 2011). The NIH-HMP are currently analysing 580 reference strains associated with the urogenital tract (NIH-HMP, 2017).

For several decades, it was generally considered that the bladder is sterile (Thomas-White *et al*., 2016). However, expanded quantitative urine culture and culture independent methods such as 16s rRNA gene sequencing has enabled the characterisation of microbiota in the urinary bladder (Brubaker and Wolfe,

2015; Thomas-White *et al*., 2016). DNA sequencing techniques have revealed diverse sets of bacterial communities resident in the urine, presumably from the bladder (Brubaker and Wolfe, 2015). DNA belonging to a variety of microbes including *Lactobacillus, Gardnerella, Streptococcus, Staphylococcus, Actinomyces, Corynebacterium* and those belonging to the family Enterobacteriaceae have all been found in urine of healthy people (Wolfe *et al*., 2012; Hilt *et al*., 2014; Pearce *et al*., 2014; Pearce *et al*., 2015; Thomas-White *et al*., 2016).

The beneficial role of microbiota and their relationship with human diseases is well established (Rajilić-Stojanović, 2013; Budden *et al*., 2017). This can be particularly seen between gastrointestinal microbiota and infections such as *Clostridium difficile* and *Helicobacter pylori* (Horwitz *et al*., 2015; Thomas-White *et al*., 2016). However, the relationship between bladder microbiota, particularly their diversity, and predisposition to infection is not fully explored (Horwitz *et al*., 2015). Through their systematic review on urinary microbiome, Aragon *et al*. (2016) have concluded that urological disorders can induce changes in healthy urinary microbiome particularly decreased diversity.

Understanding the symbiotic relationship between the urinary bladder and microbiota, and their potential pathological relationship can provide useful insights into the pathogenicity of UTIs.

1.1.4.2 Pathogens associated with UTIs

Several species of bacteria are known to cause UTIs. According to Matthews and Lancaster (2011), the most prevalent uropathogen in UTI is *Escherichia coli*. In a UK-wide multicentre study conducted by Farrell *et al.* (2003), *E. coli* was found to be the predominant pathogen isolated from patients suffering from UTI followed by *Enterococcus faecalis, Klebsiella pneumoniae* and *Proteus mirabilis*.

In a study conducted on the isolation of bacteria from urine samples, Taneja *et al.* (2008) found that 36.5% of the isolated uropathogens were extended-spectrum beta-lactamases (ESBL) producers. Other studies have also isolated ESBL producing uropathogens from urine samples of patients suffering from UTIs (Rodríguez-Baño *et al.*, 2008; Meier *et al.*, 2011; Picozzi *et al.*, 2014; Fan *et al.*, 2014). ESBLs in Gram-negative bacteria are a group of enzymes, which have the ability to hydrolyse a variety of antibiotics including third generation-cephalosporins and aztreonam. They are, however, inhibited by clavulanic acid. ESBL genes are encoded in plasmids and are transmissible. Commonly encountered strains belong to the Temoneira (TEM), sulphydryl variable (SHV) and cefotaximases-type (CTX-M) families (Thomson, 2010). Patients with comorbidities, diabetes, rUTI's, nursing home residents, elderly, male sex and those with frequent use of antibiotics are more susceptible to ESBL positive UTI's (Aboumarzouk, 2014).

Although rUTIs can be caused by any of the above-mentioned microorganisms, few studies have reported that, rUTIs are more common when the first infection is caused by *E. coli* (Lkäheimo *et al.*, 1996; Foxman *et al.*, 2000).

In a UK study conducted on the risk of CAUTIs, Melzer and Welch (2013) found that the most common CAUTI isolates were *E. coli* (43.4%), *P. mirabilis* (13.3%), *Pseudomonas aeruginosa* (10.8%) and *K. pneumoniae* (7.5%). *P. mirabilis* particularly, is of unique importance to patients suffering from CAUTIs due to their ability to form copious biofilms and crystal formation, which often leads to catheter obstruction (Nicolle, 2014).

1.1.4.3 Common uropathogens

E. coli is part of the normal microflora of the gastrointestinal tract in humans (Bien *et al.*, 2012). Traditionally, *E. coli* are classified based on the presence of antigens: O (somatic), K (capsular polysaccharide), and H (flagellar) antigens (Wiles *et al.*, 2008). Pathogenic strains of *E. coli* can also be classified as enteric and extraintestinal. The former has six 'pathotypes' including enteropathogenic, enterohemorrhagic, enterotoxigenic, enteroaggregative, enteroinvasive, and diffusely adherent *E. coli*. Extraintestinal *E. coli* are neonatal meningitis and uropathogenic (Bien *et al.*, 2012). Uropathogenic *E. coli* (UPEC) is mainly implicated in UTIs (Wiles *et al.*, 2008). *E. coli* belongs to the family Enterobacteriaceae. It is a rod-shaped, facultative anaerobic bacterium (Tenaillon *et al.*, 2010). *E. coli* has one of the highest prevalence rate in ESBL production (Sarojamma and Ramakrishna, 2011). The major

types of ESBLs found in *E. coli* are TEM-type, CTX-M, oxacillinase-type (OXA) and *Pseudomonas* extended resistance-type (PER-2) (Shaikh *et al.*, 2015).

K. pneumoniae is the medically most important species of *Klebsiella* genus due to its high prevalence in nosocomial infections. In the human body, the urinary tract is one of the most common site of *K. pneumoniae* infections (Podschun and Ullmann, 1998). The bacterium is Gram-negative, rod-shaped, facultative anaerobe and belongs to the family Enterobacteriaceae (Woldu, 2015). Along with *E. coli, K. pneumoniae* is also a major ESBL producing organism isolated worldwide (Sarojamma and Ramakrishna, 2011). It predominantly produces SHV-1 but production of other enzymes such as PER-2 and Guiana-extended-spectrum-type (GES-1) have also been reported (Shaikh *et al.*, 2015).

P. mirabilis is another Gram-negative, rod-shaped, facultative anaerobic bacterium from the Enterobacteriaceae family, commonly implicated in UTIs (Guentzel, 1996). Unlike UPEC and *K. pneumoniae*, *P. mirabilis* is not a common pathogen isolated from UTIs in healthy people but is more frequently isolated from patients with complicated UTIs (Chen *et al.*, 2012), particularly CAUTIs (Schaffer and Pearson, 2015). The main distinctive features of this bacterium are flagella-mediated swimming and swarming, and urease production (Schaffer and Pearson, 2015). The latter feature enables it to form struvites in the urinary tract (Prywer *et al.*, 2012) and crystalline biofilms which often leads to urinary catheter obstruction (Holling *et al.*, 2014). The most common ESBL enzyme identified in *P. mirabilis* is TEM (de Champs *et al.*, 2000). It has also been reported that some strains produce PEX-1 (Shaikh *et*

al., 2015). Table 1.1 lists some features, of these common uropathogens, usually implicated in the pathogenesis of UTIs.

Table 1.1: Some pathogenic characteristics of common uropathogens associated with UTIs.

(Podschun and Ullmann, 1998; Gupta *et al.*, 2003; Bien *et al.*, 2012; Baldo *et al.*, 2014; Flores-Mireles *et al.*, 2015)

Uropathogen	**Motility**	**Virulence factors**		
		Adherence	**Cytotoxicity and immune evasion**	**Other**
UPEC	Motile with peritrichous flagella or non-motile	Fimbriae (type 1, P, S F1C) Fimbrial and afimbrial adhesins	Capsular and lipopolysaccharides α-haemolysin (HlyA) toxin Cytotoxic necrotising factor 1 (CNF1) Secreted autotransporter toxin (SAT)	Flagella
K. pneumoniae	Non-motile	Pili (type 1 and type 3)	Outer membrane proteins Capsular antigen	Siderophores
P. mirabilis	Flagella mediated swimming and swarming motility	Fimbriae - mannose-resistant/*Proteus*-like (MR/P) fimbriae, *P. mirabilis* fimbriae (PMF), uroepithelial cell adhesion (UCA) (NAF), ambient-temperature fimbriae (ATF), and *P. mirabilis* P-like pili (PMP)	Toxins (haemolysin and *Proteus* toxic agglutinin)	Quorum sensing Metalloproteinase (ZapA) Flagella Urease

1.1.5 Pathogenesis

Three main routes of infection have been identified. Bacteria from the faecal flora can enter the bladder via the urethra. This is known as the ascending route and is most commonly seen in patients diagnosed with UTI (Altarac, 2015). Haematogenous route of infection is uncommon in healthy individuals and is more likely to occur in cases of persistent bloodstream infections or urinary tract obstruction. Bacteria from the surrounding organs can also gain access to the urinary tract via the lymphatic system, however the importance of lymphatic spread is unknown (Hooton, 2000).

The host-pathogen interaction during urinary tract infections is a complex process. Bacterial virulence factors (Table 1.1) and host defence mechanisms play a vital role in determining the severity of infection (Kucheria *et al.*, 2005).

The adult urinary bladder is composed of mucosal, submucosal, muscular and a perivesical soft tissue layer. As previously mentioned, the mucosal layer is also known as NHUC. This uppermost layer is histologically classified as transitional epithelium as it can distend and retract, accommodating bladder filling and emptying. During physiological homeostasis, the bladder urothelium renews itself every 3 to 6 months, however during bacterial induced pathological damage, the urothelium rapidly proliferates and regenerates completely within 72 hours (Ho *et al.*, 2012). The transitional epithelium is lined by large facet cells (Rosen *et al.*, 2007) and also expresses ion channels, ligands and receptors, which not only facilitate cell-to-cell communication but also allow microorganisms like UPEC to attach and invade the bladder mucosal

layer. It has been suggested that attachment and invasion happens via specific interactions between UPEC's FimH adhesion and urothelial cell proteins (Thumbikat *et al.*, 2009). UPs are important integral membrane proteins with varying molecular weights: 15 (UP I), 27 (UP II), 28 (UP III) and 47kDa (UP IV). They are crucial mediators in UPEC cell invasion and also serve as biomarkers for differential diagnosis of urothelial cancer (Wu *et al.*, 2009).

Several studies (Justice *et al.*, 2003; Rosen *et al.*, 2007; Garofalo *et al.*, 2007; Berry *et al.*, 2009; Robino *et al.*, 2013) have been conducted to explore the mechanism of action of UPEC on bladder epithelial cells. In a mouse model of UTI, It has been demonstrated that UPEC interacts with urothelial cells via a multi-step pathogenic pathway to form intracellular bacterial communities (IBCs) (Figure 1.2). UPEC uses type 1 pili and UP's as receptors to adhere to the bladder mucosa. Upon attachment, the bacteria are able to invade and replicate within the cytoplasm of the facet cells. Within these cells, UPEC forms large biofilm-like IBC's, which ultimately flux out, adhere to other cells and promote establishment and recurrence of UTI. During this process, urothelial cells exfoliate and a large number of neutrophils are recruited (Rosen *et al.*, 2007).

Rosen *et al.* (2008) conducted a study to investigate the utilisation of an IBC pathway in *K. pneumoniae* UTI. They found that *K. pneumoniae* also formed IBC's in urothelial cells albeit at a lower level when compared to UPEC. *P. mirabilis* exhibits a slightly different approach. They have the ability to differentiate into swarming cells, which are long filaments that possess 50 times

more flagella per unit cell surface. Flagellum is an important infection virulence factor. These swarming cells migrate away from the original colony and invade NHUC (Allison *et al.*, 1992; Liaw *et al.*, 2000).

In addition to pathogenic virulence factors that promote colonisation, various other predisposing factors assist pathogenic adherence. These include alterations to the host's immune system as seen in immunocompromised patients, co-morbidities like diabetes mellitus, age, anatomical and physiological factors, antimicrobial use, genetic susceptibility and invasive techniques such as catheterisation (Behzadi *et al.*, 2010).

Scott *et al.* (2015) have characterised three distinct stages of IBC formation in a mouse model (Figure 1.2). The early stage consists of attachment and invasion of uropathogens into NHUC. In the middle stage, IBCs mature and form closely packed tight junctions. The late stage is characterised by fluxing and release of uropathogens. In addition, uropathogens can also form reservoirs in the deeper layers of NHUC.

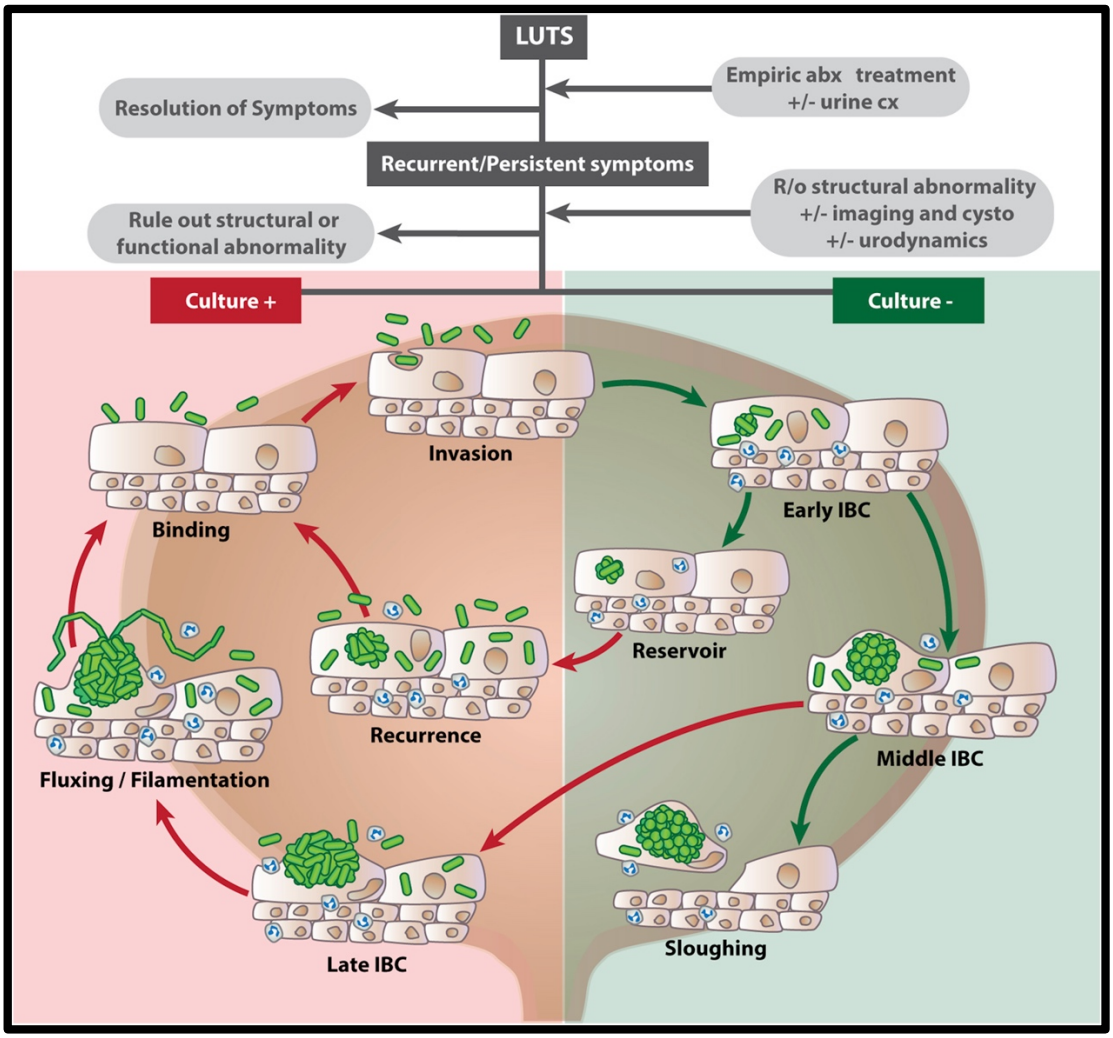

Figure 1.2: Cycle of IBC formation in the bladder urothelium.

The figure also provides a flow chart for the clinical management of lower urinary tract symptoms (LUTS). The IBC cycle consists of three stages (early, middle and late). Uropathogens can also form reservoirs in the superficial or deep layers of NHUC. The red half of the bladder shows IBC formation with intraluminal bacteria resulting in a positive urine culture (cx) whereas the green half of the bladder shows IBC formation with intracellular bacteria resulting in a negative urine cx, antibiotic resistance and rUTIs (Scott *et al.*, 2015). Reproduced with permission from Elsevier (license no: 4086321409415).

1.1.6 Treatment

Treatment of UTIs depend on the type of infection acquired by the patient. ABU, uncomplicated UTI, complicated UTI and rUTI require different initial approaches (Mazzulli, 2012). Clinically significant ABU is diagnosed on the basis of a positive urine culture of greater than 10^5 colony forming units (CFUs) per ml of urine in patients who lack typical UTI symptoms (Cormican, 2011). Antibiotic management of ABU is only significant in pregnant women and has no established value in non-pregnant women, men and children (Cormican, 2011; Mazzulli, 2012). The other types of UTIs require antibiotic treatment (Najar *et al.*, 2009). The Joint Formulary Committee (JFC) has laid out specific guidance for the antibiotic management of UTIs in the UK. Table 1.2 provides a summary of their guidance (JFC, 2016).

Table 1.2: Summary of antibiotic choices for treatment of uncomplicated, complicated and rUTIs.

Recommended by the JFC (2016), UK.

Type	Antibiotics	
Uncomplicated lower UTIs	Initial treatment	Trimethoprim, nitrofurantoin, or amoxicillin
	Resistant organisms	Co-amoxiclav (amoxicillin with clavulanic acid), an oral cephalosporin, nitrofurantoin, pivmecillinam, or a quinolone.
	Multiple-antibacterial resistant organisms	Fosfomycin [unlicensed]
	Long-term low dose therapy (rUTIs)	Trimethoprim, nitrofurantoin and cefalexin
Acute pyelonephritis	IV cefuroxime or a quinolone Gentamicin (if severely ill)	
Prostatitis	Trimethoprim, or some quinolones.	
UTIs in pregnancy	Penicillins and cephalosporins	
UTIs in children	Children under 3 months of age	IV ampicillin with gentamicin, or cefotaxime alone
	Uncomplicated lower UTIs (>3 months of age)	Trimethoprim, nitrofurantoin, a first generation cephalosporin (e.g. cefalexin), or amoxicillin
	Acute pyelonephritis (>3 months of age)	First generation cephalosporin or co-amoxiclav cefotaxime or co-amoxiclav or gentamicin (if severely ill)
	Long-term low dose therapy (rUTIs)	trimethoprim or nitrofurantoin

1.2 Catheter associated urinary tract infections

CAUTIs are seen in patients with an indwelling catheter. It has been associated with poor patient outcomes and rising healthcare costs (Hartley and Valley, 2015). It is the second most common cause of HCAIs in the UK (Fasugba *et al.*, 2017).

1.2.1 Urinary catheterisation

"Urinary catheterisation is a medical procedure used to drain and collect urine from the bladder" (NHS, 2013). Urinary catheterisation is generally used for diagnostic evaluation, monitoring urinary output and for therapeutic purposes in case of urinary retention (Ramakrishnan and Mold, 2005). In the UK, different NHS trusts have individual policies and guidelines governing the use and management of urinary catheterisation. However, they are all based on the guidelines issued by The EAU and The National Institute for Health and Care Excellence (NICE). According to NICE (2012b), the best approach to catheterisation should take into account; the clinical need, anticipated duration, patient preference and the risk of infection. The guidance also emphasises the importance of regularly reviewing the clinical need for catheterisation, documenting catheter insertion, changes and care.

1.2.1.1 Overview

There are different forms of urinary catheterisation. Based on the type of catheter used, catheterisation can be intermittent – where the catheter is inserted into the bladder and removed once the flow of urine has stopped or the most common form which is indwelling – where the catheter is held in the

bladder, positioned by a water-filled balloon (NHS, 2013). Indwelling catheterisation can be categorised further depending on the duration of catheterisation and the site of insertion. It can be short term (0-2 weeks), intermediate term (2-6 weeks) and long term (>6 weeks) (Ramakrishnan and Mold, 2005). If inserted directly into the bladder via a small incision in the wall of the abdomen, it is known as suprapubic catheterisation. If inserted through the urethra into the bladder, it is known as urethral catheterisation. The latter is the most commonly used method (NHS, 2013). Common indications for urinary catheterisation are diagnostic evaluation (obtaining urine samples for evaluation, bladder distention prior to pelvic ultrasound, cystograms, cystourethrograms, assessing vesicoureteric reflex and accurate measurement of urine output in critically ill patients), therapeutic short-term catheterisation (epidural anesthesia, acute urinary retention and instillation of chemotherapeutic agents), and therapeutic long-term catheterisation (uncorrectable bladder outlet obstruction, neurogenic bladder, palliative care and urinary incontinence) (Ramakrishnan and Mold, 2005).

As with any other medical procedure, urinary catheterisation also poses significant risks. Most of these risks are associated with long-term catheterisation (LTC) as the duration of catheterisation is strongly associated with the risk of infection. It is estimated that the risk of acquiring bacteriuria is at 5% for each day of catheterisation (Garibaldi *et al.*, 1982). One of the main risks associated with LTC is infection referred to as CAUTI. Other significant risks include, incorrect catheter insertion causing urethral strictures (Hart, 2008), catheter traction, which can lead to hematuria (Ramakrishnan and Mold,

2005) and balloon distention or incorrect positioning causing urethral rupture (Colle *et al.*, 1999). Maughan *et al.* (2010) have found that a staggering 79% of patients on LTC have had at least one complication over two years, out of which, 62% had a CAUTI, 55% reported social interference, 33% had to visit the emergency department, 32% reported discomfort related to catheter change and a further 32% complained of urinary leakage.

1.2.1.2 Present day urinary catheters

A urinary catheter is a medical device used for emptying the urinary bladder. In Greek, the term catheter means to let down into or send down. In mid 1930's, Frederic Foley, an American urologist designed and developed a standard, single use, self-retaining catheter known as the Foley catheter. It is typically 220 – 380mm in length and consists of a drainage channel at the proximal end and a two or three-way outlet at the distal end. The three outlets are used for drainage, inflation of the catheter balloon and for irrigation of bladder if needed (Warren, 2001). The catheter is positioned in the bladder by means of a 10ml retention balloon (Pickard *et al.*, 2012). Catheter size is measured in French units (F= circumference in mm) or Charrière (Ch) units (1Ch= 0.33mm in diameter) (Ramakrishnan and Mold, 2005).

Foley catheters are typically made up of latex or silicone. Latex has good elastic properties, is inexpensive and remains the standard material of manufacture. However, it is known to cause allergic reactions (Denstedt *et al.*, 1998). Latex catheters are usually coated internally and externally with polytetrafluoroethylene (PTFE), which makes it water resistant and reduces

trauma to the urethral mucosa (Pickard *et al.*, 2012). Silicone is soft, non-irritating, has smooth surfaces and is hypoallergic but is more expensive to manufacture (Pace *et al.*, 2006; Pickard *et al.*, 2012). In 2011, the NHS had to spend approximately £0.91 for a latex catheter compared to £2.07 for a silicone one (Pickard *et al.*, 2012).

Although it has been suggested that there is no difference in bacteriuria between silicone and latex catheters (Pratt *et al.*, 2001), there is some evidence that bacterial adhesion is decreased in hydrophilic-coated latex catheters than silicone catheters (Roberts *et al.*, 1993). There is also some evidence to suggest that, on deflation, the balloons in silicone catheters have a greater tendency to cuff than latex catheters, which can cause distress when the catheter is removed (Parkin *et al.*, 2002).

In the UK, approximately 15–25% of the 14.5 million patients admitted in the NHS have received catheterisation at some point of their stay. An audit conducted in 2010 that reflects current practice in the NHS suggests that, of the 15% of patients catheterised with an indwelling catheter, silicone catheter was used for 50% of these patients, 36% were fitted with PTFE coated latex catheters and in the remaining 14%, the catheter type was unknown (Pickard *et al.*, 2012).

1.2.2 Pathogenesis

The pathogenesis of CAUTI depends on the catheter material and its susceptibility to bacterial colonisation (Amalaradjou and Venkitanarayanan, 2013). The urinary tract has devised several mechanisms to protect itself from bacterial invasions. Within hours of uropathogenic invasion, the bladder epithelial cell lining begins to exfoliate causing the infected cells to shed. The innate immune response is the first line defence mechanism against the invading uropathogens. Responses include the activation of natural killer cells, leukocytes, neutrophils and macrophages. Immediately after recognising bacterial products like lipopolysaccharides and peptidoglycans, various signaling pathways are activated which initiate immune and inflammatory responses against the pathogens. The adaptive immune response is activated 7-10 days post infection. B and T lymphocytes target specific uropathogens (Davis and Flood, 2011). Additionally, high flow rates (10-20 ml/s) caused by micturition are also successful in flushing out bacteria (Cadieux *et al.*, 2008).

In contrast, catheter surfaces do not have any inert defence mechanisms. The normal immune mechanisms are disrupted in a catheterised bladder causing ineffective clearance of microorganisms (Cadieux *et al.*, 2008). The catheter itself provides route of entry for bacteria along its internal and external surfaces by creating a connection between the sterile bladder and a colonised perineum (Trautner and Darouiche, 2010). Plasma proteins are normally adsorbed on to any foreign body like the catheter, these proteins assist in microbial attachment (Danese, 2002). This supports the ‘bottle effect’ theory, which states that bacterial growth is substantially increased when they are attached to a surface

(Percival *et al.*, 2011). The orientation of the catheter balloon can lead to the formation of a urine sump in the bladder, which in addition to, urinary stasis caused by slow flow rates in catheters facilitates bacterial multiplication (Trautner and Darouiche, 2010; Cadieux *et al.*, 2008). An increase in the duration of catheterisation will facilitate bacterial adherence to the catheter surface, multiplication and the formation of a biofilm (1.2.3) (Pickard *et al.*, 2012). Numerous studies have demonstrated extensive biofilm formation on urinary catheters (Amalaradjou and Venkitanarayanan, 2013).

Typically, a single microorganism is associated with short-term catheterisation (STC), while polymicrobial infections are more frequently seen in LTC. The most commonly isolated single species is *E. coli* (Pickard *et al.*, 2012). *P. mirabilis* is also commonly implicated in CAUTIs (Jacobsen *et al*., 2008).

1.2.3 Biofilms

The ability of bacteria to develop and maintain UTI's is directly related to the formation of biofilms on indwelling catheters or within the urinary tract (Hatt and Rather, 2008). Bacteria protect themselves from hostile environments by forming biofilms. Biofilms are found in a wide range of locations including aquatic environment, medical devices and tissues (Yang *et al.*, 2011). Bacteria can live in two very different states: as planktonic (free-floating) or in biofilms (sessile form). Biofilm bacteria live in highly organised communities (Costerton *et al.* 1999). Costerton *et al.* (1999) has previously defined biofilms as "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface".

Biofilm formation is an extremely complex process resulting in the formation of highly specialised structures. In addition to cells, the biofilm matrix also consists of non-cellular material known as extra polymeric substances (EPS). EPS consists of polysaccharides, proteins, nucleic acids, lipids and account for 73-98% of the biofilm (Lawrence *et al.*, 2003). Biofilms extend a range of benefits to bacteria: cell immobilisation and efficient cell to cell communication, micro-niches rich in nutrients, protection from host immune response and antibiotics, and acquisition of new genetic traits (Flemming and Wingender, 2010).

Although the mechanism of biofilm formation is different between different bacterial species, it is generally recognised to consist of several stages. Firstly, a surface conditioning film is developed followed by the transport of microorganisms into this surface, microbial adhesion, colonisation and finally dispersal of the biofilm (Percival 2011). Figure 1.3 shows the different stages of biofilm formation.

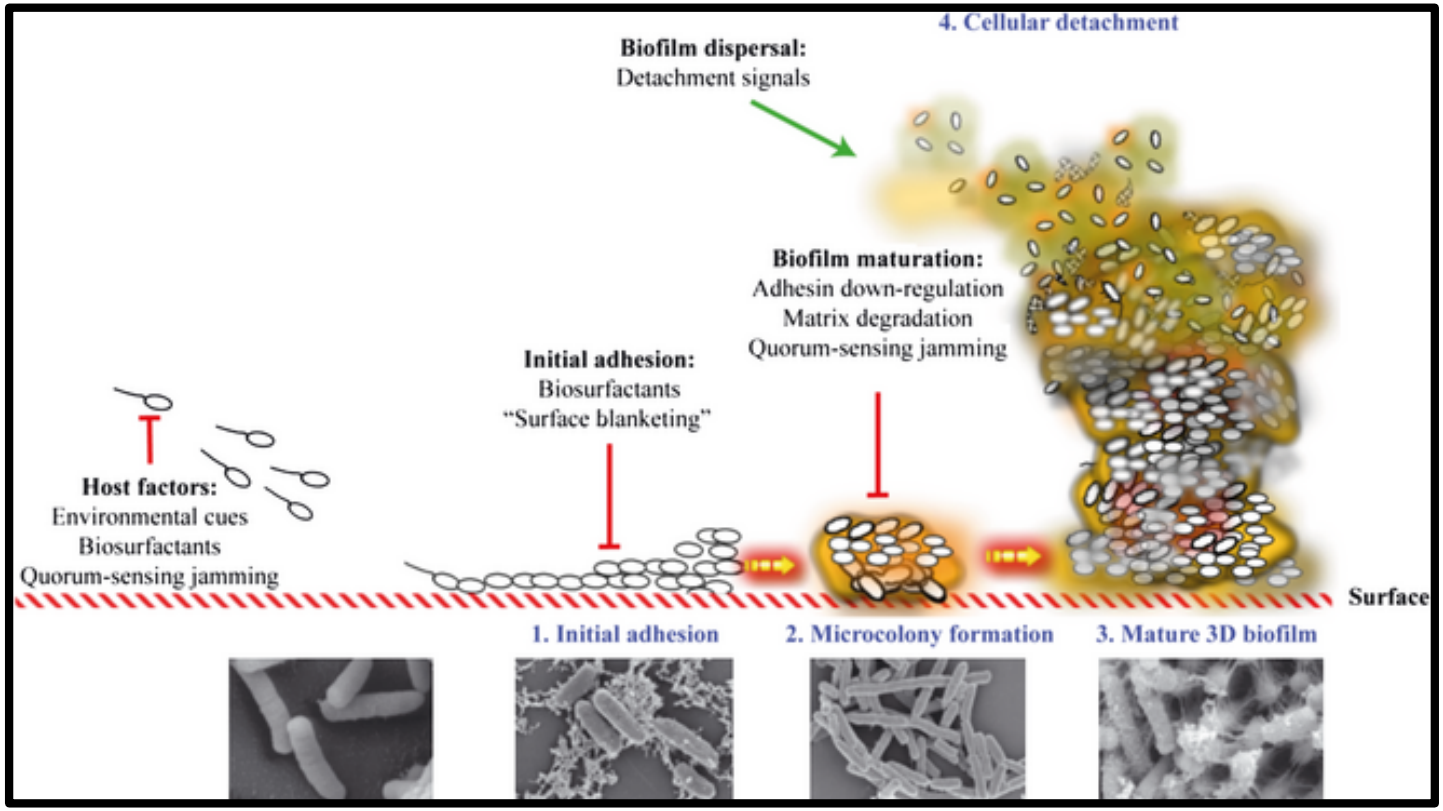

Figure 1.3: Different stages of biofilm formation.

Scanning electron microscopy images are representative of different stages shown. The formation of conditioning layer promotes initial attachment of uropathogens after which microcolonies are formed. These microcolonies group together to form a three-dimensional (3D) structure. Formation of EPS allows the biofilms to undergo maturation. The final step is dispersal, through which the bacteria regain planktonic status and colonise new areas. The figure also shows anti-biofilm molecules which act at different stages of biofilm formation (Rendueles and Ghigo, 2012). Reproduced with permission from Oxford University Press (license no: 4086620380557).

1.2.3.1 Conditioning film

Once inserted into the urinary tract, the catheter becomes exposed to urine resulting in the adsorption of urine components onto the catheter surface. This conditioning film is made up of various proteins present in the urine, like albumin, Tamm-Horsfall protein (THP) and alpha1-microglobulin. Other urine components also form part of the film (Tieszer, 1998; Raffi *et al*., 2012). This protein coat acts as a surface for bacterial adherence. Many bacteria don't have direct mechanisms by which they can adhere to catheter surfaces; hence the formation of this conditioning film is crucial for microbial adhesion (Busscher and Weerkamp, 1987).

1.2.3.2 Adhesion and attachment

The next step is the initial attachment of planktonic bacteria to the surface conditioning film. Initially, planktonic bacteria form a reversible attachment, which is then transformed to irreversible adsorption regulated by chemical and physical interactions. Attachment can be achieved only if the adhering bacteria get closer to the surface. This surface approach is possible via several physical forces, including Brownian motion, van der Waal's forces, hydrophobic interactions and using mechanisms such as sedimentation, convective and active transport using flagella and turbulent flow (Gottenbos *et al.*, 2002; Palmer *et al.*, 2007). Depending on the proximity of bacterial cells to the surface, these physical forces can be divided into long-range interactions (>50nm) and short-range interactions (<5nm) (Gottenbos *et al.*, 2002). These interactions depend on the bacterial cell surface, substrate surface and the

surrounding solution (Parent and Velegol, 2004). In order to further understand these interactions, three theoretical approaches have been studied.

The Derjaguin, Landau, Verwey and Overbeek (DLVO) theory states that when two colloidal properties are in close proximity, their electrical double layers interfere resulting in electrostatic repulsion. To overcome this and be able to aggregate, the two colloidal particles must have a large kinetic energy. Therefore, a balance between the attractive (van der Waal's forces) and repulsive interactions results in a net interaction between the two particles. In theory bacteria do not possess a large kinetic energy and hence should not be able to attach to a surface. However, the DVLO theory assumes that bacteria do not have a flat surface but have adhesins and other polymers protruding from their surface, which can overcome the energy barrier and firmly adhere to a surface (Hermansson, 1999; Jones and Isaacson, 1983).

Van Oss's (1986) extended DLVO theory describes the role of hydrophobic attraction and hydrophilic repulsion caused by acid-base interactions, in addition to the previously described electrostatic interactions (Hermansson, 1999).

Lastly, the thermodynamic theory requires an estimation of thermodynamic value for the free energy of the bacterial cell surface, substrate surface. It expresses the attractive and repulsive forces in terms of free energy. It simply states that the energy states of the bacterial-substrate should be lower than the

energy state of the bacteria and substrate in separation for bacterial adhesion to be successful (Vilinska and Rao, 2011).

Apart from these mechanisms, bacterial adhesion is also governed by various other factors mainly of bacterial origin, environmental and host derived. An important factor for cell adhesion is the recognition of proteinaceous components on the catheter surface by bacterial cell surface adhesins. Flagellum, pili and fimbriae are implicated in biofilms formed by Gram-negative bacteria (Jacobsen *et al.*, 2008).

UPEC is one of the most commonly isolated Gram-negative microorganisms in UTIs. Motility plays an important role in biofilm formation. UPEC uses its flagella to swim in both fluid and semi-fluid media. Surface virulence factors such as type 1 fimbriae, P fimbriae and S fimbriae are known to promote bacterial attachment. The lipoprotein α-HlyA is also associated with UPEC virulence. HlyA is a toxin that is widespread among Gram-negative uropathogens. This toxin is secreted by the bacteria and transported to the site of action (Bien *et al.*, 2011).

Fimbrial adhesins in *Klebsiella* are known to aid initial attachment to abiotic surfaces. Schroll *et al.* (2010) demonstrated that type 3 fimbriae are crucial for the initial surface attachment of *Klebsiella*. Studies conducted by Di Martino *et al.* (2003) and Balestrino *et al.* (2008) also indicate that cell-to-cell attachment in *Klebsiella* biofilms are aided by type 3 fimbriae.

A variety of different virulence factors are linked to *P. mirabilis* biofilm formation. These include adhesion factors, transcription factors and proteins involved in LPS production. Studies conducted on a mouse model have shown that mannose-resistant *Proteus*-like fimbriae are produced by a majority of cells in a mouse model and play a key role in colonisation. Bacterial urease and capsular polysaccharides contributes to the development of crystalline biofilm formation in *P. mirabilis*. An increase in urinary pH is seen due to urease-mediated hydrolysis of urea to ammonia and carbon dioxide. This increase in pH is essential for bacterial attachment (Jacobsen and Shirtliff, 2011).

Additionally, environmental factors such as the surface characteristics of a catheter also affect the rate of bacterial attachment. Rougher substrate surface and hydrophobic material can enhance bacterial attachment. Flow rate, temperature, presence of cations, pH of the surrounding solution also play an important role in bacterial cell to surface adhesion (von Eiff *et al.*, 2005).

The host's innate immune system and components of the urine, namely urea and antimicrobial peptides also aim to eliminate bacteria (Ryder, 2005).

Transformation of reversible attachment to an irreversible one is achieved by the upregulation of bacterial genes and phenotypic changes directing the formation of exopolysaccharides. Along with proteins, nucleic acids, humic acids and metal ions, these exopolysaccharides form a high molecular weight protective matrix known as EPS (Bales *et al.*, 2013). EPS protects the bacteria

by irreversibly attaching the cells to one another and to the catheter surface (Ryder, 2005).

1.2.3.3 Proliferation and maturation

Following irreversible attachment, bacterial cells undergo phenotypic changes and start the process of proliferation and maturation. Aggregation of attached cells, microbial growth by cell division and recruitment of planktonic cells facilitate the development of microcolonies. The microenvironment of a biofilm varies depending on local conditions such as pH and nutrient availability. The upper layers of a biofilm are exposed to high concentrations of O_2, while the lower layers exhibit anaerobic conditions (Pace *et al.*, 2006).

Following proliferation and further matrix production, the cells become embedded and immobilised in EPS. Finally, a 3D structure with tower or mushroom shaped cell clusters is formed, which appears as a continuous layer or a patchy mesh like layer on the catheter surface. As the biofilm matures, water channels connect the adjoining microcolonies and facilitate nutrient and waste exchange (Ryder, 2005). Biofilms are such sophisticated systems that its organisation can be compared to that of eukaryotic organisms. Microcolonies are the basic unit of a biofilm just like tissues in an organism and the water channels represent the circulatory system (Pace *et al.*, 2006).

Biofilm thickness is highly dependent on its local environment. Previous studies have suggested that the biofilm density is an important measure of heterogeneity and the biofilm's mechanical strength (Laspidou and Rittman,

2004). Biofilm maturity is directly proportional to its resistance and indirectly proportional to its metabolic rate. A study conducted by Wolcott *et al.* (2010) demonstrated that *P. aeruginosa* biofilms were susceptible to antibiotic treatment after 6-12 hours of growth but were completely resistant after 48 hours of growth compared with *S. aureus* biofilms which were susceptible to the antibiotic after 6-72 hours of growth and completely resistant after 92 hours of growth indicating that biofilm maturity offers an important survival strategy for microorganisms.

Cell to cell communication plays an essential role in the growth and development of all organisms. Microorganisms have the ability to sense changes in their environment and adjust their phenotypes. A network of signals, which allow the biofilm cells to communicate with each other, confers this ability. This type of cell-to-cell communication activated at high cell densities by which bacteria can regulate their genes in response to certain concentrations of signaling molecules is known as quorum sensing (QS) (Sifri, 2008). QS was first discovered in marine bacteria, *Vibrio fischeri* and *Vibrio harveyi* (Marshall, 2013).

Most Gram-negative bacteria use *N*-acyl homoserine lactones (AHLs) as signaling molecules. AHL's are autoinducers secreted into the environment by biofilm bacteria. Initially their concentration is low, however when bacterial cell density increases, the increased concentration of AHL is detected by the biofilm bacteria that can then activate or suppress target genes. Thus, the principle of QS is directly related to cell density. QS in Gram-negative bacteria involves two

regulatory components: transcriptional activator proteins (R protein) and the autoinducer molecule produced by the autoinducer synthase. When the threshold level is achieved, the autoinducer molecule binds to the R protein and activates it, ultimately inducing gene expression (de Kievit and Iglewski, 2000). QS offers a competitive advantage to the bacteria ensuring their survival and propagation in natural environments (Jayaraman and Wood, 2008). QS also co-ordinates the final stage of biofilm development (Donlan, 2002).

1.2.3.4 Detachment and dispersal

The final stage is the detachment of cells and their dispersal in to the environment in order to colonise new sites. Detachment involves the transfer of cells and other components from the biofilm to the surrounding bulk liquid (Percival *et al.*, 2011). The process can be divided into detachment of cells, translocation to a new site and re-attachment. Detachment/dispersal can occur actively, wherein the bacteria themselves initiate the mechanism or passively, wherein the process is mediated by external forces such as collision of solid particles with the biofilm, shearing due to flow dynamics, predator grazing and human intervention (Kaplan, 2010). Lack of nutrients, more supportive growth areas and quorum signaling can lead to shedding of daughter cells by biofilm bacteria (active process). Due to increased cell density, quorum signaling can cause degradation of EPS and eventually release clumps of cells into the bulk liquid (Donlan, 2002). Proposed dispersal mechanisms include continuous release of single cells or small clusters (erosion) or a sudden detachment of large parts of the biofilm (sloughing) (Kalpan and Fine, 2002). After detachment/dispersal, biofilm bacteria can reattach to other parts of the

catheter or seed the urine in the bladder and can further progress into the systemic circulation (Donlan, 2002). This final step in the development of a biofilm is clinically significant as they can cause persistent infections with increased severity (Cadieux *et al.*, 2008).

1.3 Underlying issues associated with UTIs

One of the major issues associated with UTIs is the development of highly virulent, multi-drug resistant bacterial strains (Bien *et al.*, 2012; Dash *et al.*, 2013). Uropathogens employ different mechanisms to develop antibacterial resistance (ABR), biofilm resistance and IBC resistance.

1.3.1 Resistance mechanisms

1.3.1.1 Antibacterial resistance

The widespread and inappropriate use of antibiotics particularly over the last decade has led to the development of ABR. Multidrug-resistant bacteria are considered as a major public health problem by several organisations including the US Centers for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO) (Roca *et al.*, 2015).

An increase in resistance to antibiotics has rendered Gram-negative bacteria as hugely problematic microorganisms in the healthcare sector (Domínguez-Gil *et al.*, 2016). *E. coli* and *K. pneumoniae* amongst others are considered as bacteria of international concern by the WHO due to the significant impact on public health caused by ABR patterns and because they are common etiologies for HCAIs. The global report on surveillance has presented key findings related to ABR from all WHO regions (WHO, 2014). Table 1.3 presents the national data related to commonly seen ABR patterns in *E. coli* and *K. pneumoniae* from the UK.

Table 1.3: Commonly seen ABR patterns.

UK national data for *E. coli* and *K. pneumoniae* (WHO, 2014).

Bacteria	ABR	No of tested isolates	Resistance (%)
E. coli	Third-generation cephalosporins	5182	9.6
	Fluoroquinolones	5564	17.5
K. pneumoniae	Third-generation cephalosporins	935	5.3
	Carbapenems	825	0.4

Bacteria develop ABR via different mechanisms. Genetic mechanisms of ABR may be innate or intrinsic, may be acquired through spontaneous mutations or through horizontal gene transfer (Sharma *et al.*, 2016; Yilmaz and Özcengiza, 2017) whereas the mechanistic basis of ABR include target alteration, drug inactivation, decreased permeability and increased efflux (Sun *et al.*, 2014; Yilmaz and Özcengiza, 2017).

Intrinsic ABR is the innate ability of bacteria which is independent of antibiotic selective pressure and horizontal gene transfer, and is found within the genome of all bacterial species (Cox and Wright, 2013). Owing to short generation times and an ability to adapt rapidly to their changing environments, a variety of bacterial species can also develop ABR via spontaneous de novo mutations (Woodford and Ellington, 2007). Finally, bacteria can also acquire foreign DNA material through horizontal gene transfer by utilising three main strategies: uptake of naked DNA or transformation, phage mediated transduction and conjugation (Munita and Arias, 2016).

Multiple biochemical pathways are used in the mechanistic basis of ABR. Changes in the target site can be achieved via target protection and target modification (Munita and Arias, 2016). ABR proteins bind to the antibiotic target site through protein-protein interaction and thereby prevent the antibiotic from exerting its bactericidal action. FusB-type resistance to fusidic acid (FA), and Qnr-mediated resistance to fluoroquinolones are the only two clinically significant examples identified till date (Tomlinson *et al.*, 2016). FusB-type proteins allow the ribosomes to resume translation by binding to the elongation

factor G (EF-G) which is the target site of FA (Cox *et al.*, 2012). This type of resistance is primarily seen in methicillin-resistant *Staphylococcus aureus* (MRSA) (Hanssen *et al.*, 2005; Chen *et al.*, 2011). The primary target for quinolones in Gram-negative bacteria is DNA gyrase whereas in Gram-positive bacteria it is topoisomerase IV (Nordmann and Poirel, 2005). Qnr proteins directly protect these target sites and thereby confer quinolone resistance to a variety of bacterial species (Tran *et al.*, 2005). These proteins were first identified in *K. pneumoniae* isolated from urine (Strahilevitz *et al.*, 2009). On the other hand, target modification results in a decrease in the target site affinity of the antibiotic and can be achieved via point mutations in the genes that encode the target sites, alteration of the target sites using enzymes and by bypassing the target site (Munita and Arias, 2016). Table 1.4 provides a summary of some classical examples of antibiotic resistance developed through target modification strategies.

Table 1.4: Antibiotic resistance mechanisms.

Some classical examples of ABR developed through target modification strategies (Arias *et al.*, 2008; Munita and Arias, 2016).

Strategy	Classical examples	Mechanism
Mutations of the target site	Rifampin (RIF) resistance	RIF inhibits RNA polymerase by binding to a highly conserved structure (encoded by rpoB gene) on the β subunit of RNA polymerase and thereby blocks bacterial transcription. Point mutations in the rpoB gene confers RIF resistance.
	Fluoroquinolone (FQ) resistance	FQs inhibit DNA replication by inhibiting DNA gyrase and topoisomerase IV enzymes. Mutations in the genes (gyrA-gyrB and parC-parE) encoding the above enzymes confers FQ resistance.
	Oxazolidinones resistance	These drugs inhibit protein synthesis through an interaction with the A site of bacterial ribosomes and by interfering with the positioning of the aminoacyl-tRNA. Mutations in the genes encoding the domain V of the 23S rRNA (rplC) and L3 and L4 ribosomal proteins (rplD) confers resistance.
Enzymatic alteration of the target site	Macrolide resistance	Resistance to macrolides is developed via the enzyme catalysed methylation of ribosomes causing impaired target site binding. These enzymes are encoded by the erythromycin ribosomal methylation (erm) genes.
	Linezolid resistance	Methylation of a highly conserved adenosine (A2503) in the 23S rRNA of the large ribosomal subunit by an enzyme encoded by the cfr gene confers resistance to linezolids.
Complete replacement or bypass of the target site	Methicillin resistance	Acquisition of mecA gene encoding PBP2a which is a penicillin binding protein (PBP) that has low affinity to all β-lactams.
	Vancomycin resistance	Acquisition of van gene clusters which remodel the synthesis of peptidoglycans, significantly reduces antibiotic affinity.
	Trimethoprim-sulfamethoxazole (TMP-SMX) resistance	These antibiotics inhibit folic acid synthesis in bacteria. Dihydropteroic acid synthase (DHPS), and dihydrofolate reductase (DHFR) are crucial enzymes necessary for folate synthesis. DHPS and DHFR are inhibited by TMP and SMX respectively. Mutations in the promoter regions of DNA encoding the above enzymes causes overproduction of these enzymes, overwhelming the ability of TMP-SMX to inhibit folate synthesis thereby allowing bacterial survival.

Antibiotic inactivation is another biochemical aspect of ABR. Bacteria can inactivate antibiotics via hydrolysis, group transfers and redox reactions (Shaikh *et al.*, 2015). Many antibiotics have chemical bonds that are susceptible to hydrolysis. To this end, bacteria have developed mechanisms to secrete a variety of enzymes to cleave these bonds (Shaikh *et al.*, 2015). Although several enzymes are known to inactivate antibiotics via hydrolysis, ESBLs are classical hydrolytic amidases which have the ability to hydrolyse penicillins, third-generation cephalosporins and aztreonam (Paterson and Bonomo, 2005; Shaikh *et al.*, 2015). ESBLs confer bacterial resistance to β-lactam antibiotics by cleaving the β-lactam ring (Dhillon and Clark, 2012). There are more than 200 characterised ESBLs classified on the basis of their protein homology (Ambler molecular classification) and functional similarities (Bush-Jacoby-Medeiros classification scheme) (Rawat and Nair, 2010). There are more than 130 TEM and more than 50 SHV ESBLs found in *E. coli*, *K. pneumoniae* and *P. mirabilis* amongst other members of the Enterobacteriaceae family of Gram-negative organisms (Turner, 2005). Other types of hydrolytic enzymes are esterase (macrolide) (Morar *et al.*, 2012) and carbon-phosphorus lyase complex (fosfomycin) (Kamat *et al.*, 2013). Another strategy of antibiotic inactivation is the chemical substitution of the antibiotic molecule by a group of resistant enzymes known as transferases. This covalent modification of antibiotics affecting their ability to bind to their target sites are caused by a variety of enzymes including acyltransferases, phosphotransferases, thioltransferases, nucleotidetransferases, ADP-ribosyltransferases and glycosyltransferases (Schroeder *et al.*, 2017). Oxidation and reduction of antibiotics is another, albeit infrequent, mechanism

of ABR. Oxidation of tetracycline by TetX enzyme is a well-known example (Shaikh *et al.*, 2015). Antibiotics use a lipid mediated pathway (hydrophobic molecules) or a porin mediated (general diffusion porins) pathway (hydrophilic molecules) to penetrate the outer membrane of Gram-negative bacteria. However, bacteria have devised strategies to resist antibiotics by modifying these pathways (Delcour, 2009). It has been suggested that, bacterial modification of the former pathway by reducing the net negative charge of the lipopolysaccharide (LPS) and close packing of the LPS layers decreases bacterial sensitivity to antibiotics (Gutsmann *et al.*, 2005; Delcour, 2009). Such mechanisms are observed in *S. typhimurium* and *E. coli* (Delcour, 2009). In the latter pathway, a decrease in the number of pores or in their qualitative function can affect the diffusion ability of antibiotics. This ABR mechanism is most often seen in *Klebsiella, Enterobacter, Serratia* and *Salmonella* (Nguyen and Gutman, 1994). Bacterial modification of both mechanisms leads to reduced antibiotic permeability.

Finally, increased efflux of antibiotics via efflux pumps is a chromosomally encoded ABR mechanism. Milton Saier's group have classified efflux pumps based on their protein sequences. Transporters belonging to the resistance-nodulation-division (RND), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), small multidrug resistance (SMR), and ATP-binding cassette (ABC) superfamilies or families are prominent members of efflux transporters (Li *et al.*, 2015). They differ in their subcellular organisation (Li *et al.*, 2015) and in the use of energy source for the efflux of antibacterial drugs (Anes *et al.*, 2015).

These clever bacterial devised ABR strategies are causing adverse economic and health effects. Additional treatment costs related to ABR is estimated at €1500 million per year in the European Union and causes more than 25000 deaths per year (Domínguez-Gil *et al.*, 2016). Due to these significant economic and health implications, several organisations have conducted reviews and made recommendations to tackle the threat of ABR from an economic perspective. Notable ones include the O'Neill Commission commissioned by the UK Prime Minister in 2014, a report produced by the United States President's Council of Advisors on Science and Technology in 2014 and a comprehensive policy paper published by the Infectious Diseases Society of America in 2011. Targeting the discovery and development of novel antibiotics is a recommendation that is common to all the three reports (Dodds, 2016).

1.3.1.2 Biofilm resistance

CAUTI is one of the most common HCAI in critically ill patients and plays a marked role in morbidity and mortality (Stamm, 1998; Conway and Larson, 2012). According to Pickard *et al.* (2012) the daily risk of developing bacteriuria in catheterised patients is estimated at 5%, increasing to 35% after 7 days and 70% after 14 days of catheterisation. In 1995, additional treatment costs related to CAUTI's have cost the NHS £125 million per annum (Pickard *et al.*, 2012). Biofilms are of major clinical significance due to decreased susceptibility of uropathogens to antimicrobial products and increased antimicrobial resistance caused by plasmid exchange within the matrix (Niveditha *et al.*, 2012). Biofilms

aid bacteria to overcome the effects of the immune system and create resistance towards antibiotics.

To better understand biofilm resistance, three main mechanisms have been hypothesised (Figure 1.4):

Slow or incomplete antibiotic penetration into the biofilm

Many *In vitro* studies have shown that some antibiotics readily penetrate into the biofilm matrix. However, if the antibiotic is deactivated in the surface layers of the biofilm, penetration is significantly reduced (Stewart and Costerton, 2001). Even if the antibiotic is able to diffuse across the thick biofilm matrix, it may not be able to achieve an effective concentration or be able to reach its target due to physical and chemical properties of the matrix (Paraje, 2011).

Altered chemical microenvironment

Depletion of oxygen within deep layers of the biofilm and changes in pH due to accumulation of waste products can antagonise the action of antibiotics. Alternatively, bacteria can enter a non-growing state due to depletion of nutrients or excess accumulation of waste products; such bacteria are immune from killing as antibiotics like penicillin only target growing bacteria. Additionally, changes in the local osmotic environment can reduce cell envelope permeability to antibiotics (Stewart and Costerton, 2001).

Phenotypic changes

A subpopulation within the biofilm exhibits a type of cell differentiation, which is similar to spore formation. They form a highly protected phenotypic state, which is resistant to a wide variety of antibiotics and disinfectants (Stewart and Costerton, 2001). These cells are known as persister cells and their phenotypic variations offer resistance even for high level antibiotic treatment over a prolonged period of time (Paraje, 2011).

Owing to the above mechanisms (Figure 1.4), once biofilms are formed on catheters it becomes very difficult to eradicate them using traditional antibiotics.

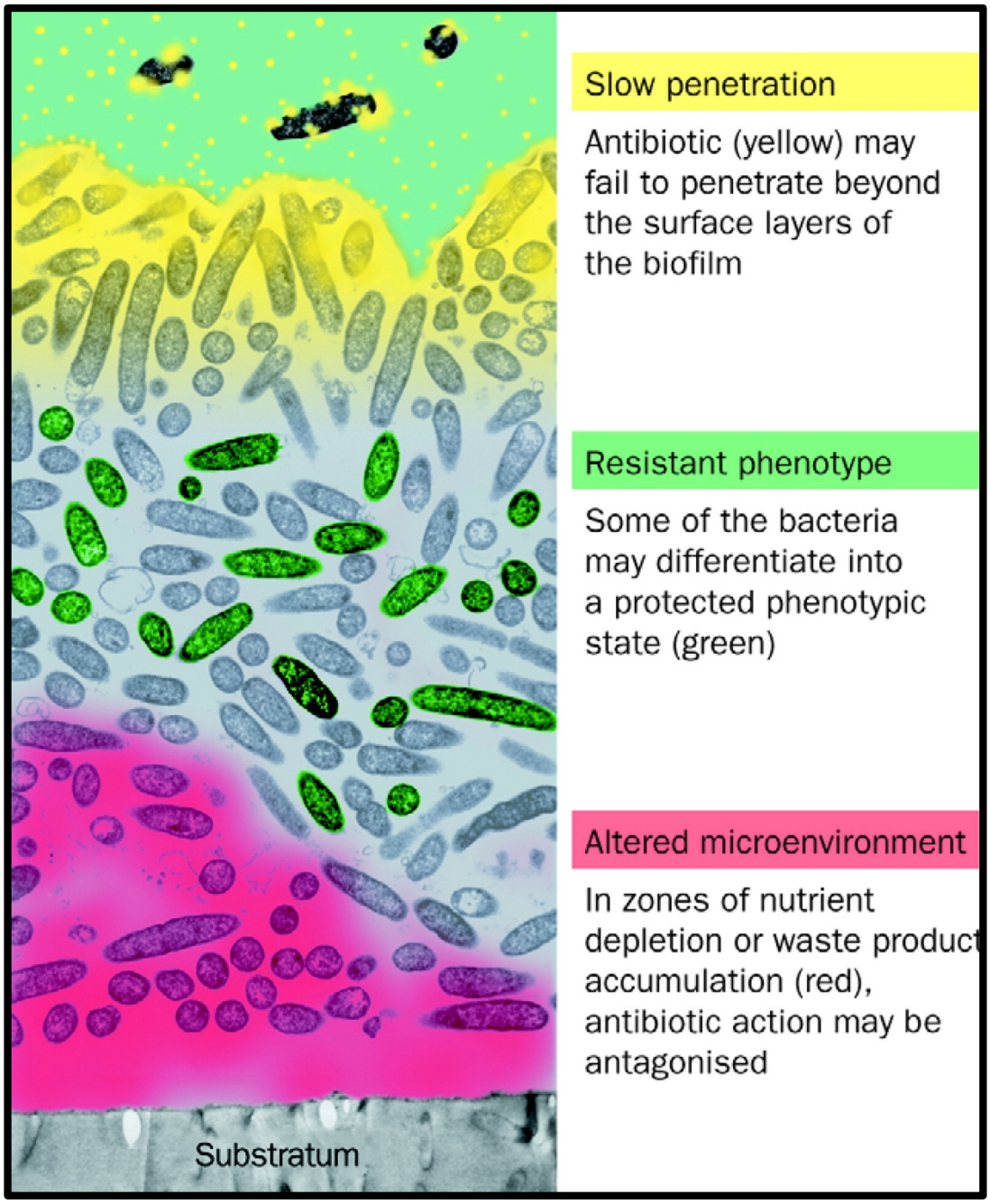

Figure 1.4: Hypothesised mechanisms of ABR in biofilms.

The substratum is the attachment surface and the aqueous phase containing the antibiotic molecules are shown at the top (green with yellow dots) (Stewart and Costerton, 2001). Reproduced with permission from Elsevier (license no: 4086620995643).

1.3.1.3 Resistance conferred by IBCs

The formation of IBCs in NHUCs are implicated in the pathogenesis of rUTIs (Anderson *et al.*, 2004; Rosen *et al.*, 2007; Robino *et al.*, 2013; Robino *et al.*, 2014; Scott *et al.*, 2015; Conover *et al.*, 2016). IBCs are also known to confer ABR (Anderson *et al.*, 2004; Barber *et al.*, 2013; Scott *et al.*, 2015). The IBC cycle (Figure 1.2) consists of association, invasion, survival and dispersal. In the survival stage, IBCs form closely packed, tight communities. This high-level organisation allows the bacteria to develop biofilm like traits making them resistant to most antibiotics (Scott *et al.*, 2015). IBCs can also form membrane-bound compartments within the deeper layers of NHUC and entangle themselves within the cellular actin filaments. This allows them to enter a dormant state and persist for long periods of time. This quiescent nature and intracellular localisation (Figure 1.2) enables them to develop resistance to most antibiotics and evade host defence mechanisms such as infiltrating neutrophils (Barber *et al.*, 2013; Lüthje and Brauner, 2016). Additionally, the prolonged course of antibiotics required for the treatment of rUTIs (Dason *et al.*, 2011) can increase the risk of ABR development in IBC strains.

Irrespective of the type of mechanism used to develop ABR, it is of paramount importance to understand the genetic and biochemical basis of resistance in order to devise novel therapeutic strategies to not only prevent UTIs but also the spread of ABR.

1.3.2 Alternative treatment strategies

An increase in ABR, ESBL positive strains, rUTIs and the sophisticated biofilm resistance mechanisms in CAUTIs has questioned the future use of antibiotics, and has stimulated significant interest in the non-antibiotic prophylaxis and treatment of UTIs (Stewart and Costerton, 2001; Beerepoot and Geerlings, 2016). Most of the current and emerging preventive strategies target bacterial colonisation and adherence (Beerepoot and Geerlings, 2016).

In vitro studies employing the use of pilicides to target type 1 pili and block bacterial adhesion, and mannosides to bind adhesion protein FimH and prevent bacterial adhesion to host receptors have shown promising results (Barber *et al.*, 2013). *Lactobacillus* preparations have also been used to limit bowel colonisation by uropathogens and thereby reduce the risk of contracting UTI via the ascending route. Clinical trials conducted in the United States and Netherlands have shown some evidence to suggest that *Lactobacillus* preparations are effective in preventing rUTIs (Foxman and Buxton, 2013). There is also some evidence to suggest that oestrogen replacement therapy in postmenopausal women increases the presence of *Lactobacilli* in the vagina, decreases *Enterobacteriaceae* vaginal colonisation and therefore decreases the incidence of UTIs (Beerepoot and Geerlings, 2016). Altarac and Papes (2013) have highlighted the prophylactic use of D-mannose in preventing rUTIs. They reported that the rates of rUTIs were significantly lower in groups receiving D-mannose as prophylaxis compared with the placebo group. D-mannose can inhibit adhesion of type 1 pili to NHUC (Beerepoot and Geerlings, 2016). It has been reported that fructose and a polymeric compound (unknown

origin) present in cranberries are effective in inhibiting *E. coli* adherence to urothelial cells. Evidence from the Cochrane review also highlights the potential use of cranberries in preventing rUTIs (Kucheria *et al.*, 2005). Several other studies have reported significant antibacterial and ant-biofilm activities of a variety of medicinal herbs (Lüthje and Brauner, 2016).

CAUTIs are responsible for prolonged hospital admissions, increased costs and significant morbidity (Makuta *et al.*, 2013). This has led to a deepened interest in developing strategies aimed at reducing or even preventing CAUTI's altogether. Targeting different stages of biofilm formation could serve as an alternative approach in preventing the formation of catheter-associated biofilms. Virtually all biofilms go through a process of adhesion, colonisation and dispersal (Figure 1.3). Inhibiting or disrupting biofilm formation at each of the above stages can prove to be effective in treating biofilm-associated infections (Kostakioti *et al.*, 2013).

Some anti-biofilm strategies currently being researched include catheter coatings, nanoparticles, iontophoresis, enzyme inhibitors, liposomes, bacterial interference, bacteriophages, quorum sensing inhibitors, low-energy surface acoustic waves and antiadhesion agents (Soto, 2014).

Developing novel urinary catheters that target biofilm formation is an interesting area of research. Silver alloy-coated latex catheters and silicone catheters impregnated with nitrofurazone are antimicrobial catheters that are widely available and routinely used in some UK hospitals. In order to measure the

efficacy of these antimicrobial catheters, the UK National Institute for Health Research (NIHR) conducted a catheter trial in NHS hospitals. Adult patients on STC were included in the study. The primary outcome of the trial demonstrated that nitrafurazone-impregnated catheters achieved an absolute CAUTI risk reduction at 2.1% compared to no risk reduction in silver alloy-coated catheters, suggesting that neither of the two catheters are effective in reducing CAUTI's (Makuta *et al.*, 2013). The disappointing outcome further stresses the importance of investigating the use of novel products (non-antibiotic) for developing antimicrobial catheters.

Jones *et al.* (2006) conducted a study using used catheterised bladder models infected with *P. mirabilis*, to investigate the efficacy of loading triclosan (antimicrobial agent) into silicone catheter balloons to determine its effect on catheter biofilm formation. They found that triclosan (10g/L) prevented catheter biofilm formation by *E. coli, K. pneumoniae, S. aureus* and *P. mirabilis* (Jones *et al.*, 2006).

Hachem *et al.* (2009) developed a novel gendine coated urinary catheter (GND-UC). Gendine is an antiseptic dye consisting of Gentian violet and chlorhexidine. Silicone urinary catheters coated with gendine were tested against seven microorganisms (*E. coli, P. aeruginosa, K. pneumoniae*, Vancomycin resistant *Eneterococcus*, *Candida albicans, Candida glabrata* and *Candida krusei*) for *in vitro* inhibition of biofilm formation. It was found that GND-UC reduced the CFUs of all organisms in comparison with uncoated catheters and silver hydrogel-coated catheters. This was further correlated in

an *in vivo* rabbit model inoculated with *E. coli.* Four days post inoculation bacteriuria was present in 60% of rabbits with uncoated catheter, 71% in those with silver hydrogel-coated catheters and none in GND-UC. The group concluded that GND-UC's were effective in preventing CAUTI's and clinical trials are needed to validate their findings.

Other studies using antiseptic coated central venous catheters including benzalkonium-chloride (Tebbs and Elliott, 1993) and chlorhexidine-silver sulfadiazine (Maaskant *et al.*, 2009) have shown promising results in eradicating biofilm formation.

Darouiche and Raad (1997) have devised a novel method of impregnating medical implants with an antimicrobial agent. Their invention uses a range of different agents (which promote penetration and enhance reactivity and receptivity of the catheter material) to prepare an effective antimicrobial composition that will penetrate the exposed surfaces and impregnate throughout the medical implant. This method can be used to develop novel antimicrobial catheters with relative ease.

Susceptibility of bacteria to antimicrobial agents on surfaces can be influenced by a number of factors including cell physicochemical properties, surface roughness, surface tension and the type of contact material (Mousavi *et al.*, 2016). Released-based coatings, contact-killing coatings and anti-adhesion coatings are some of the main approaches used in preparing antibacterial surfaces (Cloutier *et al.*, 2015). Figure 1.5 provides a graphical description of

various antimicrobial mechanisms (Singha *et al.*, 2017). The major challenges in the production of anti-biofilm catheters is uneven release kinetics and reservoir exhaustion issues (Jiao *et al.*, 2017). Several researchers have concentrated on novel drug delivery systems using non-antibiotic compounds to overcome these shortcomings. Use of liposomes, lipid and polymeric nanoparticles, and nanofibers have all been extensively researched (Mirtič *et al.*, 2016).

Quaternary ammonium compounds (QACs), chitosan, silver nanoparticles, antimicrobial peptides and antimicrobial enzymes are some of the commonly used non-antibiotic antimicrobial compounds (Jiao *et al.*, 2017).

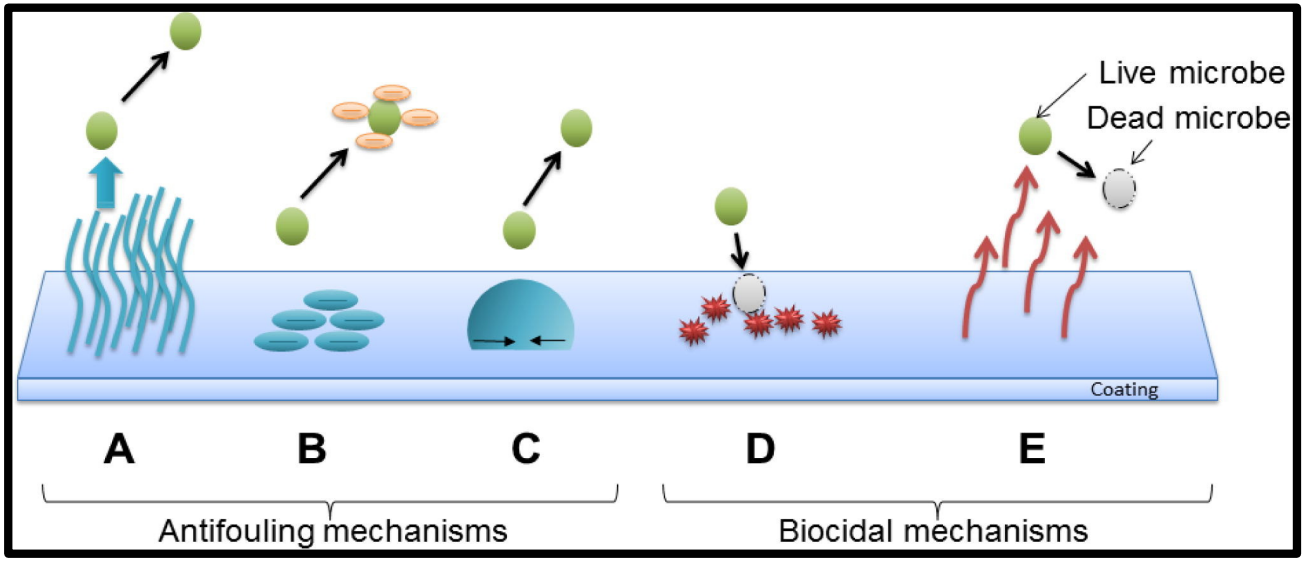

Figure 1.5: Different antimicrobial mechanisms.

Antifouling mechanisms include exclusion steric repulsion (A – physical attachment barrier), electrostatic repulsion (B – charge based attachment barrier) and low surface energy (C – low energy surfaces reduces microbial adhesion). Biocidal mechanisms include release-based coatings (D – coatings release biocides and kill microbes) and contact-killing coatings (E – coatings don't release biocides but kill microbes upon contact) (Singha *et al.*, 2017). Reproduced with permission from Elsevier (license no: 4086191248592).

1.3.2.1 Quaternary ammonium compounds

Several studies have incorporated QACs into polymers to prepare antibacterial (AB) biomaterials. This is a very promising area of research (Jiao *et al.*, 2017). QACs are a major class of cationic surfactants used as ingredients in a number of household and industrial products (Zhang *et al.*, 2015). Structurally, the representative formula for all QACs is $N^{+}R_1R_2R_3R_4X^{-}$, where N is the nitrogen atom attached to four different groups via covalent bonds. R can be a hydrogen atom, or an alkyl group or a substituted alkyl group and X represents an anion which most often belongs to the halide group. Most QACs are chloride or bromide salts and to a lesser extent iodide salts (Jiao *et al.*, 2017).

There is an increase in the utilisation of QACs in consumer products due to their broad spectrum AB activity and the ability to adapt and optimise their structure for specific functions. This has resulted in the formation of several generations of QACs (Melin *et al.*, 2014). In 1935, Domagk described the antimicrobial activity of benzalkonium chloride which is the earliest QAC that was developed as an AB compound (Kourai *et al.*, 2006). A sample of QACs currently available on the market are shown in Figure 1.6.

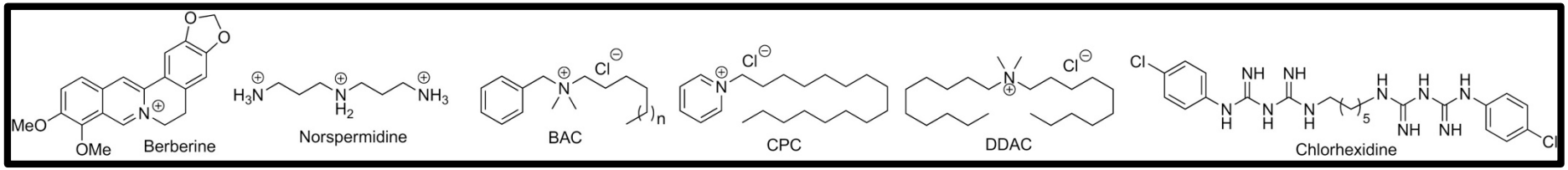

Figure 1.6: Naturally occurring and commercially available QACs.

MeO – methoxy, NH_3 – ammonia, NH_2 – amidogen, N – nitrogen, Cl – chlorine, BAC - benzalkonium chloride, CPC – cetylypyridinium chloride, DDAC - didecyldimethylammonium chloride (Minbiole *et al*., 2016). Reproduced with permission from Elsevier (license no: 4086161209269).

Bacterial cytoplasmic membranes are the primary targets of QACs. They bind to the membrane and cause disruption via their alkyl side chains (Sütterlin *et al.*, 2008). Cell leakage and membrane damage are common mechanisms shared by all QACs (Ioannou *et al.*, 2007). Factors that control the AB activity of QACs are molecular hydrophobicity, adsorbability, the electron density of the ammonium nitrogen atom and bacterioclastic activity (Kourai *et al.*, 2006). It has been previously reported that QACs with a 16-carbon hydrophobic chain length are more efficient in affecting the outer membrane of Gram-negative bacteria that shorter chain compounds (Ioannou *et al.*, 2007).

Although QACs are very potent AB agents, there have been reports of ABR to QACs especially among strains of MRSA. It has been reported that efflux pumps are responsible for QAC resistance (Minbiole *et al.*, 2016). However, the antimicrobial efficiency of QACs can be improved through modifications of the alkyl chain length, specifically via the substitution of aromatic ring hydrogen with chlorine, methyl, and ethyl groups. The newest generation of QACs that exhibit wide spectrum antibacterial action are twin-chain or dialkyl quaternary QACs (Melin *et al.*, 2014).

There is also some evidence to suggest that QACs possess anticancer (Solano *et al.*, 2015), anticandidal (Xian *et al.*, 2016) and antimalarial activity (Basilico *et al.*, 2015).

CPC is a QAC and its molecular formula is $C_{21}H_{38}ClN$ (Figure 1.6) (NCBI, 2004). Having a broad AB activity against biofilm formation and gingivitis, CPC

is commonly used in oral care products. It is also used as a disinfectant in private and public areas (Imai *et al.*, 2017). CPC is classified by the FDA as a safe and effective oral antimicrobial agent in a concentration range of 0.045% to 0.1%. It has a substantivity of between 3 to 5h (Elworthy *et al.*, 1996). CPC has a positively charged hydrophobic and hydrophilic region. It interacts with the negatively charged bacterial cell wall causing leakage of cellular components, disruption of bacterial metabolism, inhibition of cell growth, and cell death (FDA, 2003).

Oral care (Hwang *et al.*, 2013) and poultry processing (Li *et al.*, 1997; Arritt *et al.*, 2002; Beers *et al.*, 2006) are the two main areas of current research investigating the antimicrobial potential of CPC. Considering its potent antimicrobial activity and its cost effectiveness, CPC can be investigated for its potential use in treating UTIs, particularly CAUTIs.

Apart from many of the issues already discussed, bacteria have also developed other tactics to evade host immune response and bactericidal drugs. Of paramount importance is the use of *Acanthamoeba*, a single celled eukaryotic organism, as a reservoir.

1.4 Acanthamoeba

Acanthamoeba is a free-living amoeba (FLA) that exists in two forms: trophozoites or cysts. It is a ubiquitous protist that feeds on bacteria and other microorganisms. In the trophozoite stage, it replicates and can cause infections in humans. In unfavourable conditions, *Acanthamoeba* undergoes cystic transformation, which is the dormant, resistant stage of its life cycle. Its symbiotic relationship with bacteria has enhanced its importance in medical research (Siddiqui and Khan, 2012).

1.4.1 History

Antonie van Leeuwenhoek was the first person to observe free-living protozoa. The Greek word 'Protozoa' means 'first animal' and is generally used to refer to unicellular protists. Protists fall under Kingdom Protista, and amoebozoa are the largest group in this kingdom. *Entamoeba histolytica* was discovered in 1873 and since then a variety of amoebae including a number of FLAs were discovered. Figure 1.7 provides a summary of the discovery of *Acanthamoeba*. Since its discovery, a huge amount of interest is placed on *Acanthamoeba* research primarily due to its emerging medical importance (Sarmardella, 1999; Khan, 2009).

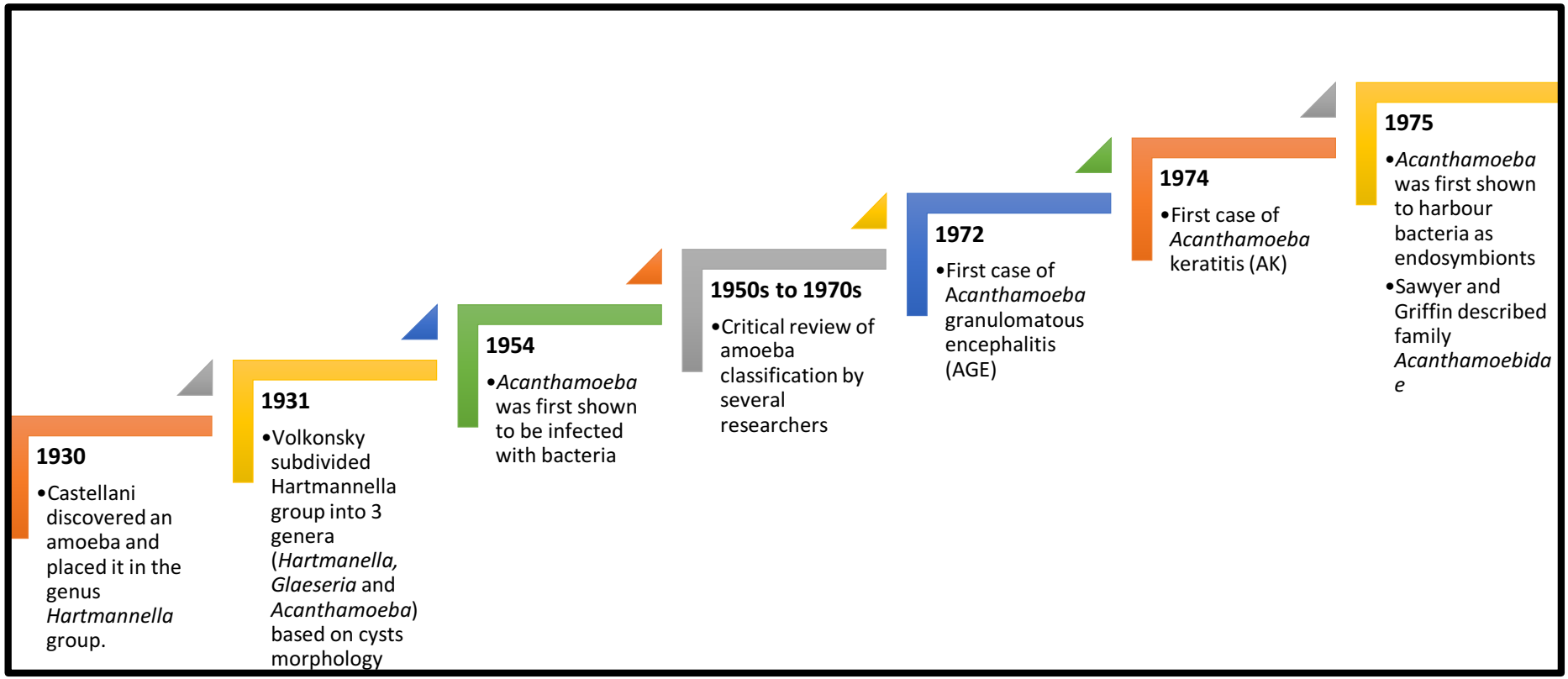

Figure 1.7: Timeline – discovery of *Acanthamoeba* (Khan, 2009).

1.4.2 Classification

The genus *Acanthamoeba* can be classified based on their morphological characteristics (cyst size and number of arms within a single cyst) into three groups (Khan 2006; Visvesvara *et al.*, 2007). This classification was established in 1931, however, it has been suggested that this classification is of limited phylogenetic value due to the effect of culture conditions on cyst morphology (Daggett *et al.*, 1985). *Acanthamoeba* can also be classified into genotypes based on their nuclear ribosomal small subunit (18s rRNA) gene sequences (Khan, 2006; Qvarnstrom *et al.*, 2013). Till date, 20 genotypes (T1 to T20) have been identified (Fuerst *et al.*, 2015). A new genotype (T21) has not yet been described in any publication but has been deposited in the NCBI GenBank with accession number CDEZ01000000 (NCBI, 2015). Several researchers are also attempting to classify *Acanthamoeba* based on its mitochondrial small subunit ribosomal RNA (16s rRNA) gene sequences (Booton *et al.*, 2001; Ledee *et al.*, 2003; Rahman *et al.*, 2013).

1.4.3 Ecology

FLAs are widely distributed in nature (de Jonckheere, 1991). Their distribution depends on a variety of factors including pH, temperature, salinity, soil texture, sulfhydric acid levels and bacterial populations (Rodríguez-Zaragoza, 1994). *Acanthamoeba* are the most common FLA found in nature, particularly in soil and water (Visvesvara, 1980). They have been isolated from air (de Jonckheere, 1991), soil (Neff, 1957; Weekers, 1993; Martinez, 1996; Kreuzer *et al.*, 2006; Reyes-Batlle, 2014) and a variety of water bodies (Lorenzo-Morales *et al.*, 2005; Liu *et al.*, 2006; Nuprasert *et al.*, 2010). Additionally,

Acanthamoeba have also been isolated from public water systems (Kilvington *et al.*, 2004; Jeong and Yu, 2005; Sente *et al.*, 2016), sewage systems (Nerad *et al.*, 1995; Schroeder *et al.*, 2001), swimming pools (Toczołowski *et al.*, 2000; Caumo *et al.*, 2009; Al-Herrawy *et al.*, 2014), bottled water (Visvesvara *et al.*, 2007; Siddiqui and Khan, 2012; Maschio *et al.*, 2015) and contact lenses (Woodruff and Dart, 1999; Ibrahim *et al.*, 2009; Gomes *et al.*, 2016). Isolation from the healthcare environment includes dialysis units (Dendana *et al.*, 2008; Hassan *et al.*, 2012) air conditioning systems (Ozçelik *et al.*, 2017), surgical instruments (Siddiqui and Khan, 2012), dental irrigation units (Trabelsi *et al.*, 2010; Hassan *et al.*, 2012; Retana-Moreira *et al.*, 2015) and the general hospital environment (Costa *et al.*, 2010; Fukumoto *et al.*, 2016). *Acanthamoeba* has also been isolated from mammals (Dyková *et al.*, 1999; Lorenzo-Morales *et al.*, 2007) including humans (Marciano-Cabral and Cabral, 2003). In the ecosystem, *Acanthamoeba* plays an important role by regulating bacterial populations including the structure of the microbial community, nutrient recycling (Siddiqui and Khan, 2012) and bacterial mineralisation of organic matter (Rodríguez-Zaragoza, 1994).

1.4.4 Lifecycle

Acanthamoeba has two stages in its life cycle: a vegetative trophozoite stage and a dormant cyst stage (Figure 1.8). The trophozoite stage is maintained under favorable environmental conditions such as temperature (30°C), pH (neutral) and osmolarity (50 to 80 mOsmol). The trophozoites measure from 12 to 40µm. They are uninucleated and contain other eukaryotic organelles such as mitochondria, ribosome, endoplasmic reticulum and Golgi complex. They

also have a contractile vacuole for osmoregulation. Pseudopodia known as acanthapodia assist the trophozoites with locomotion, surface adherence and prey capture. Maintenance of trophozoites also depends on abundant food supply. *Acanthamoeba* are known to feed on bacteria, yeast and algae, and contain numerous food vacuoles in their cytoplasm. They reproduce asexually via binary fission (Martinez, 1996; Visvesvara *et al.*, 2007; Khan, 2009; Siddiqui and Khan, 2012).

Under harsh environmental conditions such as extreme temperature, pH, osmolarity and lack of nutrients, *Acanthamoeba* undergoes encystation (Figure 1.8). The encystation process results in the formation of cysts with decreased levels of RNA, proteins, triacylglycerols and glycogen. A decrease in weight and diameter is also observed. The cysts measure from 5 to 20μm. They have thin, wrinkled double walls known as endo and exocysts. The former is primarily made up of cellulose whereas the latter consists of proteins and other polysaccharides. The inner wall makes contact with the outer wall at a number of points forming pores known as ostioles. Changes in the environment are detected via these pores. Cysts are highly resistant to antimicrobials and other chemical and physical treatments. They are airborne and can remain viable for several years (Page, 1967; Martinez, 1996; Dudley *et al.*, 2005; Niyyati *et al.*, 2013; Khan 2009).

Favorable conditions and availability of nutrients allow the cysts to undergo excystation and form trophozoites (Mazur *et al.*, 1995; Lloyd, 2014).

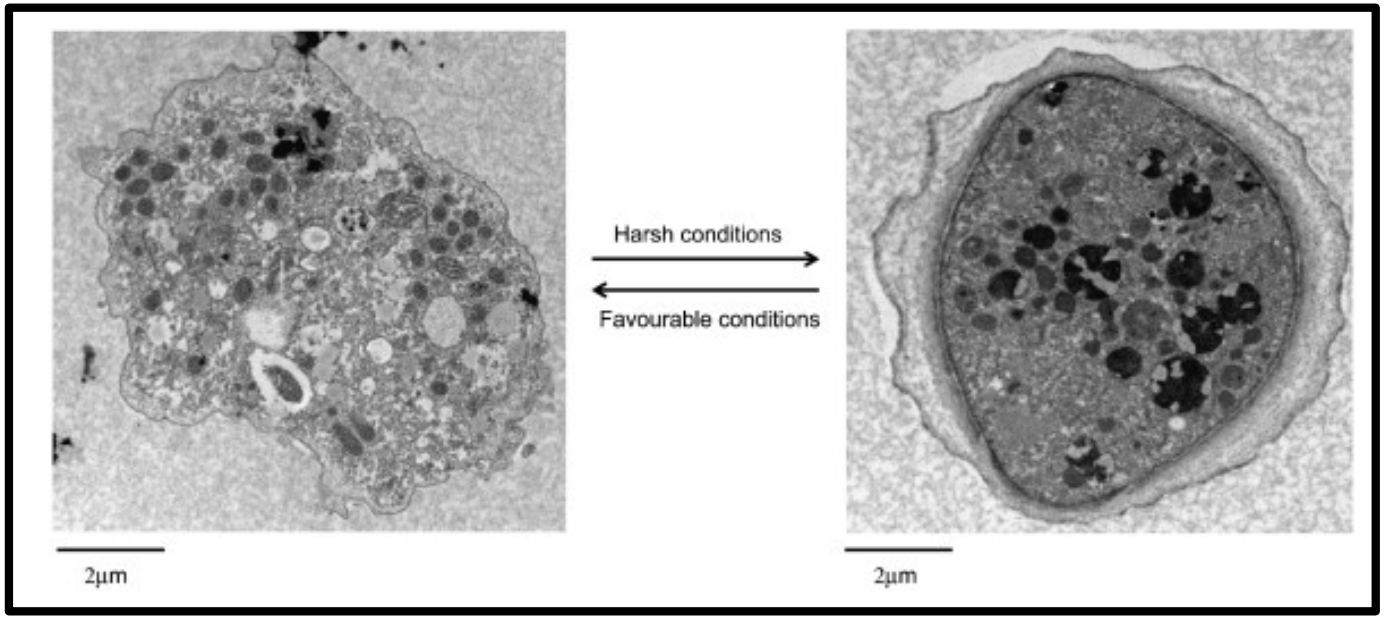

Figure 1.8: *Acanthamoeba* life cycle.

Under harsh conditions, the protozoa undergoes encystation and transforms into a dormant, double walled cyst. Cysts are highly resistant and can survive for several years. Under favourable conditions, *Acanthamoeba* can transform into a trophozoite via excystation. The trophozoites are highly infectious and divides via binary fission (Siddiqui and Khan, 2012). Reproduced under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0).

1.4.5 Feeding

Acanthamoeba actively feed in their trophozoite stage using phagocytosis and pinocytosis for nutrient uptake (Khan, 2009). They not only feed on bacteria, algae and fungi but also non-nutritive particles such as latex spherules. However, they exhibit the ability to distinguish between digestible and indigestible matter post particulate ingestion (Khan *et al.*, 2014). They can uptake solutes of varying molecular weights including leucine, glucose and albumin (Alsam *et al.*, 2005a). It is well known that *Acanthamoeba* preferentially grazes on Gram-negative bacteria (Rosenberg *et al.*, 2009) and prefer the water-air interface (Preston *et al.*, 2001). Amongst Gram-negative bacteria, *E. coli* and *K. pneumoniae* serve as excellent food sources (Weekers *et al.*, 1993; Huws *et al.*, 2005; Alsam *et al.*, 2006). It is therefore, suggested that sensations of taste and smell may play a role in *Acanthamoeba* feeding habits (Khan *et al.*, 2014).

Acanthamoeba uses pseudopodia to capture prey or ingests particulate matter via food-cup formation. Digestion happens in the food vacuoles and phagolysosomes (Abd *et al.*, 2009). Phagocytosis is similar in mechanism to that of human macrophages (Yan *et al.*, 2004). The mechanism involves recognition and binding of particles to cell surface receptors followed by engulfment via actin polymerisation (Lock *et al.*, 1987). It has also been suggested that phagocytosis is mediated by *Acanthamoeba* mannose receptors which recognises mannose rich elements in the food particles (Allen and Dawidowicz, 1990).

In the absence of particles in the culture medium, *Acanthamoeba* uses non-specific pinocytosis through membrane invagination for nutrient uptake (Bowers and Olszewski, 1972; Khan, 2006). Small vesiculation is the major pathway used and most vesicles occur at the surface level with a high surface to volume ratio. Water excretion via contractile vacuoles reduces the excessive water load on the amoeba caused by pinocytosis (Bowers and Olszewski, 1972).

Phagocytosis and pinocytosis in *Acanthamoeba* are partially independent processes and are subject to different controls (Chambers and Thompson, 1976; Bowers, 1977).

1.4.6 Pathogenesis

Factors promoting *Acanthamoeba* pathogenicity can be divided into contact-dependent and contact-independent mechanisms (Bennett *et al.*, 2014).

1.4.6.1 Contact-dependent mechanisms

Contact-dependent mechanisms include binding and phagocytosis (1.4.5) (Khan, 2009). Several studies have shown that adhesion or binding of *Acanthamoeba* to the surface of host tissues is mediated by a carbohydrate recognition system primarily mannose binding protein (MBP) (Yang *et al.*, 1997; Shin *et al.*, 2001; Alsam *et al.*, 2003; Imbert-Bouyer *et al.*, 2004; Yoo and Jung, 2012). The MBP is a 400kDa transmembrane protein with multiple subunits in the range of 130kDa (Panjwani, 2010) and is a major virulence protein (Garate *et al.*, 2005). Many studies have also shown that

Acanthamoeba binding is inhibited by free mannose and not any other carbohydrate (Allen and Dawidowicz 1990; Morton *et al.*, 1991; Alsam *et al.*, 2003) further strengthening the role played by MBPs. Previous studies have shown that pathogenic strains of *Acanthamoeba* that exhibit abundant MBPs readily binds to host cells and induces potent cytopathic effects in comparison with non-pathogenic strains (Garate *et al.*, 2006). Several other studies have reported the ability of *Acanthamoeba* to preferentially bind to fibronectin, laminin and type IV collagen (Gordon *et al.*, 1993; Hong *et al.*, 2004; Rocha-Azevedo *et al.*, 2009; Clarke *et al.*, 2013) making it easier to bind to host cells, invade and cause significant destruction (Sissons *et al.*, 2005).

1.4.6.2 Contact-independent mechanisms

Contact-independent mechanisms include extracellular proteases (EPs), phospholipases (PLs) and other indirect virulence factors (Khan, 2009). *Acanthamoeba* produces three types of proteases: serine, cysteine and metalloproteases (Lorenzo-Morales *et al.*, 2013). Serine proteases of varying molecular weight: 12, 40, 42, 55, 70, 85, 97, 107, 130, 133 and 230 kDa are the predominant type of proteases secreted by various *Acanthamoeba* genotypes (Dudley *et al.*, 2008). These proteases play an important role in tissue invasion, migration (Khan, 2000) catabolism of host proteins, cytoadherence, and both stimulation and evasion of host responses (Sissons *et al.*, 2006). They induce damage to collagen and degrade glycoproteins (de Souza Carvalho *et al.*, 2011). It has also been suggested that the EPs are important for survival, multiplication and cellular transformation of *Acanthamoeba* (Dudley *et al.*, 2008). Although serine proteases are

predominant, metalloproteases also play a role in *Acanthamoeba* pathogenicity. It has been previously suggested that a metalloprotease of approximately 150kda produced by *A. castellanii* is implicated in human infections (Alsam *et al.*, 2005b). Another type of contact-independent mechanism is the secretion of PLs. It has been suggested that PLs may be involved in cell membrane disruption and cell lysis due to their ability to hydrolyse ester bonds in glycerophospholipids (Matin and Jung, 2011; Lorenzo-Morales *et al.*, 2015). A1, A2, B, C, and D are the five major known PLs (Matin and Jung, 2011). Tripathi *et al.* (2013) have previously shown that PL A2 from *A. castellanii* trophozoites induces apoptosis in corneal epithelial cells. It has previously been reported that, at 37°C and at neutral pH, *Acanthamoeba* exhibits optimal PL activity. This observation further strengthens the physiological relevance of these enzymes (Matin and Jung, 2011). Further characterisation of these PLs may provide important targets for anti-*Acanthamoeba* therapeutics. Other indirect virulence factors are related to the ability of *Acanthamoeba* to survive in varied temperature, osmolarity and pH ranges, their cell morphology, phenotype switching, drug resistance and ubiquity (Khan, 2009).

1.4.7 Human infections

Acanthamoeba is known to cause three human infections: granulomatous amoebic encephalitis (GAE), *Acanthamoeba* keratitis (AK) and a disseminated infection (DI) (CDC, 2013).

1.4.7.1 Granulomatous amoebic encephalitis

GAE is a rare infection, first identified in 1972 (Jager and Stamm, 1972). It involves the central nervous system (CNS), is typically limited to immunocompromised patients and is usually fatal (Walochnik *et al.*, 2008). Less than 200 cases of GAE have been described worldwide (Aichelburg *et al.*, 2008). Entry into the CNS can be via the olfactory route (nasal cavity – olfactory bulb) and the haematogenous route (skin cuts – blood vessels; nasal cavity – lungs – alveolar blood vessels) (Martinez, 1991; Walochnik *et al.*, 2008). Either way, the amoeba has to interact with the blood brain barrier (BBB) and invade the CNS (Khan, 2008). Confusion, headaches, seizures, sensory problems, increased intracranial pressure and changes in personality are typical symptoms (Stalin *et al.*, 2013; Salameh *et al.*, 2015). GAE is characterised by brain abscesses and lesions including multinucleated giant cells, histiocytes and inflammatory cells, necrotising vasculitis, and inflammatory perivascular infiltrate (Martinez *et al.*, 2000). A combination of antimicrobial drugs is the current treatment strategy. Sulfamethoxazole, rifampin, and ketoconazole; and pentamidine, sulfadiazine, flucytosine, fluconazole, or itraconazole are some combinations that have been previously used (Webster *et al.*, 2012). Neurosurgical intervention may also be required (Parija *et al.*, 2015).

1.4.7.2 *Acanthamoeba* keratitis

The first case of AK was described in 1974 (Nagington *et al.*, 1974). It is a rare but severe sight-threatening infection of the cornea. Although a high number of reported cases are related to the use of contact lens, AK can affect non-contact

lens wearers as well (Lorenzo-Morales *et al.*, 2015). The main clinical manifestations are corneal ulceration, diffuse stromal infiltration, and ring infiltration (Sun *et al.*, 2006) causing eye redness, ocular pain, photophobia, blurred vision and excessive tearing (Lorenzo-Morales *et al.*, 2013). AK can be divided into three stages. In the early stage, lesions are primarily located in the corneal epithelium and are characterised by pseudodendritic or punctuate lesions, dot infiltrations and recurrent erosions. The advanced stage is characterised by deep stromal ulcers with pale or yellowish-white purulent infiltrations and tissue necrosis. Increase in diameter of stromal ulcers, anterior chamber empyema, and thinning and perforation of corneal ulcers are characteristic of the late stage (Jiang *et al.*, 2015). Treatment strategies include a combination of anti-acanthamoeba agents, debridement and cauterisation (Sun *et al.*, 2006).

1.4.7.3 Disseminated infection

DI is a rare infection of extracerebral organs characterised by widespread granulomatous infiltration (Aichelburg *et al.*, 2008). It mainly affects the skin, sinuses and lungs independently or in combination (CDC, 2013). It usually occurs in immunocompromised patients and is generally fatal since most reported cases of DI has progressed to GAE (Slater *et al.*, 1994). Although there is no established therapeutic protocol for DI, 5-fluorocytosine, pentamidine and itraconazole (Koide *et al.*, 1999); pentamidine isethionate, topical chlorhexidine gluconate, and 2% ketoconazole cream (Slater *et al.*, 1994), and miltefosine (Aichelburg *et al.*, 2008) have been previously used with varying degrees of success.

From a clinical aspect, these protozoans have become increasingly important in the recent years due to a rise in the number of cases related to human infections (Gomes *et al.*, 2016).

1.4.8 Underlying issues

Apart from the isolation of *Acanthamoeba* from human samples related to the diseases already mentioned (1.4.7), the amoeba has also been isolated from humans not suffering from any *Acanthamoeba*-related infections. The amoeba has been previously isolated from human faeces (Mergeryan, 1991), nasal mucosa and throats of healthy people (Cerva *et al.*, 1973; Mergeryan, 1991; Newsome *et al.*, 1992; Michel *et al.*, 1997). It has also been isolated from sputum samples of patients suffering from pneumonia (Bradbury *et al.*, 2014). Anti-*Acanthamoeba* antibodies have also been detected in apparently healthy people (Gillespie and Pearson, 2003). These observations suggest that *Acanthamoeba* can live in the human body without causing any of the well-known *Acanthamoeba* infections.

It is also well known that bacteria have developed different tactics to resist phagocytosis and multiply within *Acanthamoeba*. Several studies have confirmed that bacteria can indeed use *Acanthamoeba* as a Trojan horse. Abd *et al.* (2003) have shown that, the highly infectious, facultative bacterium *Franisella tularensis* has the ability to grow and survive intracellularly in *A. castellanii.* They found the bacterium in intact trophozoites, excreted vesicles and cysts. Many other studies have been conducted to demonstrate the endosymbiotic relationship between *Acanthamoeba* and bacteria. *Vibrio*

cholera (Abd *et al.*, 2004), *E. coli* (Alsam *et al.*, 2006) and neuropathogenic *E. coli* (Yousuf *et al.*, 2014) have all been able to survive and multiply within *Acanthamoeba*.

This special relationship can facilitate a variety of things including increasing the pathogenicity of intracellular bacteria, gene transfer between bacteria and amoeba, microbial survival in inhospitable environments and increased protection from immune response and antibiotics (Lovieno *et al.*, 2010; Bennett *et al.*, 2014). Ultimately, this can lead to fulminant infections in humans (Bennett *et al.*, 2014).

More recently, *Acanthamoeba* has been isolated from human urine samples. Santos *et al.* (2009) conducted a study based on the hypothesis that uropathogenic bacteria can survive the effects of antimicrobial drugs, disinfectants and host immunity by potentially using *Acanthamoeba* as a protective tool. They evaluated 63 urine samples collected from indwelling catheters of critically ill patients. In an interesting finding, 23% of these samples tested positive for the presence of *Acanthamoeba spp*. This further suggests that *Acanthamoeba* can coexist with bacteria in humans without causing any of the well known *Acanthamoeba*-related infections. The type of relationship they develop within their co-existence is of considerable interest. Further research on this endosymbiotic relationship should address the issue of the actual impact of this relationship on human diseases, which, at the moment, remains unclear.

1.5 Aims and objectives

ESBL positive and negative strains of *E. coli* and *K. pneumoniae*, and *P. mirabilis* are uropathogens which are commonly associated with UTIs including rUTIs and CAUTIs. The main pathogenetic mechanisms are formation of IBCs in urothelial cells and formation of biofilms on urinary catheters. *Acanthamoeba* is a FLA widely distributed in the environment and acts as a reservoir for a range of pathogens. It causes life threatening human infections and has also been isolated from apparently healthy people. From a clinical perspective, Its endosymbiotic relationship with bacteria is of considerable interest. The primary issue explored in this thesis is the relationship between uropathogens and pathogenic *A. castellanii* (T4), and to determine if this association may be attributed to a cause-and-effect relationship with regards to UTIs.

The main aims of this thesis are to

- Investigate the biofilm forming ability of uropathogens
- Investigate uropathogenic IBC formation and its cytotoxic effect on NHUC
- Explore the endosymbiotic relationship between uropathogens and *A. castellanii* (T4)
- Investigate the presence of *Acanthamoeba* in urine samples obtained from patients diagnosed with UTI
- Explore the antimicrobial efficacy of CPC and its potential use in treating UTIs particularly CAUTIs

Chapter 2

Evaluation of biofilm formation by uropathogens

2 Evaluation of biofilm formation by uropathogens

2.1 Introduction

UTIs are the second most common HCAI in England accounting for 17.2% of the total HCAIs (HPA, 2011). UTIs are caused by a variety of pathogens including *E. coli, K. pneumoniae* and *P. mirabilis* (Flores-Mireles *et al.,* 2015). UPEC are the most commonly isolated bacteria in UTI (Flores-Mireles *et al.,* 2015).

"Urinary catheterisation is a medical procedure used to drain and collect urine from the bladder" (NHS, 2013) and is used for a variety of purposes including diagnosis and therapy (Ramakrishnan and Mold, 2005). In a prevalence survey conducted in 66 European hospitals, 17.5% of patients had a urinary catheter making it the most commonly used indwelling medical device (Nicolle, 2014). In England 43% of nosocomial infections were associated with the use of an indwelling catheter (HPA, 2011) costing the NHS millions of pounds (Pickard *et al.*, 2012). The use of indwelling catheters is directly related to the occurrence of CAUTIs (Meddings *et al.,* 2013) because of the ability of bacteria to readily form biofilms on catheters (Hatt and Rather, 2008). CAUTIs lead to significant complications in patient care particularly when the catheter lumen gets blocked by crystalline biofilms caused by *P. mirabilis* infections which can often lead to pyelonephritis and septicaemia (Stickler, 2008). A thorough understanding of the mechanism involved in biofilm formation is key to tackling this notorious problem (Stewart and Costerton, 2001).

Bacteria can live in a free-floating, planktonic state or in a sessile biofilm mode. Bacterial communities in biofilms are highly organised due to the formation of specialised structures (Costerton *et al*., 1999). Biofilms consist of host components, bacteria and their extracellular products (Trautner and Darouiche, 2004). They form a matrix with extracellular materials known as EPS which primarily consists of biopolymers such as proteins, polysaccharides, lipids and nucleic acids (Lawrence *et al.,* 2003). EPS forms an interconnected 3D polymer mesh-like network and provides mechanical stability to the biofilms (Flemming and Wingender, 2010). Apart from facilitating the acquisition of new genetic traits between biofilm cells via horizontal gene transfer and providing a nutrient rich micro-niche for the bacteria, EPS also confers resistance to host responses, antimicrobials and environmental stresses (Kokare *et al.,* 2009; Flemming and Wingender, 2010).

Biofilm formation is an extremely complex process and the mechanism differs between bacterial species. However, it is generally recognised to consist of five stages: 1) formation of a conditioning layer (usually by host components), 2) initial reversible microbial adhesion, 3) irreversible microbial adhesion and microcolony formation 4) maturation and 5) detachment and dispersal (Percival *et al*., 2011; Rendueles and Ghigo, 2012).

Apart from planktonic and biofilm states, flagellated bacteria can also enter highly motile states such as twitching, gliding, sliding, swimming and swarming (Kearns, 2010). The latter two modes of motility are commonly linked to biofilm formation (Maya-Hoyos *et al.,* 2015). Swimming motility is linked to individual

cells and is an adaptive chemotactic response whereas swarming is regarded as a behavioural response which requires bacteria to undergo differentiation into specialised swarmer cells (Calvio *et al.,* 2005). Besides motility and environmental factors, physical and chemical surface properties and chemical communication between cells also influence biofilm formation (Renner and Weibel, 2010).

Although a variety of strategies normally aimed at prevention, weakening, disruption and killing of biofilms (Bjarnsholt *et al.,* 2013) are being tried and tested, biofilms continue to be a great cause for concern. Understanding the complexities of these multicellular communities and the factors which promote their existence is necessary to develop effective therapeutic strategies.

In this chapter, the biofilm forming ability of uropathogens were investigated. Bacterial strains isolated from patients diagnosed with UTIs were identified using polymerase chain reaction (PCR) analysis and their antimicrobial susceptibility patterns were explored. Artificial urine medium (AUM) was used in lieu of human urine and AUM's efficacy in terms of bacterial growth was tested. The main methodological approach taken in this study is based on the various stages of biofilm formation. Firstly, the planktonic and biofilm growth patterns of uropathogens were studied. Then, the effect of a conditioning layer and nutrient availability on biofilm formation was investigated and finally the differential expression of proteins in planktonic and biofilm bacteria was examined. Furthermore, bacterial motility assays were conducted to investigate any possible links between motility and biofilm formation.

2.2 Materials and methods

All chemicals were purchased from Sigma unless otherwise stated. The total volume of media was made up to 1000µl (per well) in all 24-well plate experiments unless otherwise stated.

2.2.1 Bacterial strains

Clinical pathogens isolated from patients diagnosed with UTIs were kindly provided by Dr. Tony Elston from Colchester Hospital University Foundation Trust (CHUFT). *E. coli* pathogenic (ESBL negative), *E. coli* (ESBL positive), *K. pneumoniae* (ESBL negative)*, K. pneumoniae* (ESBL positive) and *P. mirabilis* were used in this study. All strains were maintained on Cysteine lactose electrolyte deficient (CLED) agar and refreshed every week. All strains were refreshed every month from frozen stock.

2.2.2 Bacterial culture

For all experiments (unless otherwise stated), the bacterial culture was grown at 37°C for 4 to 6h (log or exponential growth phase) in Luria Bertani (LB) broth. The optical density (OD) of bacteria was measured in LB broth using a spectrophotometer [Cecil CE 2041] at a wavelength of 595nm and adjusted to give an OD of 0.22 (~ 10^8 CFU/ml). For each experiment (unless otherwise stated), a sufficient amount (~ 10^6 CFU/well) of bacteria was transferred into a 24-well plate and the final volume in each well was made up to 1000µl by adding the required medium.

2.2.3 Urinary catheters

Silicone and latex (PTFE and hydrogel) urinary catheters (Ch 12) were kindly provided by Dr. Tony Elston from CHUFT. Catheters were aseptically cut into approximately 1cm pieces, sterilised in 70% ethanol and air-dried. The catheter pieces were stored in sterile petri dishes at room temperature (RT).

2.2.4 Artificial urine medium

AUM was prepared based on the analysis of human urine constituents (Brooks and Keevil, 1997). The medium corresponds to dilute urine. All components (Table 2.1) were mixed on a magnetic stirrer and the pH was adjusted to 6.5. The medium was then sterilised by passing through a 0.2µm nylon membrane filter under vacuum suction. AUM was then aliquoted into 50ml tubes and freeze stored.

Table 2.1: AUM composition (Brooks and Keevil, 1997).

Component	Quantity (g)
Peptone L37	1
Yeast extract	0.005
Lactic acid	0.1
Citric acid	0.4
Sodium bicarbonate	2.1
Urea	10
Uric acid	0.07
Creatinine	0.8
Calcium chloride.$2H_20$	0.37
Sodium chloride	5.2
Iron II sulphate.$7H_20$	0.0012
Magnesium sulphate.$7H_20$	0.49
Sodium sulphate.$10H_20$	3.2
Potassium dihydrogen phosphate	0.95
Di-potassium hydrogen phosphate	1.2
Ammonium chloride	1.3
Distilled sterilised (ds) water	to 1L

2.2.5 Polymerase chain reaction

2.2.5.1 DNA extraction

To extract DNA, overnight cultures of uropathogens grown in LB broth at 37°C were boiled in a water bath (95 ºC) for 15min, centrifuged (1000 x g, 15min) and the supernatants were used for PCR analysis.

2.2.5.2 PCR cycling setup

Specific primers (Table 2.2) were used for PCR amplification. PCR was performed in 50µl reaction mixtures (buffer 10X containing 5µl (KCl, Tris-$MgCl_2$), 3µl $MgCl_2$, 1µl 10mM dNTPs, 1µl forward and reverse primers, 1µl DNA, 0.5µl Taq polymerase, and 38µl ds water). The PCR cycle profile was set up as listed in Table 2.3. The total PCR product with sample loading buffer (5:1) was run on safe view DNA-stained 0.5% agarose gel at 150v for 15min. Finally, PCR product was detected in the gel under UV light.

Table 2.2: Gene and primer sequences.

Target gene	Primer (nucleotide sequence)	Product length (bp)	Reference
E. coli 16S rRNA	F 5'-CCCCCTGGACGAAGACTGAC-3'	450	Wang *et al.,* 2002
	R 5'-ACCGCTGGCAACAAAGGATA-3'		
TEM-1	F 5'-ATGAGTATTCAACATTTCCG-3´	850	Chroma and Kolar, 2010; Kolar *et al.,* 2010
	R 5´-CCAATGCTTAATCAGTGAGC-3´		
CTXM	F 5'-ATGTGCAGYACCAGTAARGT-3'	544	Ramadan *et al.,* 2016
	R 5'-TGGGTRAARTARGTSACCAGA- 3'		
SHV	F 5'-TTAACTCCCTGTTAGCCA-3'	768	Varkey *et al.,* 2014
	R 5'-GATTTGCTGATTTCGCCC-3'		
UreC	F 5'-CCGGAACAGAAGTTGTCGCTGGA-3'	533	Takeuchi *et al.,* 1996
	R 5'-GGGCTCTCCTACCGACTTGATC-3'		

Table 2.3: PCR cycling setup.

E. coli 16s rRNA (Wang *et al.,* 2002), TEM-1 (Chroma and Kolar, 2010; Kolar *et al.,* 2010), CTXM (Ramadan *et al.,* 2016), SHV (Varkey *et al.,* 2014) and UreC (Takeuchi *et al.,* 1996).

Setup	***E. coli* 16s rRNA**		**TEM-1**		**CTXM**		**SHV**		**UreC**	
	30 cycles		30 cycles		32 cycles		35 cycles		30 cycles	
	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)
Initial denaturation	95	08:00	95	05:00	94	05:00	94	03:00	94	03:00
Denaturation	95	00:30	94	00:30	94	00:45	94	00:30	94	01:00
Annealing	59	00:30	55	00:60	50	00:40	50	00:30	63	00:30
Extension	72	00:30	70	00:60	72	01:00	72	02:00	72	01:00
Final extension	72	07:00	75	07:00	72	10:00	72	10:00	72	07:00

2.2.6 EUCAST susceptibility test

Disk diffusion method standardised by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) was used. The antibiotic discs were chosen based on the recommendations made by the EUCAST expert rules in antimicrobial susceptibility testing (Leclercq *et al.,* 2011). Overnight inoculum suspension of uropathogens grown in LB broth at 37°C was spectrophotometrically standardised to a density of approximately 10^8 CFU/ml. Within 15min of preparation, a sterile cotton swab was dipped into the adjusted inoculum and extra fluid was remove by pressing the swab against the inside of the container. The inoculum was spread evenly on Mueller Hinton (MH) agar by swabbing in three different directions. Within 15min of inoculation, antibiotic discs were applied and the plates were incubated for 18-24h at 37°C. After incubation, the diameters of zones of inhibition was measured with a ruler (EUCAST, 2015).

2.2.7 Growth assays in differential media

Uropathogens (~10^6 CFU/ml) were inoculated in differential media (as listed below) in a 24-well plate. The plate was incubated at 37°C for 24h. After the incubation period, the planktonic suspension was diluted and cultured on CLED agar and the CFUs were counted after 18h of incubation at 37°C. The following media were used: LB broth, Peptone yeast extract glucose (PYG) broth, Human urine (HU), AUM, AUM without urea (urea –ve), AUM without uric acid (uric acid –ve) and AUM without creatinine (creatinine –ve).

Human urine was collected from healthy male volunteers (18 – 35 age group). The urine samples were streaked on to CLED agar and incubated at 37°C for 24h. All samples that were negative for bacterial growth were mixed, aliquoted into 50ml tubes and freeze stored. To determine the effect of urea, uric acid and creatinine on uropathogens, samples of AUM without the aforementioned components were prepared as described in section 2.2.4.

2.2.8 Microtitre plate assays

Uropathogens (~10^6 CFU/ml) were inoculated in AUM in a 24-well plate and the plate was incubated at 37°C for various time periods (1h, 2h, 3h, 4h, 5h, 6h, 12h, 24h, 48h and 72h).

2.2.8.1 Planktonic assays

After each incubation period (2.2.8), the suspension in each well was diluted and cultured on CLED agar and the CFUs were counted after 18h of incubation at 37°C.

2.2.8.2 Biofilm viability assays

After each incubation period (2.2.8.2), the supernatant from each well was discarded, wells were washed twice with 500µl PBS and the biofilm was scraped using a cell scarper and enumerated on CLED agar.

2.2.8.3 Biofilm biomass assays

The biofilm biomass was assayed by crystal violet (CV) staining. Each well (2.2.8) was washed three times with 500µl PBS and air-dried. The bacteria

were then fixed by adding 250µl of 99% methanol for 15min, aspirated and then allowed to air dry. The wells were then stained with 1% CV solution for 5min. Excess stain was removed by washing three times with 500µl PBS. When the wells had dried, light microscope images were taken. The stain was then resolubilised in 160µl of 33% glacial acetic acid and absorbance was read at an OD of 570nm using a plate reader (Verhoeven *et al.,* 2010).

2.2.9 Catheter biofilm assays

Bacterial biofilms were grown on catheter pieces by placing each piece in a 24-well plate containing approximately 10^6 bacteria per well in their log or exponential growth phase. The final volume in each well was made up to 1ml by adding AUM. The plate was incubated for 24h at 37°C to allow biofilm formation. After incubation, the catheter pieces were washed once with PBS, sonicated in an ultrasonic bath at 38.5 KHz for 5min and vortexed for 30s. The suspension was diluted and cultured on CLED agar and the CFUs were counted after 18h of incubation at 37°C (Kadurugamuwa *et al.,* 2003).

2.2.9.1 Bacterial adherence assays

An artificial bladder model was used to form a conditioning layer on PTFE latex urinary catheters. The model (Figure 2.1) consisted of an inlet used as a reservoir for AUM connected to a peristaltic pump. The pump ensured flow of urine at the rate of 1ml per minute. The pump was connected to a plastic bottle, which served as an artificial bladder. A latex catheter was inserted into the bottle through an outlet at the base and the catheter was connected to a beaker for drainage. AUM was pumped into the plastic bottle so that a residual volume

collected below the catheter eyepiece before flowing through the catheter and collecting in the beaker. The entire model including the tubing used to connect the different parts was sterilised by autoclaving. The plastic bottle and the catheter were maintained at 37°C in an incubator (Stickler *et al.,* 1999). The bladder model was assembled and supplied with AUM for 1h after which the catheter was removed from the model. Catheters with and without a conditioning film were aseptically cut into approximately 1cm pieces. Uropathogens (~10^6 CFU/ml) were inoculated in AUM in a 24-well plate. Sterile catheter pieces (~ 1cm) with and without conditioning layer were added into each well and the plate was incubated for 3h at 37°C. After the incubation period catheter biofilms were enumerated on CLED as described above (2.2.9 (Kadurugamuwa *et al.,* 2003).

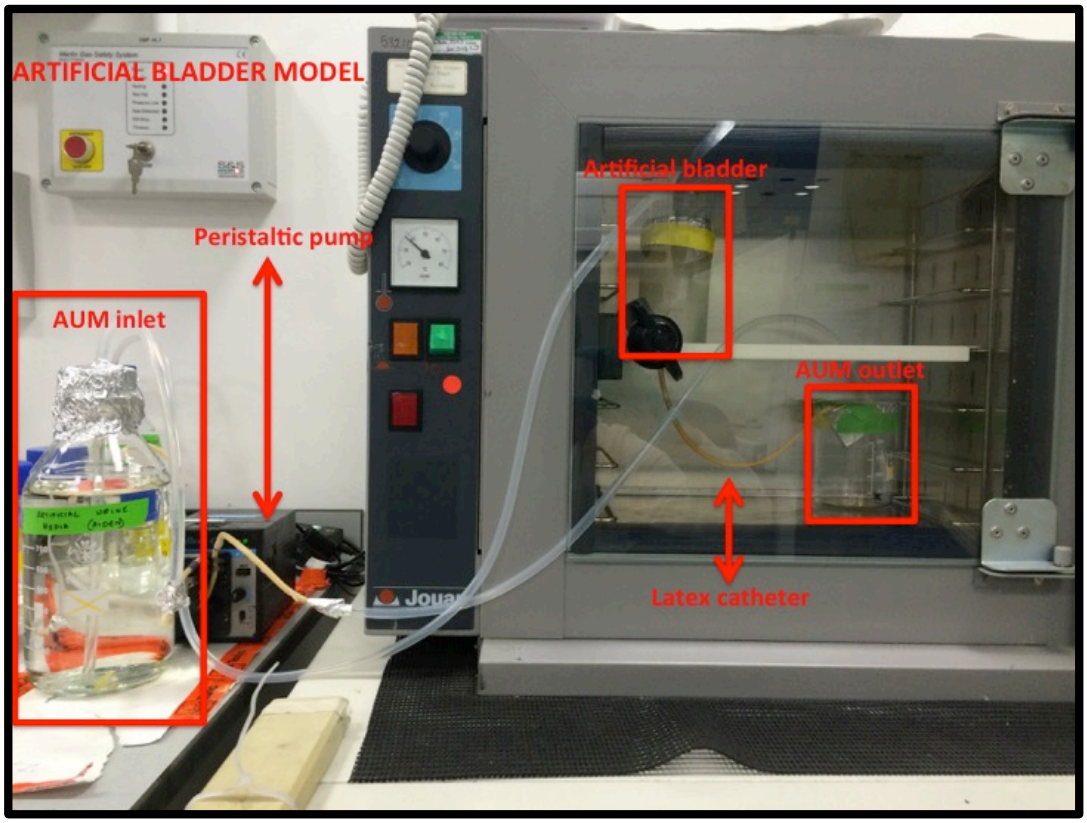

Figure 2.1: Artificial bladder model (Stickler *et al.,* 1999).

2.2.10 Biofilm nutrition assays

Biofilms were grown in 24-well plates as described above (2.2.8) for 24h, 48h and 72h. After each incubation period, the medium was discarded and the wells were washed with PBS. Fresh AUM (control) or ds water was added and the plates were incubated at 37°C for 24h. For biofilm dispersal, after the incubation, an absorbance value of the planktonic bacteria was measured spectroscopically at 595nm using AUM as blank. For biofilm biomass, CV assays were performed as described in 2.2.8.3 (Reuter *et al.,* 2010).

2.2.11 Biofilm EPS

2.2.11.1 EPS extraction

Uropathogens (~10^6 CFU/ml) were inoculated in AUM in T25 flasks. The final volume in each flask was made up to 5ml by adding AUM. The flasks were incubated at 37°C for various time periods (24h, 48h and 72h) to allow bacterial biofilm formation. After each incubation period, the medium was collected (for planktonic suspension) from each flask, the flasks were washed in 5 ml PBS and the biofilms were scraped using sterile pipette tips. The scraped biofilms (suspended in 5ml PBS) and the supernatants (planktonic suspension) were centrifuged (3700 x g, 15min). The pellets were re-suspended in 10ml 0.22% formaldehyde in 8.5% sodium chloride and incubated for 2h at 4°C. After incubation, the samples were centrifuged (3700 x g, 15min, 4°C) and the pellets containing EPS were further re-suspended in 10ml ds water. Each of this suspension was centrifuged (3700 x g, 15min, 4°C) again to wash away any non-EPS material and the weight of the pellets were determined. Fifty ml distilled water per gram of the pellet was added to each pellet and the purified

EPS in the pellets were collected by centrifugation (3700 x g, 15min, 4°C). The pelleted EPS were resuspended in 5ml 10^{-2}M potassium chloride and 10ml cold ethanol and the EPS were precipitated by incubating overnight at 4°C. After incubation, the EPS mixtures were centrifuged (3700 x g, 15min, 4°C), medium decanted and 10ml ds water was added to each of the final pellets. This method is known as the ethanol extraction method (Gong *et al.,* 2009). Following extraction, the composition of EPS was evaluated using colorimetric methods.

2.2.11.2 Protein estimation

Protein concentration in the EPS extract was measured using Bradford assay kit (Bio-Rad) according to manufacturer's instructions. Briefly, five different concentrations (0.125, 0.25, 0.5, 1 and 2 mg/ml) of bovine serum albumin (BSA) were prepared. Bradford reagent was added to EPS samples and BSA standard solutions in a 96-well microtitre plate. The absorbance was quantified in a plate reader at 595nm. A standard curve was plotted using the absorbance values and the unknown protein concentration was calculated (Gong *et al.,* 2009).

2.2.11.3 Carbohydrate estimation

Sugar quantification was performed using the Phenol-Sulphuric Acid (PSA) method. Fifty microliter of 80% (w/w) phenol solution and 5ml of highly concentrated sulphuric acid (95.5%) were added to 2ml of the EPS solution and incubated at RT for 10min followed by incubation in a water bath (30°C) for 20min. Ds water was used as blank. The resulting dark yellow to brown

solutions were incubated at RT for 4h to ensure stabilisation. The absorbance of the solution was quantified spectroscopically at 480nm (Gong *et al.,* 2009).

2.2.12 Biofilm protein profiling

2.2.12.1 Intracellular protein extraction

Biofilms were grown in T25 flasks as described in section 2.2.11.1. After 24h, 48h and 72h of incubation, the supernatants were discarded and biofilms scraped from the surface of the flasks using sterile pipette tips. The flasks were washed with PBS and the entire content (PBS + scraped biofilm) was centrifuged (5000 x g, 10min). One ml of lysis buffer (Appendix D) was added to the pellets, mixtures were vortexed, incubated in RT for 15min and centrifuged (14100 x g, 25min). The supernatants were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Zhou *et al*., 2006).

2.2.12.2 SDS-PAGE

A Bio-Rad mini-protein electrophoresis unit was used. A 12% running gel and 5% stacking gel (Appendix D) were prepared and run according to manufacturer's (Bio-Rad) instructions. Briefly, the running gel solution was loaded into the gel cassette, overlaid with 70% ethanol and allowed to polymerise. Post polymerisation, the stacking gel solution was loaded into the gel cassette (above the running gel) and a 10-well plastic comb was inserted and the gel was again allowed to polymerise. The gel was then placed into the electrophoresis tank and loaded with the sample (supernatant from section 2.2.12.1) mixed with sample buffer (Appendix D). The tank was filled with the

running buffer and the gel was run for ~2 to 3h at 115V. The gel was stained with Coomassie blue stain (Appendix D) (~50ml) for 1h at RT with gentle shaking. The stain was discarded and the gel was de-stained with de-stain buffer (Appendix D) (~50ml) until the bands were clear. The de-stain buffer was then discarded and the gel was placed in ds water.

2.2.13 Bacterial motility assays

2.2.13.1 Swimming and swarming assays

Swimming motility was characterised on 0.3% LB agar plates. Two microliter drops of overnight bacterial cultures were stabbed into the centre of each plate. The plates were incubated at 37°C for 24h. After incubation, the distance migrated from point of inoculation was measured. Swarming motility was characterised on 1.5% LB agar plates. Ten microliter drops of overnight bacterial cultures were inoculated into the centre of each plate and the culture was allowed to soak into the agar at RT. The plates were incubated at 37°C for 24h. After incubation, the distance migrated from point of inoculation was measured with a ruler (Jones *et al.,* 2004).

2.2.13.2 Catheter bridge migration assays

A catheter bridge model was used to determine the ability of uropathogens to migrate over surfaces of urinary catheters. Plates of 1.5% LB agar were dried, and 0.8-cm-wide channels were cut across their centres. A 10µl drop of 4h cultures of uropathogens were inoculated at the edge of the channel. After the inoculum had dried into the agar, sections (1 cm) of urinary catheters were placed as bridges between the agar blocks, adjacent to points of inoculation.

As a control, the corresponding area of the opposing agar block was isolated by a 0.5-cm channel. Plates were incubated at 37°C for 24h. After incubation, the distance migrated on the uninoculated halves of the plate, adjacent to catheter bridges was measured with a ruler (Jones *et al.,* 2004).

2.2.14 Software and statistics

Microsoft Excel (v15.30) was used for general data analysis and preparation of charts. Paired two-tailed *t* test was used for statistical analysis unless otherwise stated.

2.3 Results

2.3.1 PCR analysis

Chromosomal DNA from all strains and five sets of primers (Table 2.2) were used to amplify the 16s rRNA, TEM, CTXM, SHV and UreC genes. The 16s rRNA band with an approximate size of 450bp was observed in all bacterial strains (Figure 2.2A). To identify the genes in ESBL producing *E. coli* and *K. pneumoniae*, PCR products were amplified using the TEM, CTXM and SHV primers. ESBL negative strains of *E. coli* and *K. pneumoniae* were used as control. As seen in Figure 2.2B, a single band was observed for *E. coli* ESBL strain confirming the presence of TEM-1 gene (approximate size of 850bp) whereas *K. pneumoniae* ESBL strain was positive for SHV gene (approximate size of 768bp) (Figure 2.2C). Both ESBL positive strains were negative for CTXM (Figure 2.2C). Control strains had no bands for any of the ESBL genes. PCR product from *P. mirabilis* was amplified using UreC primer and a band with an approximate size of 533bp was observed (Figure 2.2D).

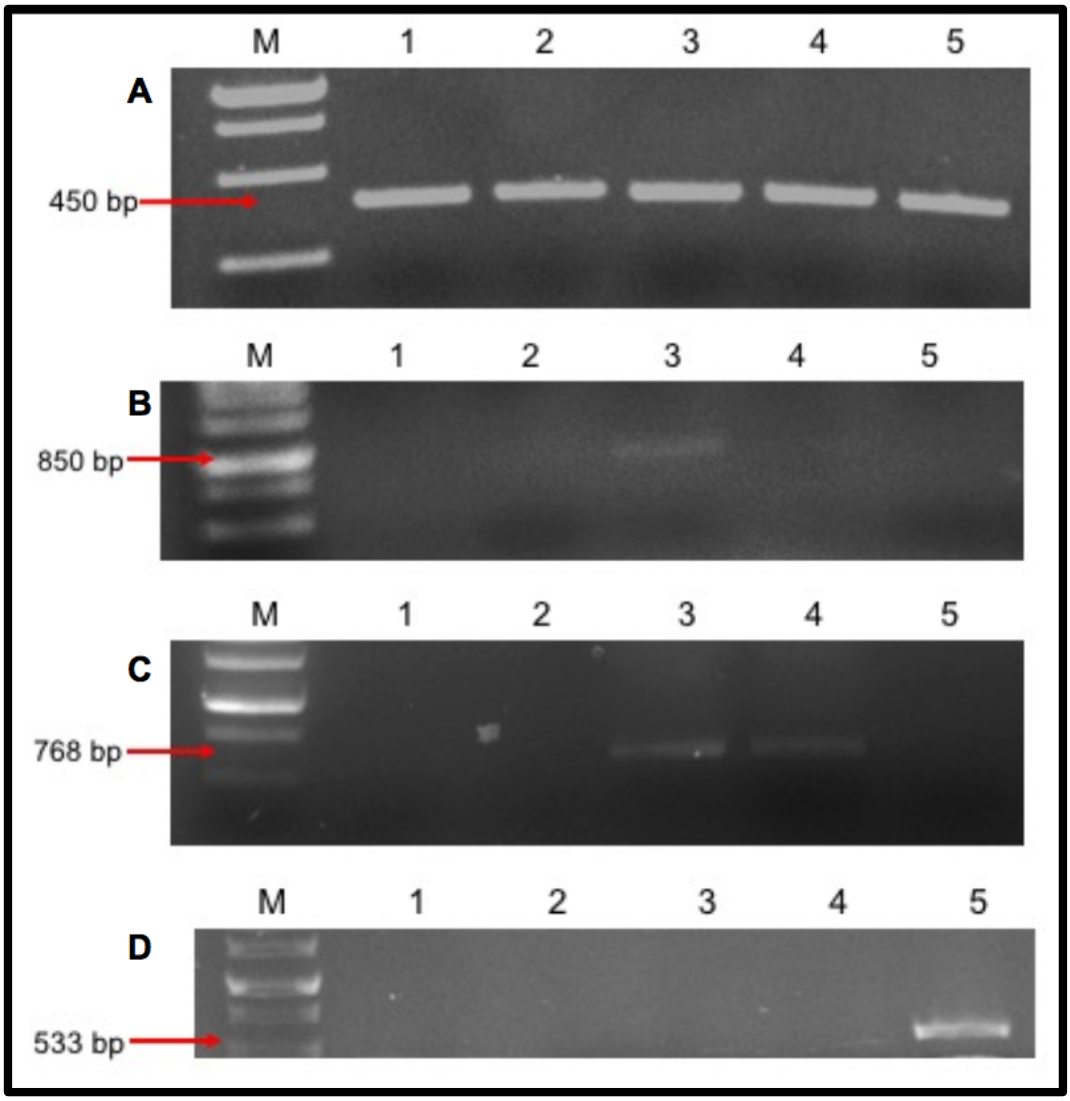

Figure 2.2: PCR products amplified using various primers.

A - 16s rRNA primer, bands are observed in all lanes with an expected size of approximately 450bp; 1 – *E. coli*, 2 – *E. coli* ESBL, 3 – *K. pneumoniae* ESBL, 4 – *K. pneumoniae*, 5 – *P. mirabilis*. B - TEM-1 primer, a single band is observed in lane 3 with an expected size of approximately 850bp; 1 – *E. coli*, 2 – *K. pneumoniae* ESBL, 3 – *E. coli* ESBL, 4 – *K. pneumoniae*, 5 – *P. mirabilis.* C - SHV primer, a single band is observed in lane 3 with an expected size of approximately 768bp (SHV); 1 – *E. coli*, 2 – *E. coli* ESBL, 3 – *K. pneumoniae* ESBL, 4 – *K. pneumoniae*, 5 – *P. mirabilis*. D - UreC primer, a single band is observed in lane 5 with an expected size of approximately 533bp. 1 – *E. coli*, 2 – *E. coli* ESBL, 3 – *K. pneumoniae* ESBL, 4 – *K. pneumoniae*, 5 – *P. mirabilis*; M – 1kbp DNA ladder.

2.3.2 EUCAST susceptibility tests

The disk diffusion method standardised by EUCAST was used to determine uropathogenic antibiotic susceptibility. Seven antibiotics were chosen based on the recommendations made by EUCAST for complicated and uncomplicated UTIs (for routine tests in a diagnostic laboratory). The diameters of zones of inhibition were measured after overnight incubation and compared with the EUCAST reference range. As seen in Table 2.4, *E. coli* ESBL negative and *P. mirabilis* were sensitive to all antibiotics with a higher susceptibility seen for Imipenem (28.6mm) and Ciprofloxacin (29.3mm) respectively. *E. coli* ESBL positive strain was resistant to all antibiotics except for Imipenem (28mm) whereas *K. pneumoniae* ESBL positive strain was sensitive to Imipenem (24.3mm), Gentamicin (16.6mm) and Cefoxitin (19.3). *K. pneumoniae* ESBL negative strain on the other hand was only resistant to Ampicillin, Amoxicillin-clavulanate (2:1) and Ceftazidime.

Table 2.4: EUCAST antimicrobial susceptibility test.

Diameters of zones of inhibition was measured to the nearest millimeter with a ruler and results were interpreted by reference to EUCAST breakpoint tables. R – Resistant, S – Sensitive, Breakpoint – chosen concentration of antimicrobial agent used to determine the sensitive and resistant patterns of bacteria.

Antimicrobial agent	**Disc content (µg)**	**EUCAST reference range (mm)**		**Zone diameter breakpoint (mm)**				
		S ≥	R <	*E. coli*	*E. coli* ESBL	*K. pneumoniae*	*K. pneumoniae* ESBL	*P. mirabilis*
Ampicillin	10	14	14	18 (S)	0 (R)	0 (R)	0 (R)	24 (S)
Amoxicillin-clavulanate (2:1)	30	19	19	19 (S)	8 (R)	0 (R)	0 (R)	23 (S)
Cefoxitin	30	19	19	23 (S)	18 (R)	19 (S)	19 (S)	22 (S)
Ceftazidime	30	22	19	23 (S)	12 (R)	11 (R)	8 (R)	28 (S)
Ciprofloxacin	5	22	19	27 (S)	0 (R)	26 (S)	18 (R)	29 (S)
Gentamicin	10	17	14	18 (S)	0 (R)	18 (S)	17 (S)	18 (S)
Imipenem	10	22	16	29 (S)	28 (S)	26 (S)	24 (S)	23 (S)

2.3.3 Growth assays in differential media

To determine the efficacy of AUM, growth of uropathogenic bacteria in AUM was compared with growth in LB broth, PYG and HU. Viable planktonic bacteria were enumerated on CLED agar after 24h of incubation. All uropathogens displayed high growth rates in LB broth compared with the other media (Figure 2.3). The increase in LB broth growth rates compared with AUM is highly significant ($p \leq 0.01$). There is no significant difference in HU and AUM growth rates ($p \leq 0.36$). *E. coli* ESBL negative strain (2.1×10^8 CFU/ml) displayed highest growth rates in HU whereas *P. mirabilis* (2.1×10^8 CFU/ml) did for AUM.

To determine the effect of urea, uric acid and creatinine on bacterial growth, samples of AUM without the above mentioned components were prepared and bacterial growth was evaluated after 24h of incubation. It is evident (Figure 2.4) that samples of AUM without urea have significantly ($p \leq 0.05$) increased bacterial growth compared with AUM samples without uric acid and creatinine. *P. mirabilis* (37.5% increase in growth) has the highest preference for AUM without urea compared with the other uropathogens. Both strains of *K. pneumoniae* have displayed similar growth patterns in AUM and AUM without urea (103% and 101% growth for ESBL positive and negative respectively). Between ESBL negative and positive strains of *E. coli*, the former has displayed a higher growth rate in AUM without urea (122% and 109% growth for ESBL positive and negative respectively). AUM without uric acid and creatinine has significantly ($p \leq 0.01$) decreased planktonic growth for all uropathogens. For both samples of AUM, *K. pneumoniae* ESBL negative has exhibited the lowest reduction in growth (5% and 28% reduction in growth for AUM without uric acid

and creatinine respectively) compared with *P. mirabilis* which has which has displayed a greater than 50% reduction in planktonic growth.

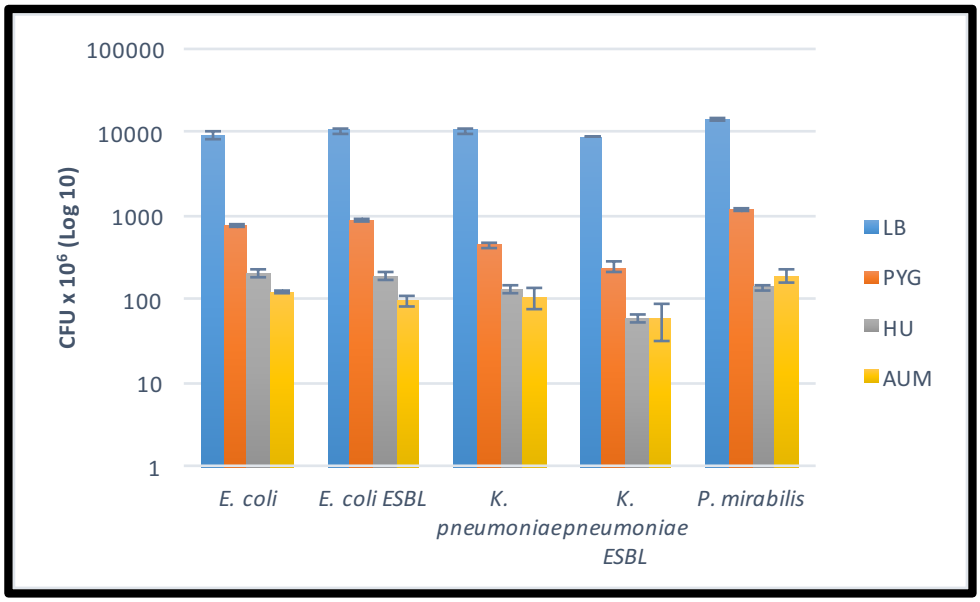

Figure 2.3: Planktonic bacterial growth of various uropathogens in differential media.

Viable bacteria were counted after 24h of incubation at 37°C. The left vertical scale is measured in log 10. Results are the mean of three independent experiments. Error bars represent standard error. LB significant at p ≤0.05 (paired two-tailed *t* test).

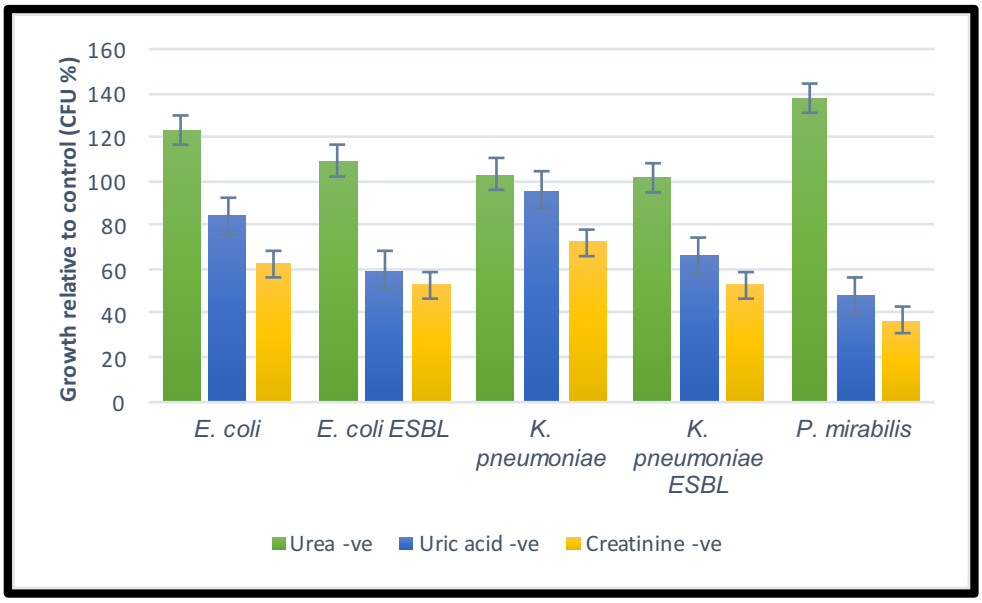

Figure 2.4: Planktonic cell growth of various uropathogens in differential AUM.

Viable bacteria were counted after 24h of incubation at 37°C. The percentage growth is relative to growth in AUM (control). Results are the mean of three independent experiments. Error bars represent standard error. AUM without urea is significant at p ≤0.05 and AUM without uric acid and creatinine is significant at p ≤0.01 (paired two-tailed *t* test).

2.3.4 Microtitre plate assays

2.3.4.1 *E. coli* (ESBL positive and negative)

Microtitre plate planktonic assays were performed to investigate *E. coli* (ESBL negative and positive strains) planktonic growth in AUM. As seen in Figure 2.5A, there is a steady increase in planktonic growth from 0h (~33 x 10^6 CFU/ml) to 5h (~161 x 10^6 CFU/ml) after which the growth slightly increases till 12h (~203 x 10^6 CFU/ml). A significant ($p \leq 0.001$) decrease is seen between 12h and 24h (~162 x 10^6 CFU/ml). On the other hand, for the ESBL positive strain there is a steady increase in planktonic growth from 0h (~48 x 10^6 CFU/ml) to 12h (~242 x 10^6 CFU/ml) after a which a significant ($p \leq 0.05$) decrease can be seen (~142 x 10^6 CFU/ml at 24h). It should be noted that after 72h of incubation, the number of viable bacteria is still significantly higher than the initial inoculum (0h) for ESBL negative *E. coli* (~50% higher, $p \leq 0.05$) whereas it is slightly lesser (-1% change, not significant) than initial inoculum (0h) for its ESBL positive counterpart.

To assess the biofilm forming ability of *E. coli* (ESBL negative and positive strains), viable bacteria were scraped from the biofilm formed on the bottom of a 24-well plate after various periods of incubation. As evident in Figure 2.5B, for ESBL negative strain there is an increase in the number of bacteria recruited by the biofilm from 1h (~0.3 x 10^6 CFU/ml) to 24h (~5.3 x 10^6 CFU/ml). At 48h the number of viable biofilm bacteria significantly ($p \leq 0.001$) decreases (~3 x 10^6 CFU/ml). On the other hand, the number of bacteria recruited by ESBL positive *E. coli* biofilm increases from 1h (~0.2 x 10^6 CFU/ml) to 12h (~4 x 10^6 CFU/ml) and then at 24h (~2 x 10^6 CFU/ml) a significant ($p \leq 0.01$) decrease in

the number of viable biofilm bacteria can be seen. It should be noted that after 72h of incubation, the number of viable biofilm bacteria is lower than the 1h old biofilm for both strains (~56% and ~67% reduction for ESBL negative and positive respectively). However, this difference is not significant.

The quantity of *E. coli* (ESBL negative and positive) biofilm biomass was indirectly assessed over a period of 72h by measuring the biofilm absorbance of CV stain. From Figure 2.5C it is evident that the biomass of *E. coli* ESBL negative biofilm increases from 1h (~0.2 abs) to 48h (~0.7 abs) after which a significant ($p \leq 0.05$) decrease can be seen (~0.4 abs at 72h). On the other hand, for its ESBL positive counterpart a similar trend can be seen (~0.2 abs at 1h to ~0.7 abs at 48h) with a significant ($p \leq 0.01$) decrease seen at 72h (~0.3 abs). Biomass of the 72h biofilms is still higher than the 1h levels for both strains (~35% and ~21% increase for ESBL negative and positive respectively). However, this difference is significant ($p \leq 0.01$) for ESBL negative strain only.

Finally, to visualise the biofilm biomass, CV stained biofilms of both strains of *E. coli* were examined under a light microscope. As evident in Figure 2.6, 48h biofilms possess the highest amount of biomass for both strains.

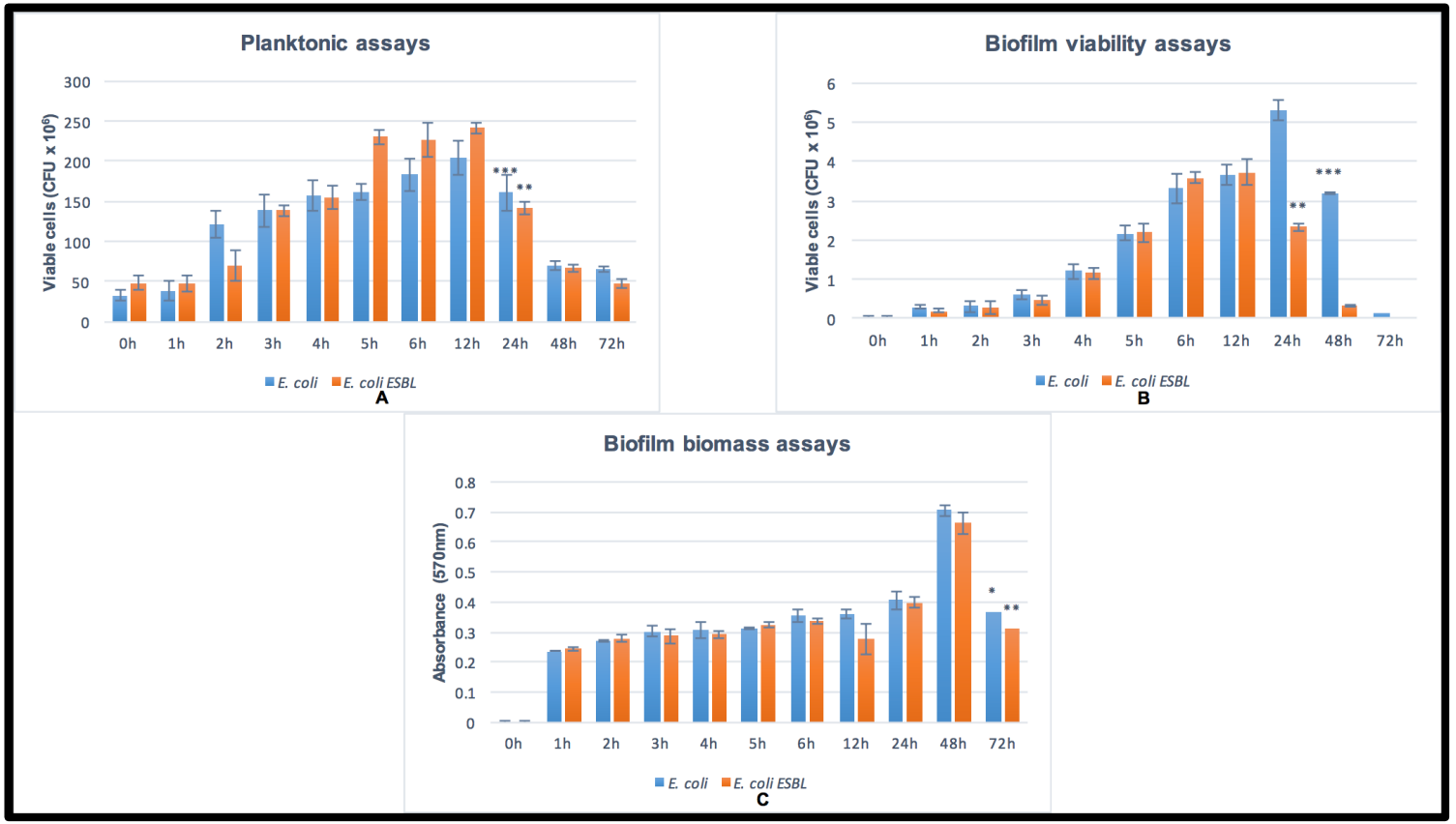

Figure 2.5: *E. coli* (ESBL negative and positive) maintained at 37°C in AUM for various periods of incubation.

A – Viable bacteria from planktonic suspension; B – Viable bacteria scraped from the biofilm; C – CV absorbance values of the biofilm biomass (bacteria with EPS) measured spectroscopically at 570nm. Results are the mean of three independent experiments. Error bars represent standard error. *** significant at p≤0.001; ** significant at p≤0.01; * significant at p≤0.05 (paired two-tailed *t* test).

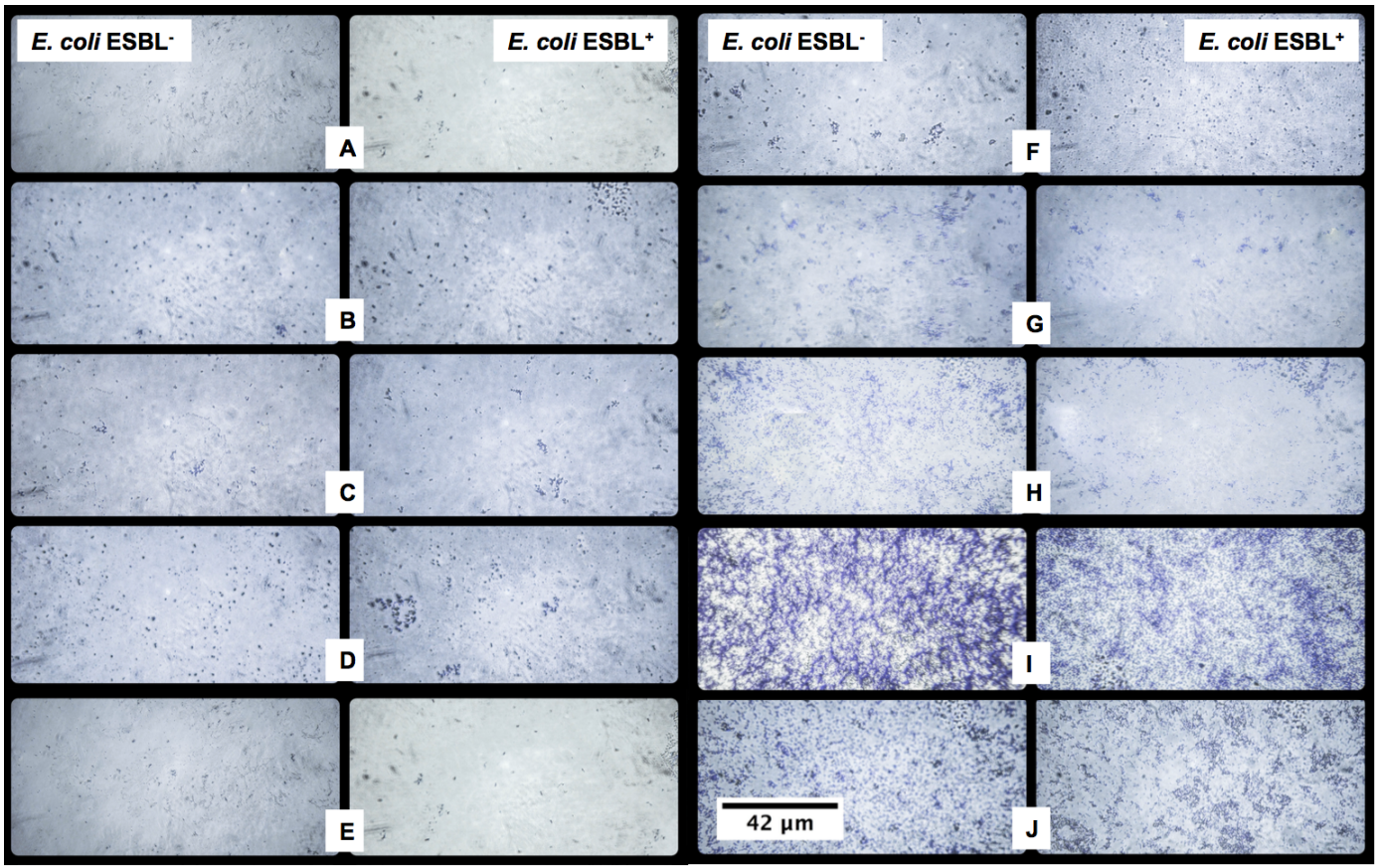

Figure 2.6: CV stain showing *E. coli* (ESBL negative and positive) biofilm biomass (bacteria and EPS) formation in AUM.

Biofilm biomass was stained with 1% CV after various periods of incubation (A – 1h, B – 2h, C – 3h, D – 4h, E – 5h, F – 6h, G – 12h, H – 24h, I – 48h and J – 72h) at 37°C. The figure is representative of images obtained from a bright field microscope (scale bar: 42µm).

2.3.4.2 K. pneumoniae (ESBL negative and positive)

Microtitre plate planktonic assays were performed to investigate *K. pneumoniae* (ESBL negative and positive strains) planktonic growth in AUM. As seen in Figure 2.7A, there is a steady increase in planktonic growth from 0h (~38 x 10^6 CFU/ml) to 12h (~377 x 10^6 CFU/ml). A significant ($p \leq 0.05$) decrease is seen between 12h and 24h (~153 x 10^6 CFU/ml). On the other hand, for the ESBL positive strain there is a steady increase in planktonic growth from 0h (~18 x 10^6 CFU/ml) to 12h (~213 x 10^6 CFU/ml) after a which a decrease can be seen (~86 x 10^6 CFU/ml at 24h, not significant). It should be noted that after 72h of incubation, the number of viable bacteria is still higher than the initial inoculum (0h) for ESBL negative *K. pneumoniae* (~31% higher, not significant) whereas it is lesser ~75% decrease, not significant) than initial inoculum (0h) for its ESBL positive counterpart.

To assess the biofilm forming ability of *K. pneumoniae* (ESBL negative and positive strains), viable bacteria were scraped from the biofilm formed on the bottom of a 24-well plate after various periods of incubation. As evident in Figure 2.7B, for ESBL negative strain there is an increase in the number of bacteria recruited by the biofilm from 1h (~3 x 10^6 CFU/ml) to 12h (~83 x 10^6 CFU/ml). At 24h the number of viable biofilm bacteria significantly ($p \leq 0.001$) decreases (~50 x 10^6 CFU/ml). On the other hand, the number of bacteria recruited by ESBL positive *K. pneumoniae* biofilm increases from 1h (~1 x 10^6 CFU/ml) to 12h (~45 x 10^6 CFU/ml) and then at 24h (~4 x 10^6 CFU/ml) a significant ($p \leq 0.01$) decrease in the number of viable biofilm bacteria can be seen. It should be noted that after 72h of incubation, the number of viable

biofilm bacteria is lower than the 1h old biofilm for both strains (~67% and ~34% reduction for ESBL negative and positive respectively). However, this difference is only significant ($p \leq 0.01$) for ESBL negative *K. pneumoniae*.

The quantity of *K. pneumoniae* (ESBL negative and positive) biofilm biomass was indirectly assessed over a period of 72h by measuring the biofilm absorbance of CV stain. From Figure 2.7C it is evident that the biomass of *K. pneumoniae* ESBL negative biofilm increases from 1h (~0.4 abs) to 24h (~1.9 abs) after which a significant ($p \leq 0.01$) decrease can be seen (~0.8 abs at 48h). On the other hand, for its ESBL positive counterpart a similar trend can be seen (~0.3 abs at 1h to ~1.2 abs at 24h) with a significant ($p \leq 0.01$) decrease seen at 48h (~0.6 abs). Biomass of the 72h biofilms is still higher than the 1h levels for both strains (~50% and ~43% increase for ESBL negative and positive respectively). This difference is significant ($p \leq 0.01$) for both strains.

Finally, to visualise the biofilm biomass, CV stained biofilms of both strains of *K. pneumoniae* were examined under a light microscope. As evident in Figure 2.8, 24h biofilms possess the highest amount of biomass for both strains.

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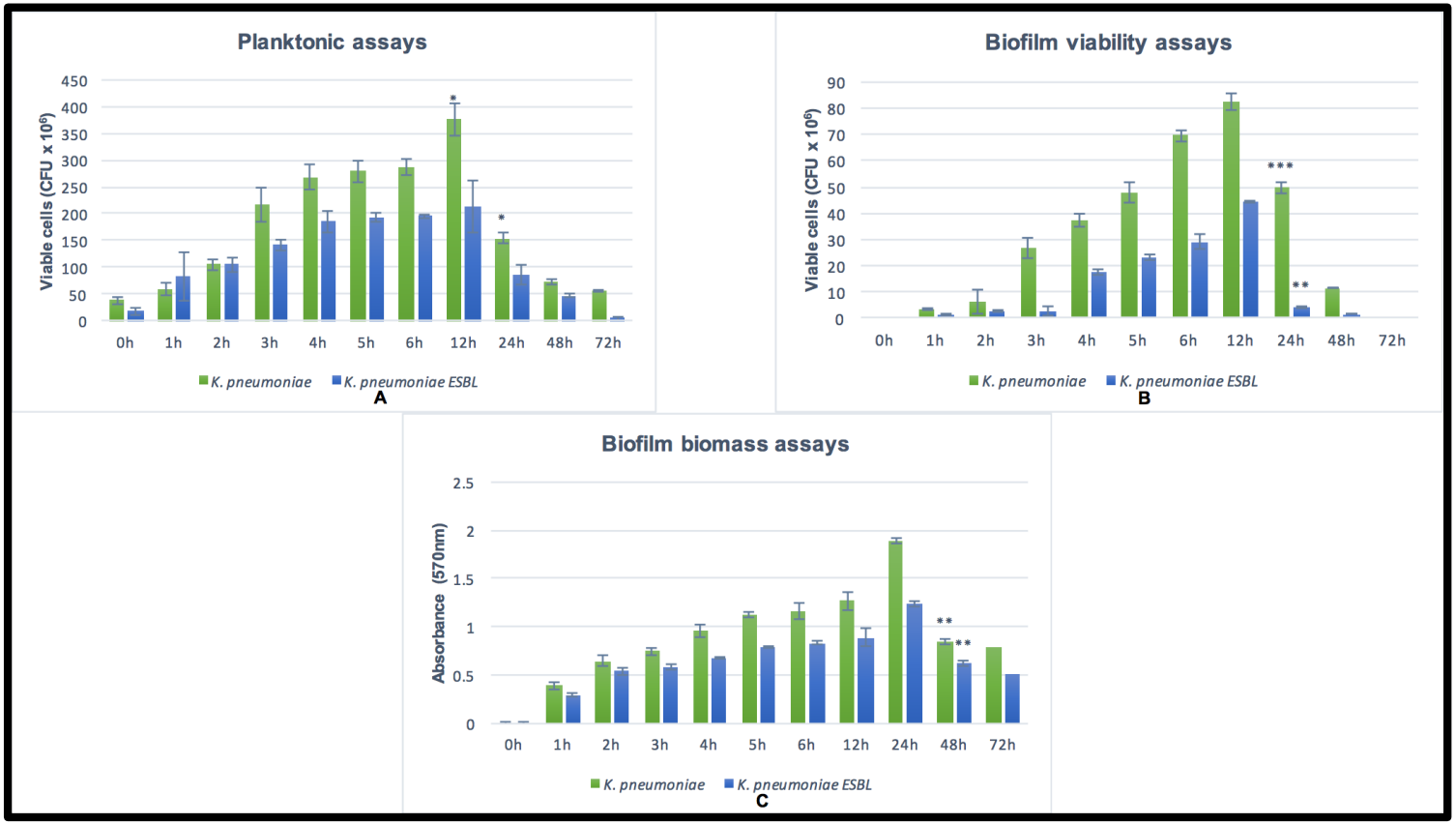

Figure 2.7: *K. pneumoniae* (ESBL negative and positive) maintained at 37°C in AUM for various periods of incubation.

A – Viable bacteria from planktonic suspension; B – Viable bacteria scraped from the biofilm; C – CV absorbance values of the biofilm biomass (bacteria with EPS) measured spectroscopically at 570nm. Results are the mean of three independent experiments. Error bars represent standard error. *** significant at p≤0.001; ** significant at p≤0.01; * significant at p≤0.05 (paired two-tailed *t* test).

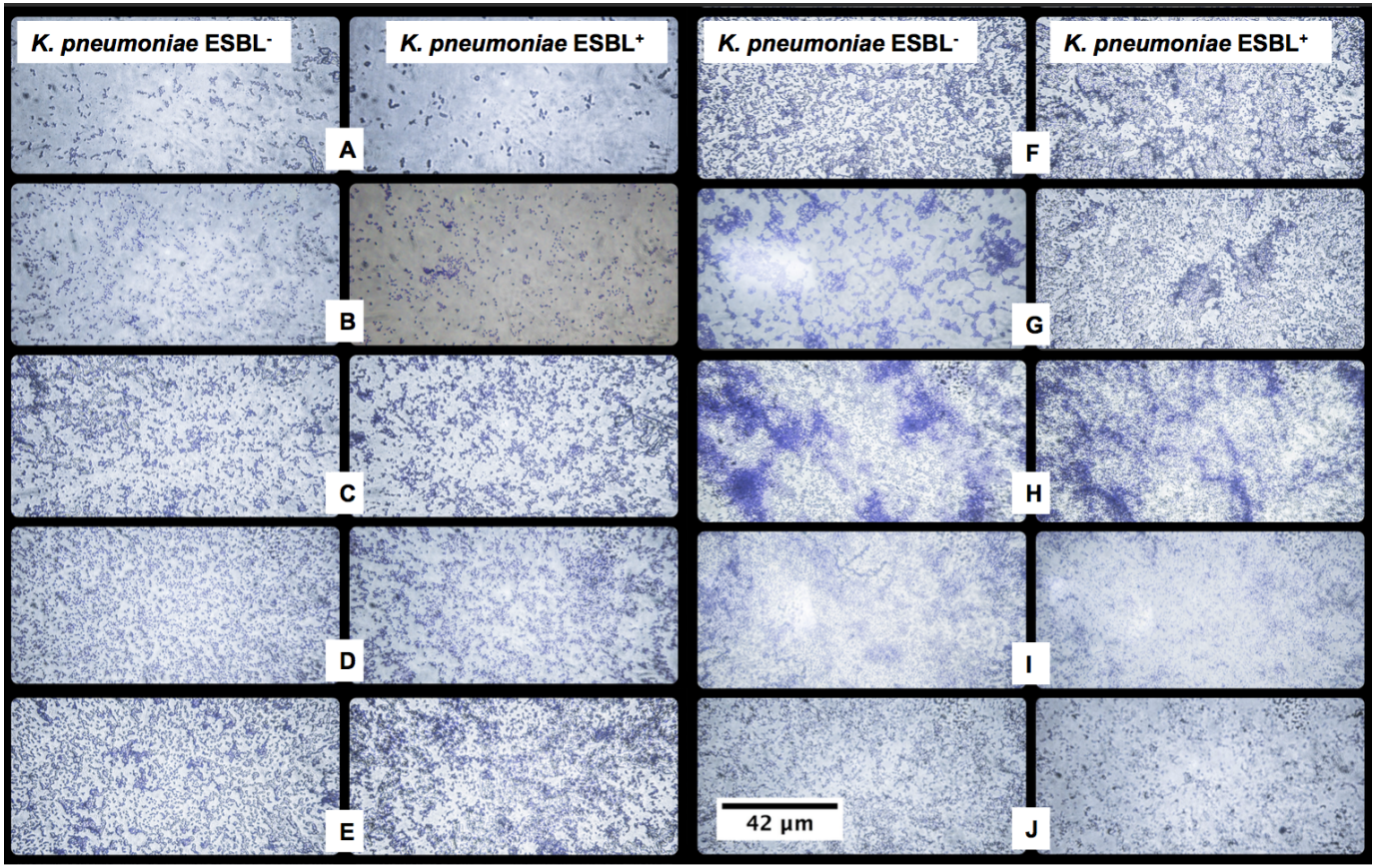

Figure 2.8: CV stain showing *K. pneumoniae* (ESBL negative and positive) biofilm biomass (bacteria and EPS) formation in AUM.

Biofilm biomass was stained with 1% CV after various periods of incubation (A – 1h, B – 2h, C – 3h, D – 4h, E – 5h, F – 6h, G – 12h, H – 24h, I – 48h and J – 72h) at 37°C. The figure is representative of images obtained from a bright field microscope (scale bar: 42µm).

2.3.4.3 P. mirabilis

Microtitre plate planktonic assays were performed to investigate *P. mirabilis* planktonic growth in AUM. As seen in Figure 2.9A, there is a steady increase in planktonic growth from 0h (~23 x 10^6 CFU/ml) to 12h (~238 x 10^6 CFU/ml). A significant ($p \leq 0.05$) decrease is seen between 12h and 24h (~213 x 10^6 CFU/ml). Although a decrease can be seen at 24h (~213 x 10^6 CFU/ml, not significant) it is not as high as the decrease seen between 24h and 48h (~42 x 10^6 CFU/ml, significant at $p \leq 0.01$). It should be noted that after 72h of incubation, the number of viable bacteria is slightly lower (2.8%) than the initial inoculum (0h). However, this decrease is not significant. In comparison with *E. coli* (ESBL negative), there is a steady increase in *E. coli* planktonic growth from 0h (~33 x 10^6 CFU/ml) to 5h (~161 x 10^6 CFU/ml) after which the growth slightly increases till 12h (~203 x 10^6 CFU/ml). A significant ($p \leq 0.001$) decrease is seen between 12h and 24h (~162 x 10^6 CFU/ml).

To assess the biofilm forming ability of *P. mirabilis*, viable bacteria were scraped from the biofilm formed on the bottom of a 24-well plate after various periods of incubation. As evident in Figure 2.9B, there is an increase in the number of bacteria recruited by the biofilm from 1h (~0.6 x 10^6 CFU/ml) to 6h (~6 x 10^6 CFU/ml). At 12h the number of viable biofilm bacteria significantly ($p \leq 0.01$) decreases (~4.2 x 10^6 CFU/ml). It should be noted that after 72h of incubation, the number of viable biofilm bacteria is still higher (~56%) than the 1h old biofilm. However, this difference is not significant. In comparison with *E. coli* (ESBL negative), there is an increase in the number of bacteria recruited by the *E. coli* biofilm from 1h (~0.3 x 10^6 CFU/ml) to 24h (~5.3 x 10^6 CFU/ml).

At 48h the number of viable biofilm bacteria significantly ($p \leq 0.001$) decreases (~3 x 10^6 CFU/ml).

The quantity of *P. mirabilis* biofilm biomass was indirectly assessed over a period of 72h by measuring the biofilm absorbance of CV stain. From Figure 2.9C it is evident that the biomass of *P. mirabilis* biofilm slightly increases from 1h (~0.3 abs) to 5h (~0.4 abs). Between 5h and 24h (~0.5 abs) an initial decrease and then an increase can be seen. At 48h (0.4 abs), there is a significant ($p \leq 0.05$) decrease in the biomass of P. mirabilis biofilms. Biomass of the 72h biofilms is still higher than the 1h levels (~32%, not significant). In comparison with *E. coli* (ESBL negative), the biomass of *E. coli* ESBL negative biofilm increases from 1h (~0.2 abs) to 48h (~0.7 abs) after which a significant ($p \leq 0.05$) decrease can be seen (~0.4 abs at 72h).

Finally, to visualise the biofilm biomass, CV stained biofilms of both strains of *P. mirabilis* were examined under a light microscope. As evident in Figure 2.10, biofilms possess similar biomass across the incubation period. In comparison with *E. coli* (ESBL negative), 48h *E. coli* biofilms possess the highest amount of biomass.

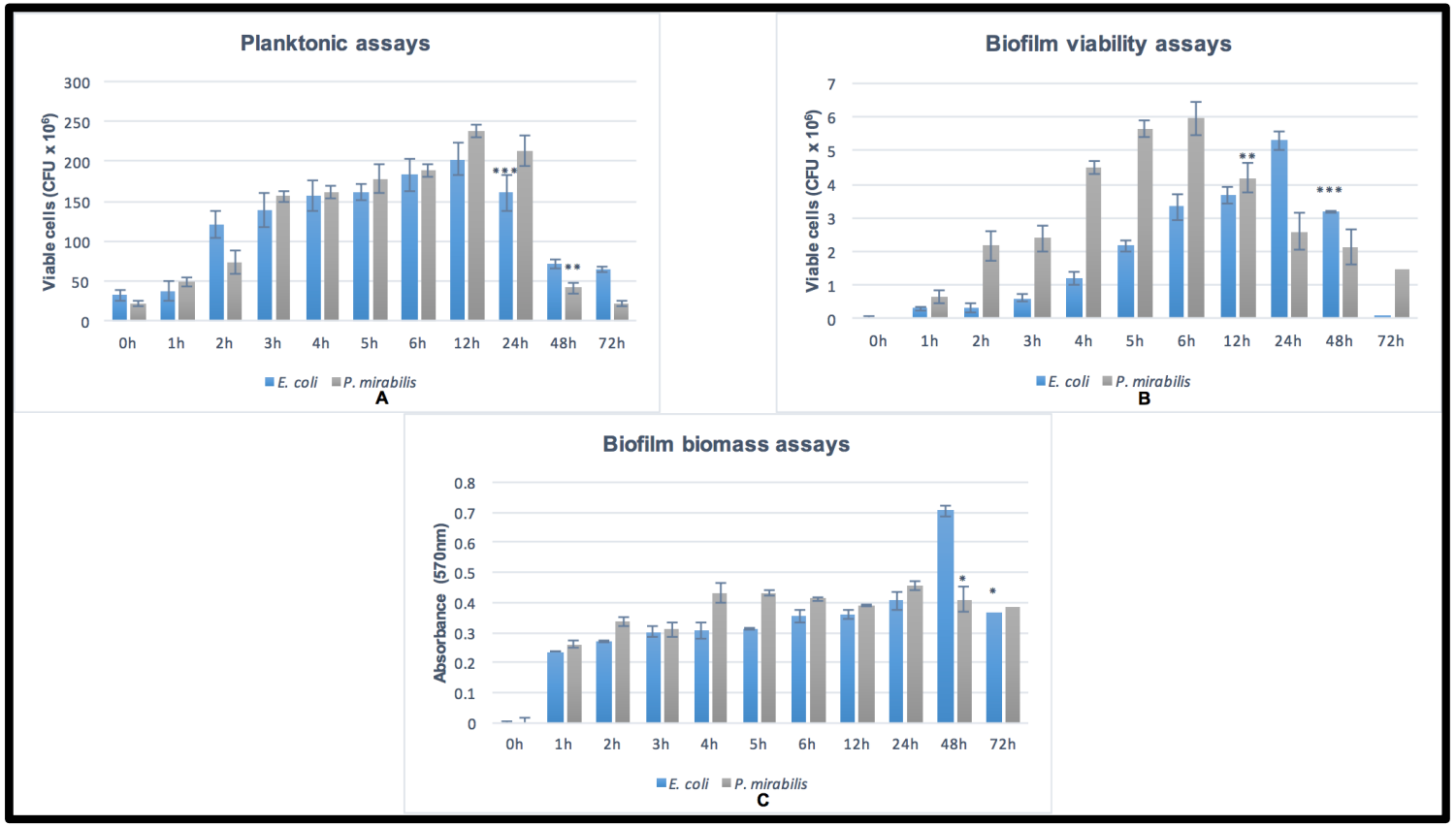

Figure 2.9: *P. mirabilis* in comparison with *E. coli* ESBL negative maintained at 37°C in AUM for various periods of incubation.

A – Viable bacteria from planktonic suspension; B – Viable bacteria scraped from the biofilm; C – CV absorbance values of the biofilm biomass (bacteria with EPS) measured spectroscopically at 570nm. Results are the mean of three independent experiments. Error bars represent standard error. *** significant at p≤0.001; ** significant at p≤0.01; * significant at p≤0.05 (paired two-tailed *t* test).

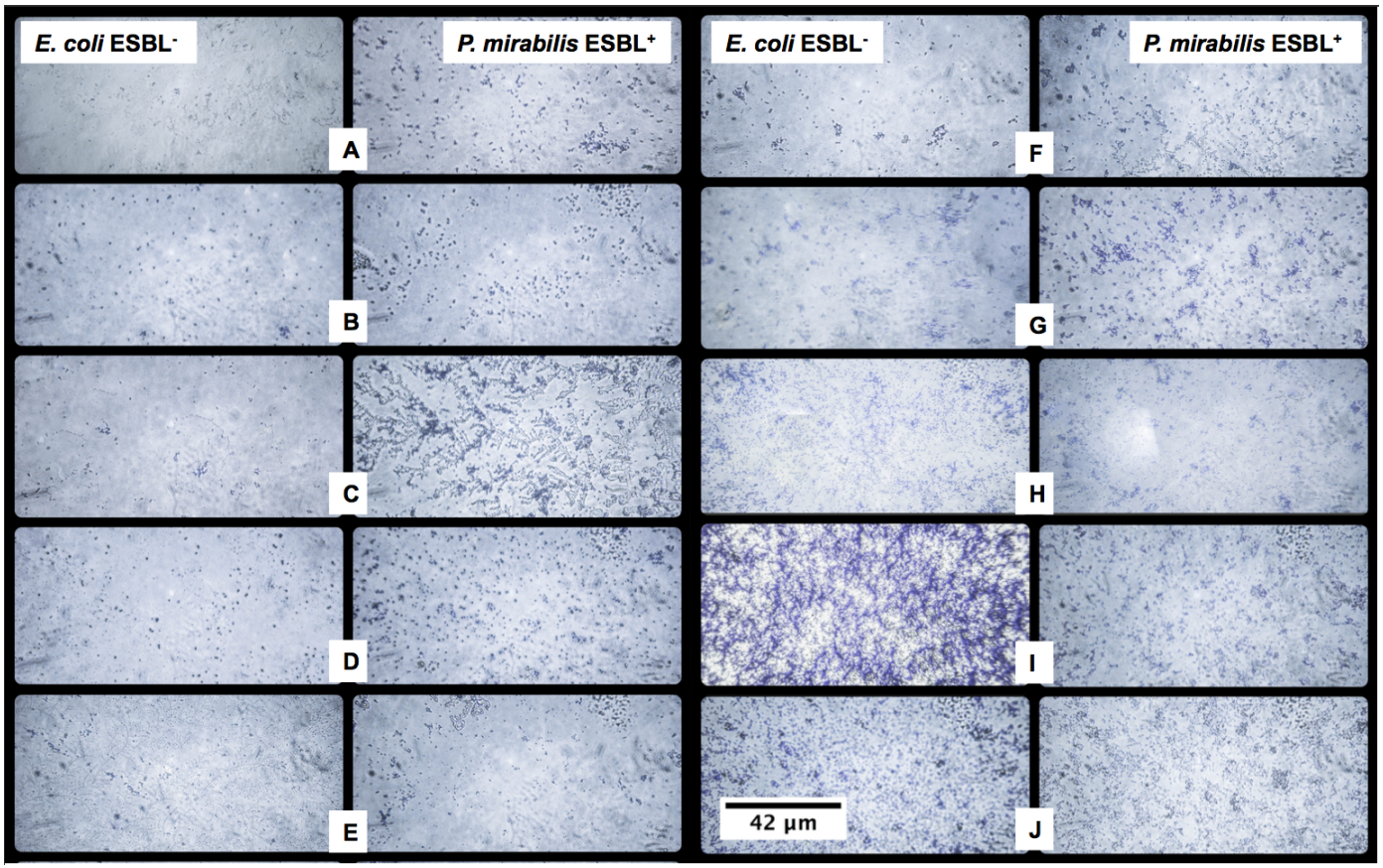

Figure 2.10: CV stain showing *P. mirabilis* in comparison with *E. coli* ESBL negative biofilm biomass (bacteria and EPS) formation in AUM.

Biofilm biomass was stained with 1% CV after various periods of incubation (A – 1h, B – 2h, C – 3h, D – 4h, E – 5h, F – 6h, G – 12h, H – 24h, I – 48h and J – 72h) at 37°C. The figure is representative of images obtained from a bright field microscope (scale bar: 42µm).

2.3.5 Catheter biofilm assays

Catheter biofilm assays were performed to evaluate the biofilm forming ability of uropathogens on latex (PTFE and hydrogel) and silicone catheters (Ch 12). Uropathogens were allowed to form biofilms for 24h. Figure 2.11 compares the biofilm formation on three types of urinary catheters. As evident in the figure, all uropathogens were able to form biofilms on the three tested catheters. On PTFE latex and silicone catheters, *K. pneumoniae* (ESBL negative) has displayed the greatest ability to form biofilms (4.04×10^6 CFU/ml and 5.05×10^6 CFU/ml respectively) whereas its ESBL positive counterpart (0.01×10^6 CFU/ml and 0.32×10^6 CFU/ml) has struggled to form biofilms in comparison with the other uropathogens. On hydrogel latex catheters, *E. coli* ESBL positive has formed the highest amount (1.6×10^6 CFU/ml) of biofilm compared with *P. mirabilis* which has formed the lowest (0.09×10^6 CFU/ml). The difference in biofilm formation between ESBL negative and positive strains of *E. coli* is only significant ($p \leq 0.05$) for hydrogel latex catheters whereas for *K. pneumoniae* the difference between the ESBL negative and positive strains is significant for PTFE ($p \leq 0.01$) and silicone ($p \leq 0.05$) catheters. Comparing *E. coli* ESBL negative and *P. mirabilis*, a significant difference ($p \leq 0.001$) in biofilm formation is seen for silicone catheters only. Although there isn't any specific trend, all uropathogens have formed lesser biofilms on hydrogel latex catheters compared with PTFE and silicone.

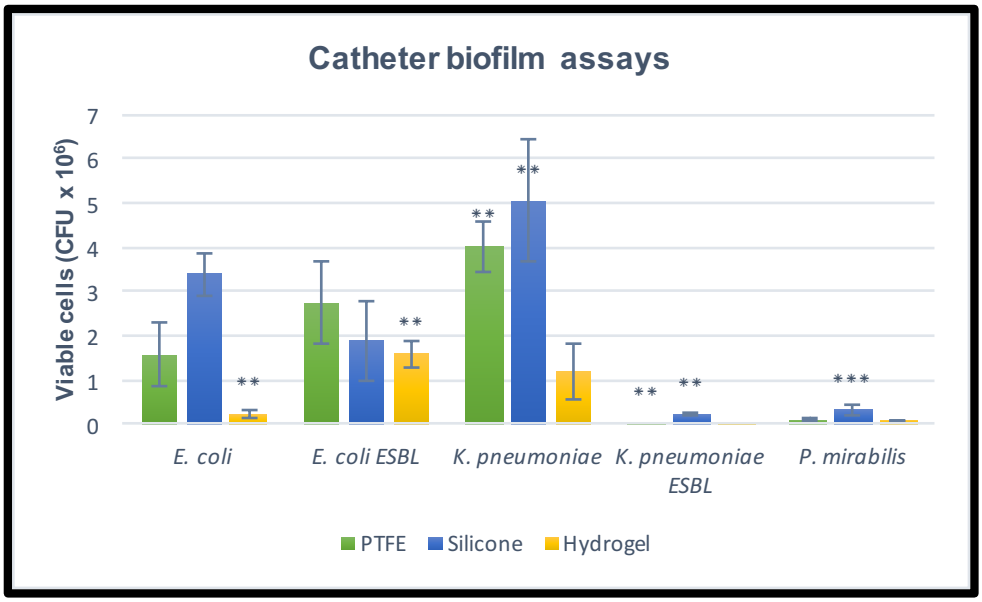

Figure 2.11: Biofilm formation on latex (PTFE and hydrogel) and silicone catheters (Ch 12).

Viable bacteria were counted after 24h of incubation at 37°C. Results are obtained from at least three independent experiments. Error bars represent standard error. *** significant at p≤0.001; ** significant at p≤0.01; * significant at p≤0.05 (paired two-tailed *t* test).

2.3.6 Bacterial adherence assays

To determine the effect of a conditioning layer on bacterial attachment, bacterial adherence on PTFE latex catheters (Ch12) with and without a conditioning layer was evaluated after 3h of incubation. The conditioning layer was formed using AUM. All uropathogens have exhibited (Figure 2.12) greater affinity to catheters with a preformed conditioning layer. The difference in adherence between pre-conditioned and unconditioned catheters is significant ($p \leq 0.01$). The highest adherence to the conditioning layer is exhibited by *E. coli* ESBL negative (~2.5 x 10^6 CFU/ml) whereas the lowest is seen for *P. mirabilis* (~1 x 10^6 CFU/ml).

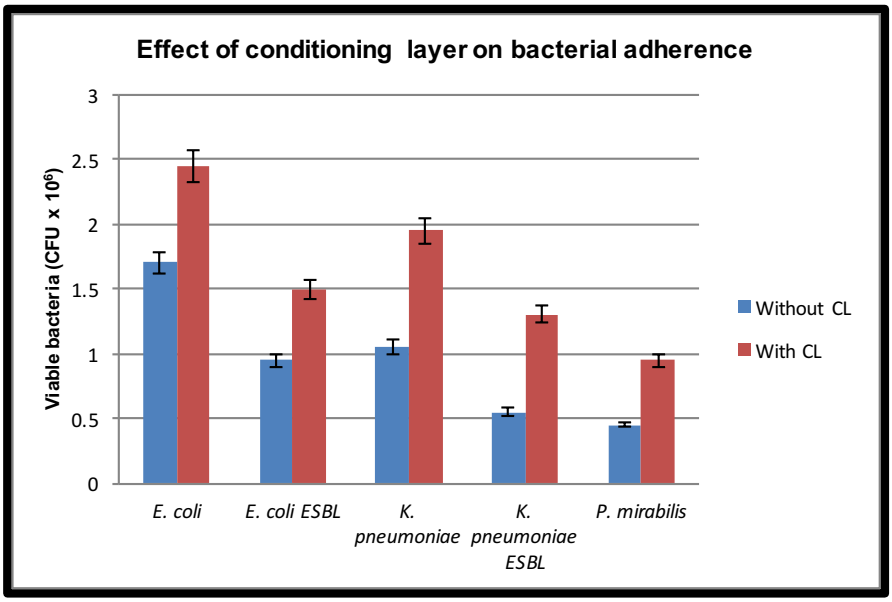

Figure 2.12: Bacterial adherence to PTFE latex catheters (Ch 12) with and without preformed condioning layer in AUM.

Viable bacteria were counted after 3h of incubation at 37°C. Results are obtained from at least three independent experiments. CL - conditioning layer. Error bars represent standard error. Results are significant at $p \leq 0.05$ (paired two-tailed *t* test).

2.3.7 Biofilm nutrition assays

To determine the role of nutrient availability on biofilm dispersal (shedding of planktonic bacteria), 24h, 48h and 72h biofilms were subjected to fed (AUM) and unfed conditions (ds water) for 24h. Figure 2.13 shows that in unfed conditions, dispersal is nearly similar for all uropathogens across 24h, 48h and 72h biofilms. The difference in dispersal between fed and unfed conditions is significant ($p \leq 0.01$). From the figure it is also evident that dispersal under fed conditions is highest from 24h biofilms indicating an age dependent response from biofilms. Among all uropathogens, *P. mirabilis* (0.62 abs) has displayed the highest proluferation from 24h biofilms compared with *K. pneumoniae* ESBL positive (0.22 abs) which has displayed the lowest dispersal. Difference between the ESBL negative and positive strains of *E. coli* and *K. pneumoniae* for 24h dispersal is not significant.

To determine the role of nutrient availability on the formation of biofilm biomass, 24h, 48h and 72h biofilms were subject to the same conditions as mentioned above. It is evident from Figure 2.14 that biofilm biomass is higher under fed conditions for all uropathogens albeit at varying levels. The difference in the biomass between fed and unfed conditions is significant for 24h ($p \leq 0.05$) and 72h ($p \leq 0.01$) biofilms. Among 24h, 48h and 72h biofilms, the highest biomass under fed conditions is observed for 48h *E. coli* (0.8 abs and 0.47 abs for ESBL negative and positive respectively) and ESBL positive *K. pneumoniae* (0.5 abs) biofilms whereas for ESBL positive *K. pneumoniae* (0.8 abs) and *P. mirabilis* (0.42 abs) the highest biomass is observed for 72h biofilms.

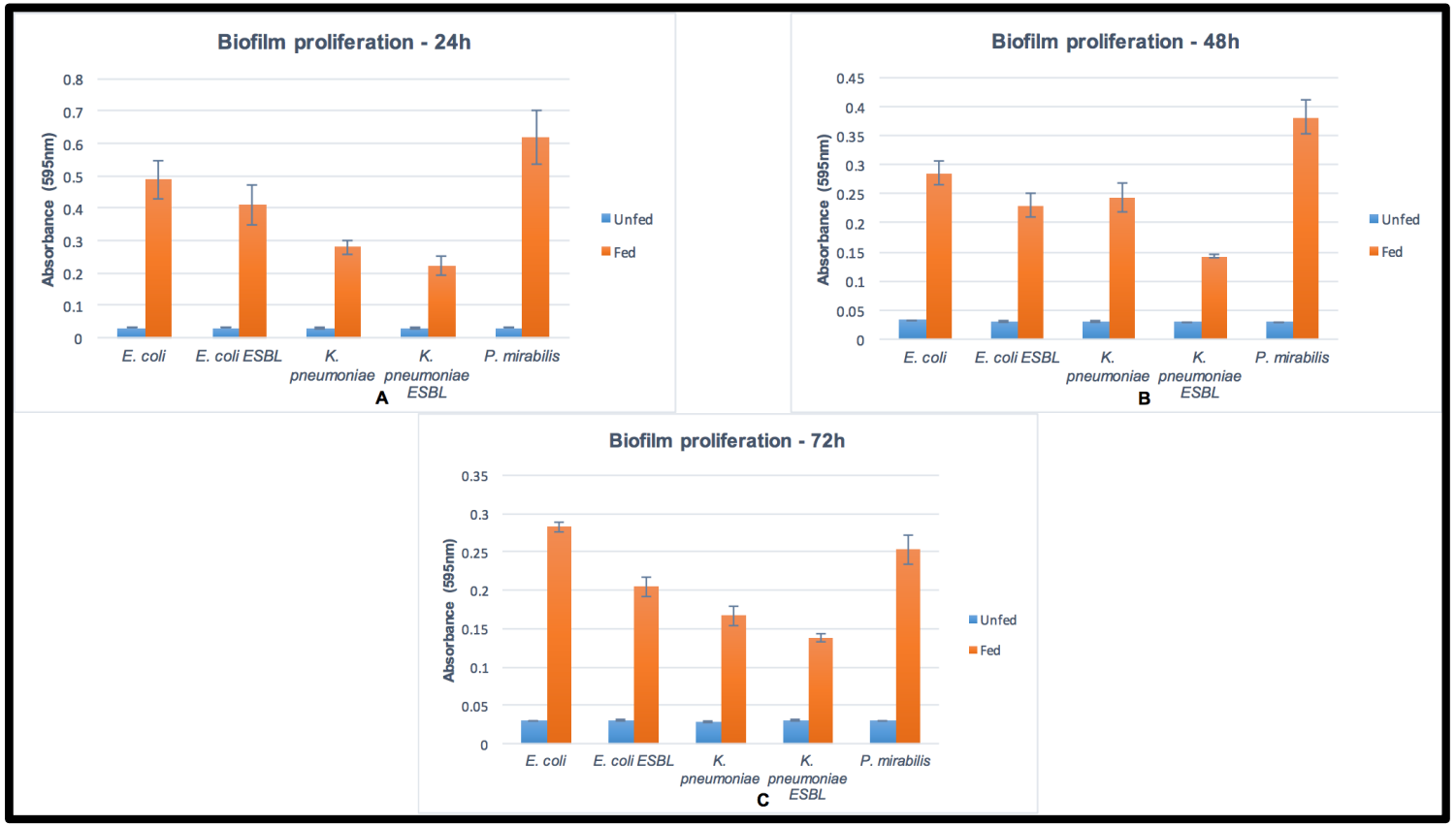

Figure 2.13: Dispersal of planktonic bacteria by uropathogenic fed and unfed biofilms.

24h (A), 48h (B) and 72h (C) old biofilms were subjected to fed (AUM) and unfed (ds water) conditions for 24h. Absorbance values were measured spectroscopically at 595nm. Results are obtained from at least three independent experiments. Error bars represent standard error. A, B and C are significant at $p \leq 0.01$ (paired two-tailed *t* test).

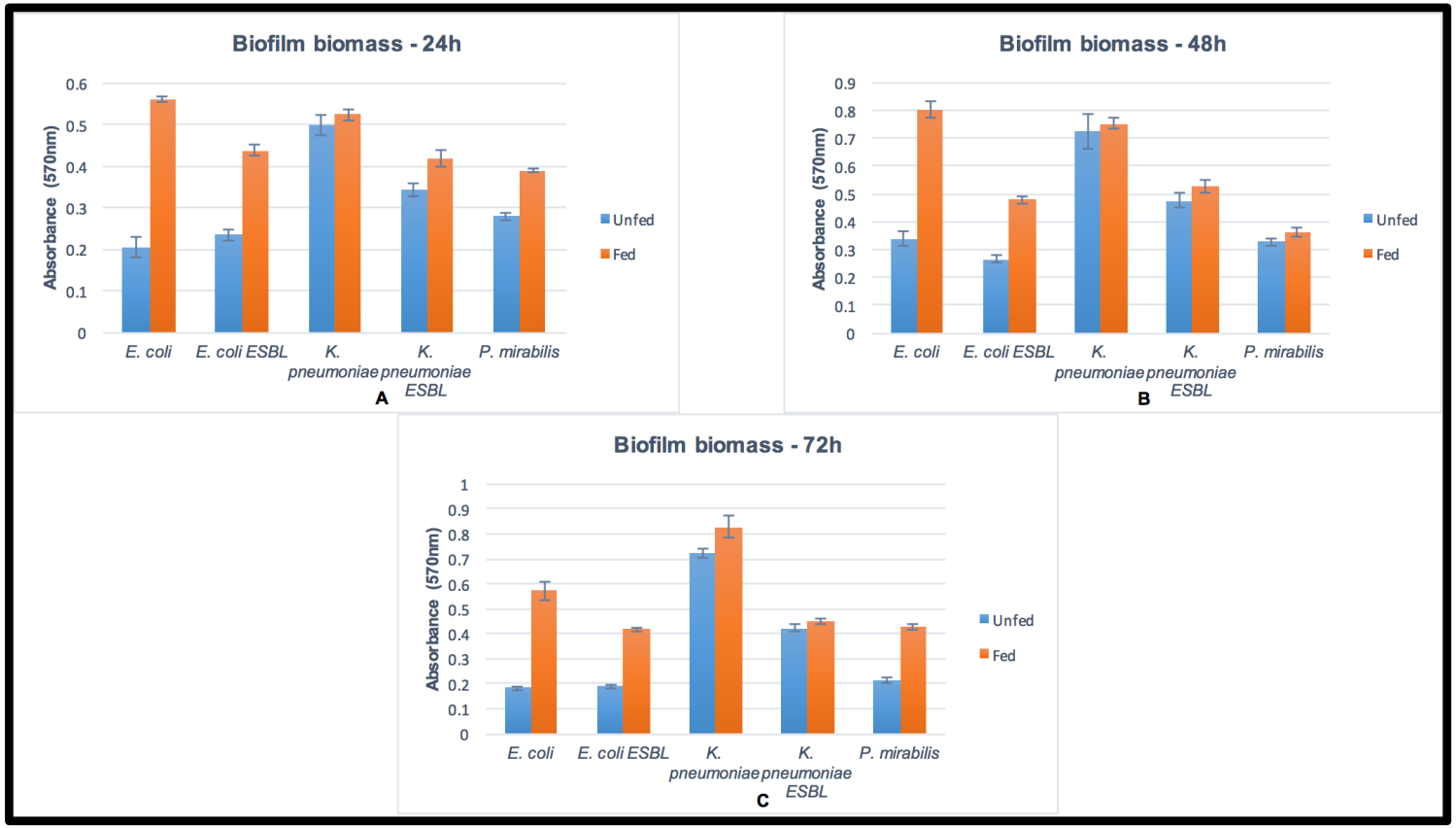

Figure 2.14: Biomass of uropathogenic fed and unfed biofilms.

24h (A), 48h (B) and 72h (C) old biofilms were subjected to fed (AUM) and unfed (ds water) conditions for 24h. CV absorbance values of the biofilm biomass (bacteria and EPS) measured spectroscopically at 570nm. Results are obtained from at least three independent experiments. Error bars represent standard error. A is significant at $p \leq 0.05$ and C at $p \leq 0.01$ (paired two-tailed *t* test).

2.3.8 Analysis of biofilm EPS

Biochemical analysis was performed on extracted biofilm EPS to evaluate the protein and carbohydrate composition of 24h, 48h and 72h uropathogenic biofilms. Figure 2.15 and Figure 2.16 experimental data on protein and carbohydrate estimation respectively.

As seen in Figure 2.15, the highest protein content was estimated for 48h biofilms. Among all uropathogens tested, 48h biofilms of *K. pneumoniae* (0.969 mg/ml and 0.967 mg/ml for ESBL negative and positive respectively) contained the highest amount of protein. On the other hand, the lowest amount was estimated for *P. mirabilis* (0.869 mg/ml). The difference in protein estimation of 24h and 48h biofilms is not significant, however, the difference is significant ($p \leq 0.01$) between 48h and 72h biofilms for all uropathogens.

From Figure 2.16 we can see that, ESBL negative strains of *E. coli* (0.027 mg/ml) and *K. pneumoniae* (0.025 mg/ml), and *P. mirabilis* (0.022 mg/ml) biofilms were estimated to contain the highest amount of carbohydrate in their 48h biofilms whereas ESBL positive strains of *E. coli* (0.021 mg/ml) and *K. pneumoniae* (0.026 mg/ml) exhibited the highest amount of carbohydrate in their 72h biofilms. The difference in carbohydrate estimation of 24h and 48h biofilms is significant ($p \leq 0.05$), however, the difference is not significant between 48h and 72h biofilms for all uropathogens.

Overall, it has been estimated that uropathogens contain a higher amount of proteins in their biofilms in comparison with carbohydrate.

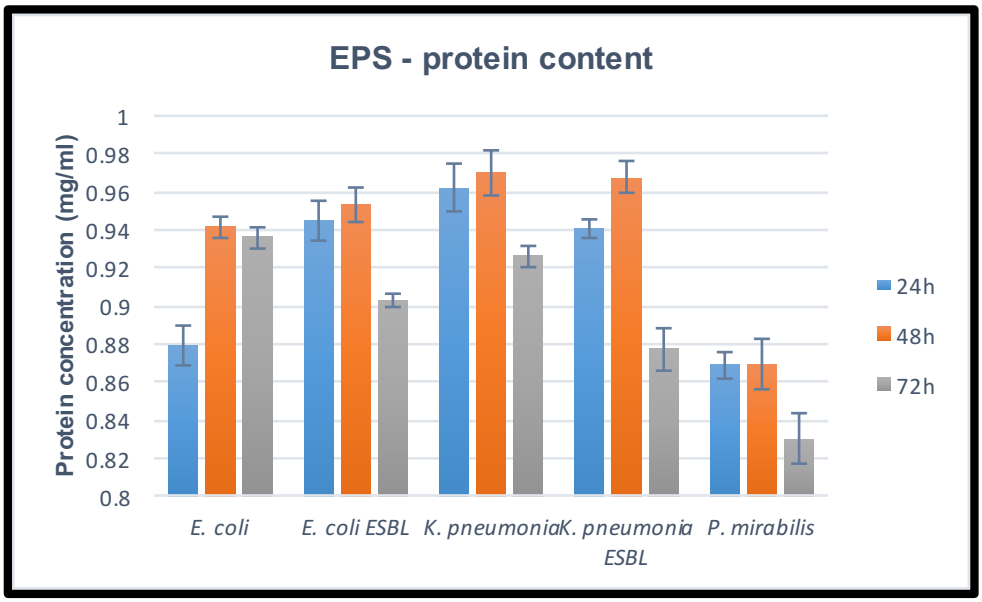

Figure 2.15: Protein content in the EPS of uropathogenic biofilms.

Protein concentration in the extracted EPS was measured using the Bradford assay and values are in mg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. Difference between protein estimation of 48h and 72h biofilms is significant at p≤0.01 (paired two-tailed *t* test).

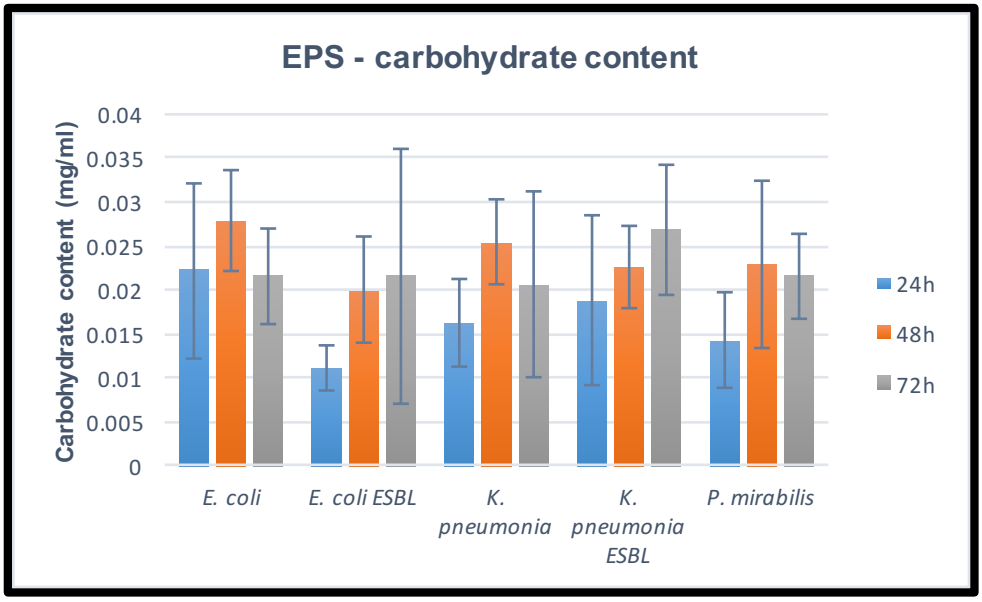

Figure 2.16: Carbohydrate content in the EPS of uropathogenic biofilms.

Carbohydrate concentration in the extracted EPS was measured using the Phenol-sulphuric acid assay and values are in mg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. Difference between carbohydrate estimation of 24h and 48h biofilms is significant at p≤0.01 (paired two-tailed *t* test).

2.3.9 Expression of biofilm proteins

To determine the differences in protein expression of planktonic and biofilm bacteria, SDS-PAGE analysis was used and gels were stained with Coomassie blue stain. As seen in Figure 2.17 (red boxes indicate higher protein expression) planktonic bacteria displayed a lower protein expression compared with biofilm bacteria for all uropathogens. The highest difference in protein expression is seen for ESBL negative and positive strains of *K. pneumoniae* and *E. coli*. On the other hand, *P. mirabilis* displayed a slightly higher expression of proteins in their planktonic form. Overall, more protein bands are seen in *E. coli* and *P. mirabilis* strains compared with *K. pneumoniae* strains.

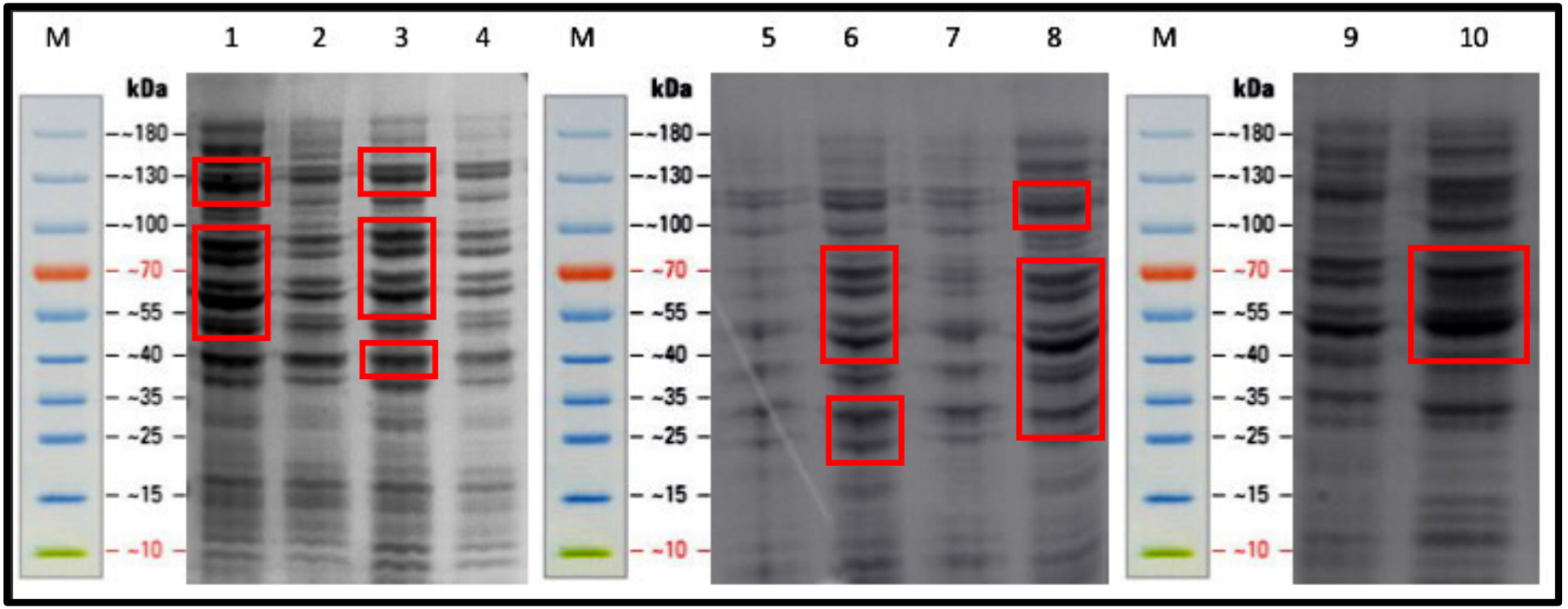

Figure 2.17: Protein profiles of uropathogens (planktonic bacteria and biofilm bacteria) using SDS-PAGE.

The gel was stained using Coomassie blue and 15µl (~1mg/ml) of sample was loaded into each well. M – Prestained protein ladder, 1 – *E. coli* biofilm bacteria, 2 – *E. coli* planktonic bacteria, 3 – *E. coli* ESBL biofilm bacteria, 4 – *E. coli* ESBL planktonic bacteria, 5 – *K. pneumoniae* planktonic bacteria, 6 – *K. pneumoniae* biofilm bacteria, 7 – *K. pneumoniae* ESBL planktonic bacteria, 8 – *K. pneumoniae* ESBL biofilm bacteria, 9 – *P. mirabilis* biofilm bacteria, 10 – *P. mirabilis* planktonic bacteria. Red box indicates higher expression of proteins.

2.3.10 Bacterial motility assays

Swimming, swarming and catheter bridge migration assays were performed to determine the motility of uropathogens.

To determine the motility of uropathogens in an aqueous environment, swimming assays were performed on 0.3% LB agar and as evident in Figure 2.18 only *K. pneumoniae* ESBL negative strain and *P. mirabilis* could swim from the point of inoculation over a period of 24h with the latter exhibiting higher swimming abilities (13mm). Prolonged incubation (data not shown) of both strains of *E. coli* used in this study displayed swimming ability. However, in order to compare motility with 24h biofilms, only 24h motility data were used in the study.

Swarming assays were performed on 1.5% LB agar to determine the surface motility of uropathogens in multicellular aggregates. Only *P. mirabilis* could swarm across the LB agar displaying a bull's eye type of swarming (Figure 2.19).

Catheter bridge migration assays were also performed on 1.5% LB agar and 1cm catheter pieces were used as bridges between the agar blocks (Figure 2.20 and Figure 2.21). All uropathogens could migrate further on hydrogel catheters compared with PTFE latex and silicone catheters (Table 2.5). The latter supported low levels of migration. Out of the five strains, *P. mirabilis* displayed a greater ability to migrate across all three catheters (Figure 2.21 5 & 6).

Table 2.5: Assessment of swimming, swarming and migration abilities of uropathogens in LB agar.

Swimming – 0.3% agar, swarming and catheter bridge migration – 1.5% agar. a – farthest swimming distance, b – farthest swarming distance, c – farthest migration distance (*P. mirabilis* – swarming migration)

Uropathogens	**Agar swimming distance[a] (mm)**	**Agar swarming distance[b]**		**Catheter bridge migration distance[c] (mm)**		
		Distance (mm)	**Type**	**Silicone**	**PTFE**	**Hydrogel**
E. coli	0	0	Non-swarming	1	2	3
***E. coli* ESBL**	0	0	Non-swarming	1	2	5
K. pneumoniae	7	0	Non-swarming	1	2	8
***K. pneumoniae* ESBL**	0	0	Non-swarming	3	5	6
P. mirabilis	13	42	Bull's eye	9	21	20

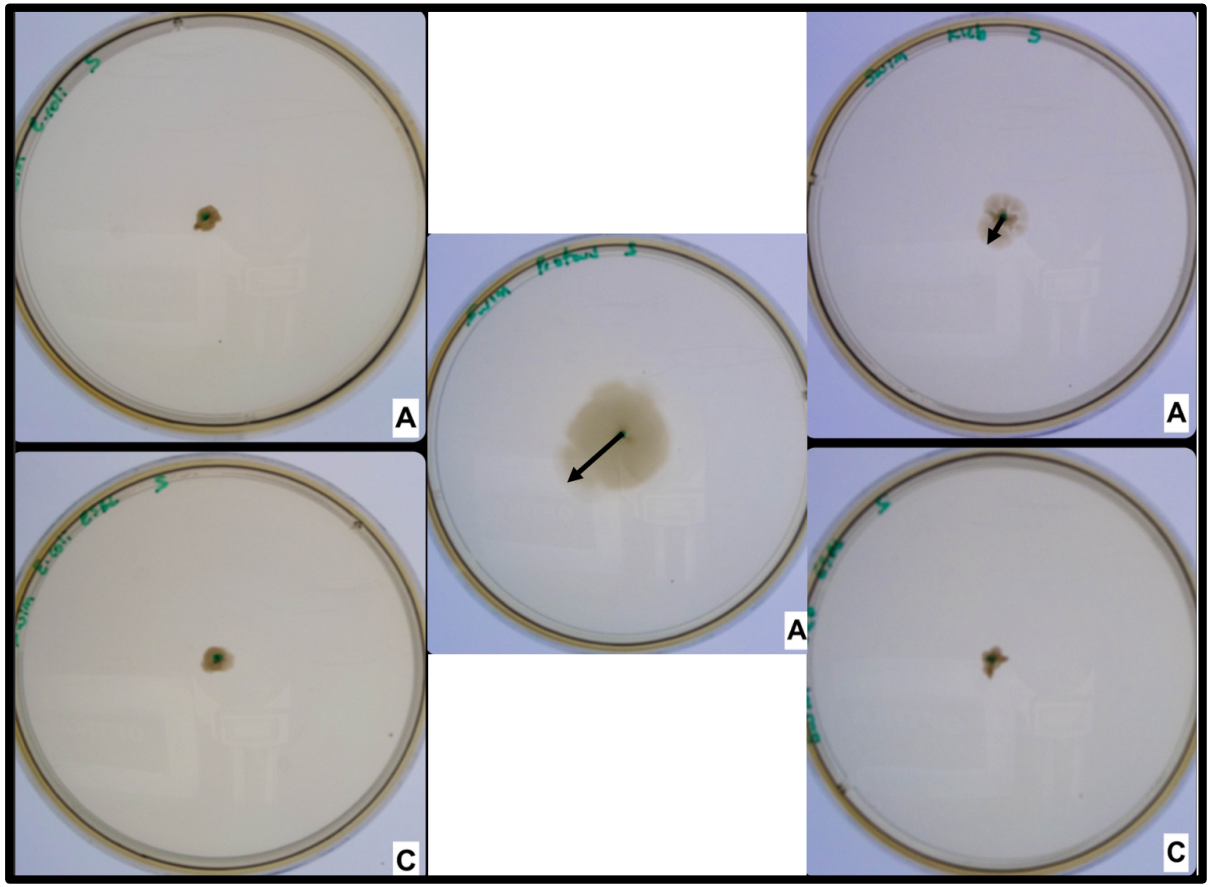

Figure 2.18: Assessment of swimming abilities of uropathogens in 0.3% LB agar.

Farthest distance travelled (swam – indicated by black arrows) was measured from point of inoculation with a ruler. Plates were incubated for 18 to 24h. A – *E. coli,* B – *E. coli* ESBL, C – *P. Mirabilis*, D – *K. pneumoniae*, E – *K. pneumoniae* ESBL.

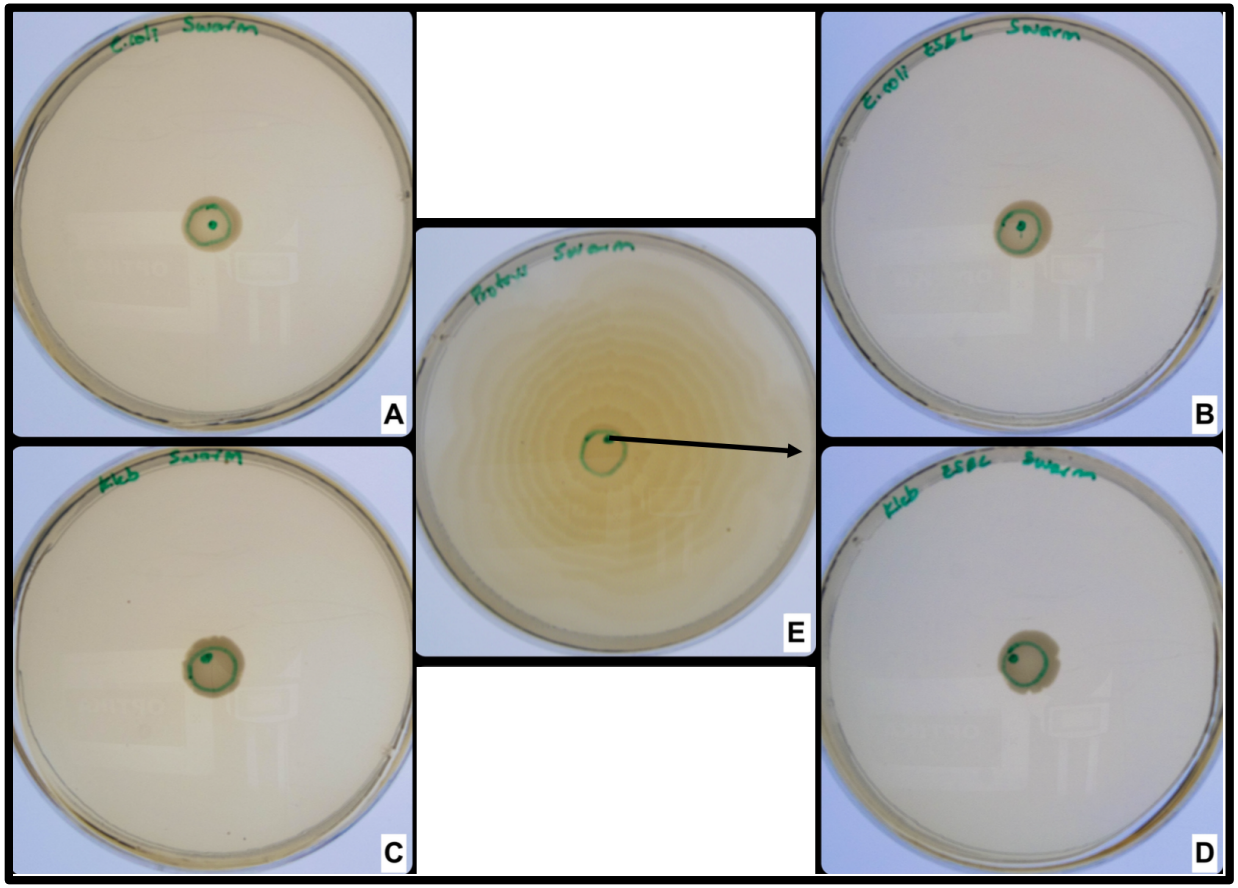

Figure 2.19: Assessment of swarming abilities of uropathogens in 1.5% LB agar.

Farthest distance travelled (swarmed – indicated by black arrows) was measured from point of inoculation with a ruler. Plates were incubated for 18 to 24h. A – *E. coli*, B – *E. coli* ESBL, C – *P. Mirabilis*, D – *K. pneumoniae*, E – *K. pneumoniae* ESBL.

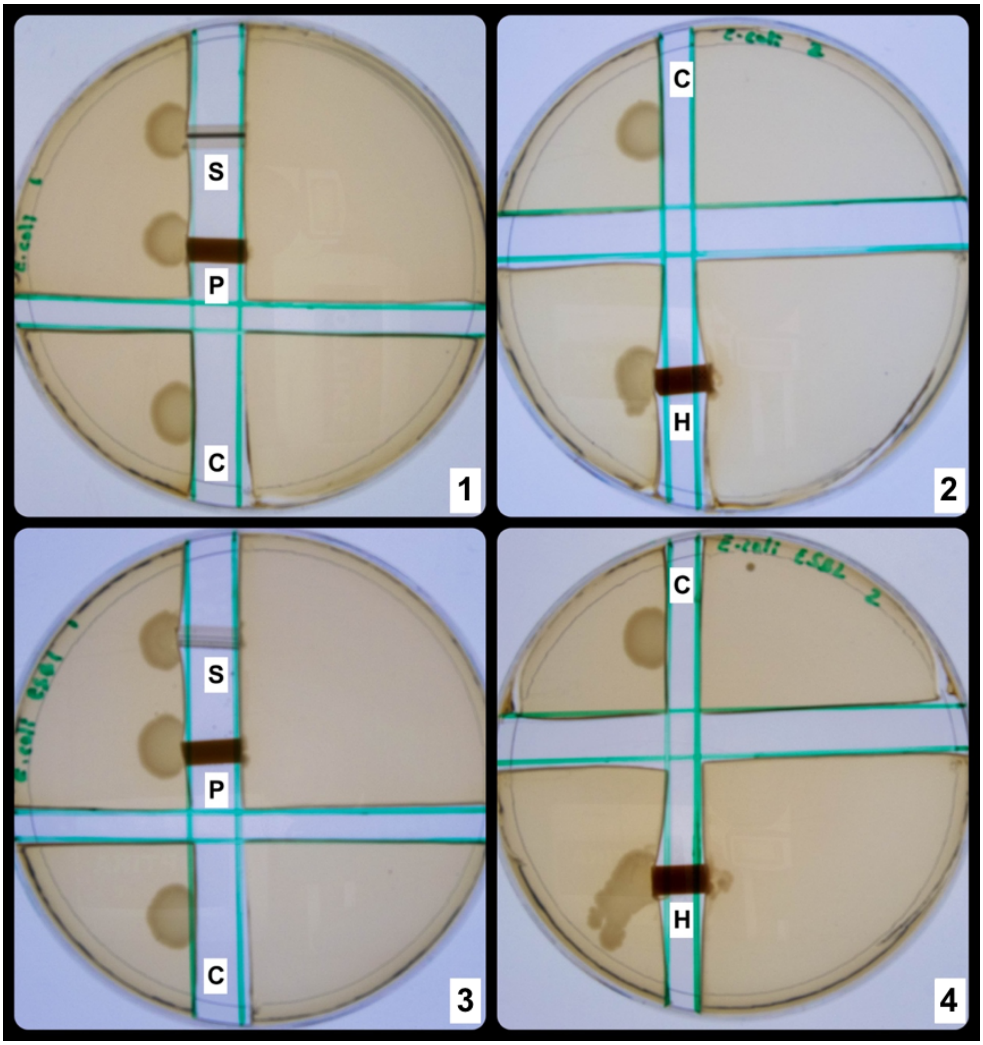

Figure 2.20: Assessment of catheter bridge migration of *E. coli* ESBL negative and positive in 1.5% LB agar.

Four hour cultures of uropathogens were inoculated on the left agar block and a 1cm catheter piece was placed as a bridge between the left and right agar blocks. After 24h incubation, the distance migrated on the uninoculated halves of the plate, adjacent to catheter bridges was measured with a ruler. C – control (no catheter bridge), S – silicone catheter, P – PTFE coated latex catheter, H – hydrogel coated latex catheter, 1 & 2 – *E. coli*, 3 & 4 – *E. coli* ESBL.

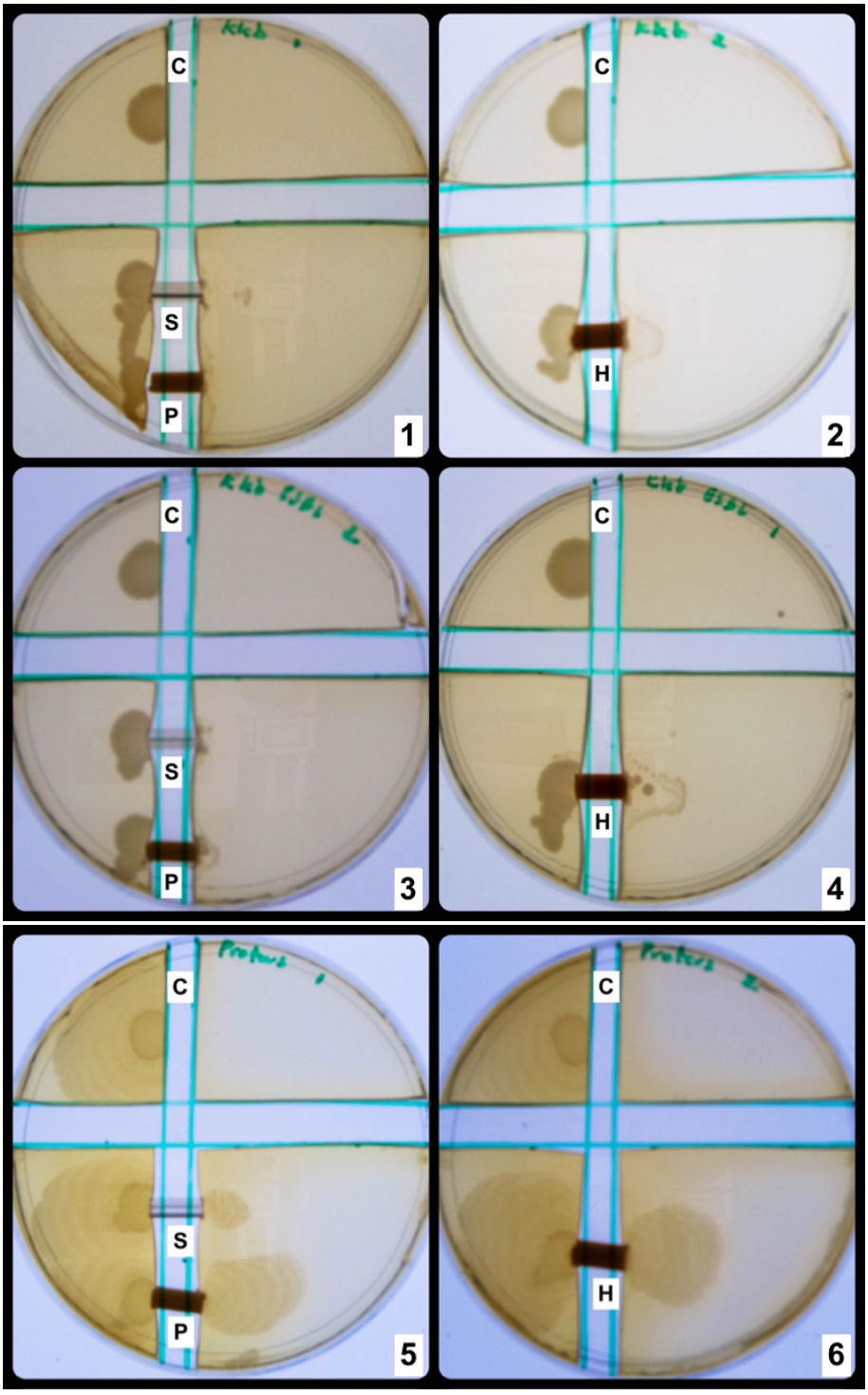

Figure 2.21: Assessment of catheter bridge migration of K. pneumoniae (ESBL negative and positive) and *P. mirabilis* in 1.5% LB agar.

4h cultures of uropathogens were inoculated on the left agar block and a 1cm catheter piece was placed as a bridge between the left and right agar blocks. After 24h incubation, the distance migrated on the uninoculated halves of the plate, adjacent to catheter bridges was measured with a ruler. C – control (no catheter bridge), S – silicone catheter, P – PTFE coated latex catheter, H – hydrogel coated latex catheter, 1 & 2 – *K. pneumoniae*, 3 & 4 – *K. pneumoniae* ESBL, 5 & 6 – *P. mirabilis*.

2.3.11 Comparison of various characteristics of uropathogens

Table 2.6 provides a comparison of the various characteristics of uropathogens investigated in the present study. The best planktonic growth patterns were seen when LB broth was used as the growth medium (Figure 2.3). Microtitre plate assays have revealed different growth patterns (Figure 2.5, Figure 2.7 and Figure 2.9). For planktonic growth in AUM, all uropathogens displayed the highest growth at 12h of incubation. Microtitre biofilm assays have revealed that for *E. coli* (ESBL positive) and *K. pneumoniae* (ESBL negative and positive), the highest number of viable biofilm cells were found at 12h of incubation whereas for *E. coli* (ESBL negative) it was 12h and for *P. mirabilis* it was 6h of incubation. Both strains of *E. coli* biofilms had the highest biomass at 48h whereas the other uropathogenic biofilms contained the highest biomass at 24h. Under fed conditions (Figure 2.13 and Figure 2.14), rate of biofilm dispersal for all uropathogens was high from 24h biofilms whereas 48h biofilms for all uropathogens except *P. mirabilis* (72h) contained the highest biomass. In terms of EPS content (Figure 2.15 and Figure 2.16), all uropathogens displayed high protein and carbohydrate content in 48h biofilms except *K. pneumoniae* ESBL positive (48h for protein and 72h for carbohydrate). Motility assays (Table 2.5) have revealed that only *P. mirabilis* was able to swim and swarm. However, all uropathogens were able to migrate across all tested catheters with increased migration patterns seen in hydrogel latex catheters (PTFE for *P. mirabilis*). It is evident from catheter biofilm assays (Figure 2.11) that all uropathogens displayed high biofilm formation on silicone catheters except for *E. coli* ESBL positive which seems to have preferred PTFE latex catheters.

Table 2.6: Comparison of various characteristics of uropathogens.

[a] Best media for highest planktonic cell growth (24h); [b&c] Best incubation period (high number of viable bacteria); [d] Best incubation period (high quantity of biomass); [e] Best incubation period for highest number of dispersed bacteria in fed state; [f] Best incubation period for highest quantity of biomass in fed state; [g] Best incubation period for highest concentration of EPS; [h] Motility (swimming and swarming distance); [h1] Best catheter for migration and [i] Best catheter for biofilm formation.

Features		***E. coli***	***E. coli* ESBL**	***K. pneumoniae***	***K. pneumoniae* ESBL**	***P. mirabilis***
Media[a]		LB broth	LB broth	LB broth	LB broth	LB broth
Planktonic bacteria[b]		12h	12h	12h	12h	12h
Biofilm viability[c]		24h	12h	12h	12h	6h
Biofilm biomass[d]		48h	48h	24h	24h	24h
Biofilm dispersal[e] (fed state)		24h	24h	24h	24h	24h
Biofilm biomass[f] (fed state)		48h	48h	48h	48h	72h
EPS[g]	**Protein**	48h	48h	48h	48h & 72h	48h
	Carbohydrate					
Motility[h]	**Swimming**	0mm	0mm	7mm	0mm	13mm
	Swarming	0mm	0mm	0mm	0mm	42mm
	Migration[h1]	Hydrogel	Hydrogel	Hydrogel	Hydrogel	PTFE
Biofilm	**Catheter[i]**	Silicone	PTFE	Silicone	Silicone	Silicone

2.4 Discussion

UTIs are amongst the most common nosocomial infections (Kalsi *et al.,* 2003). A wide range of uropathogens employs the method of forming biofilms to evade host defence mechanisms and antibacterial drugs causing ABR and recurrent infections (Roilides *et al.,* 2015; Hoiby, 2010). The purpose of the current study was to investigate the ability of uropathogens to form biofilms and explore factors that can influence biofilm formation.

Five uropathogens isolated from patients diagnosed with UTIs were used in this study. To confirm the identity of these bacteria and to show the genetic differences between ESBL positive and negative strains, PCR analysis was performed (Figure 2.2). PCR products from all strains displayed bands for the 16s rRNA gene, whereas *E. coli* and *K. pneumoniae* ESBL positive strains displayed bands for TEM-1 and SHV genes respectively. Additionally, bands for UreC gene were observed for *P. mirabilis.* These results are consistent with previous studies (Wang *et al.,* 2002; Chroma and Kolar, 2010; Kolar *et al.,* 2010; Ramadan *et al.,* 2016; Varkey *et al.,* 2014; Takeuchi *et al.,* 1996) for all the primers (Table 2.2) used. TEM and SHV are most commonly found in *E. coli* and *K. pneumoniae*, and the majority of ESBLs are derived from TEM and SHV enzymes (Sharma *et al.,* 2010). TEM-1 is a plasmid-mediated β-lactamase gene predominantly found in *E. coli* (Shahi *et al.,* 2013) whereas SHV is a chromosomally encoded gene specific to *K. pneumoniae* (Chaves *et al.,* 2001). ESBLs are one of the major causes of ABR in Gram-negative bacteria (Rawat and Nair, 2010). UreC is a protein involved in urea degradation

pathway and is encoded by the UreC gene commonly found in *P. mirabilis* and is often implicated in *P. mirabilis* induced toxicity (Shi *et al.,* 2016).

ESBL producing bacteria have the ability to hydrolyse a variety of antibiotics including third generation-cephalosporins and aztreonam (Thomson, 2010). To analyse the antibiotic susceptibility range of uropathogens, seven antibiotics were chosen based on the recommendations made by EUCAST for complicated and uncomplicated UTIs (Leclercq *et al.,* 2011). *E. coli* ESBL positive strain was resistant to all antibiotics except imipenem whereas *K. pneumoniae* ESBL positive strain was sensitive only to imipenem, gentamicin and cefoxitin showing that they are more difficult to treat when compared with ESBL negative strains (Table 2.4). *E. coli* and *K. pneumoniae* are on the list of seven pathogens considered as bacteria of international concern by the WHO. This is mainly due to their ability to develop ABR via ESBL genes. The WHO global report on surveillance has reported that *E. coli* and *K. pneumoniae* are resistant to third-generation cephalosporins in addition to fluoroquinolones (*E. coli*) and Carbapenams (*K. pneumoniae*) (WHO, 2014). ABR causes significant economic and health impacts worldwide (WHO, 2014; Cars, 2014).

In 1863, Pasteur first reported that bacterial growth is readily supported by human urine (Asscher *et al.,* 1966). Several studies have suggested that urine supports growth of facultative Gram-negative bacilli better than other uropathogens (Wilson, 2005). It has been previously reported that presence of glycine betaine, an osmoprotectant produced by the renal medulla, in urine confers some degree of osmoprotection to *E. coli* in the urinary tract (Chambers

and Kunin, 1985; Kunin *et al.,* 1992). However, a variety of factors in urine such as urea, organic acids (Chambers *et al.,* 1999), pH (Erdogan-Yildirim *et al.,* 2011) and the presence of secretory immunoglobulins (Wilson, 2005) can have an inhibitory effect on bacterial growth. Based on a recipe (Table 2.1) prepared by Brookes and Keevil (1997), AUM was prepared and its bacterial growth efficacy was compared with that of HU. There was no significant difference in the ability of uropathogens to grow and multiply in AUM and HU (Figure 2.3). AUM has been successfully used before in a range of experiments as a bacterial growth medium (Chutipongtanate and Thongboonkerd, 2010) and based on the present results (Figure 2.3) it is effective in mimicking the growth of uropathogens in natural human urine.

To determine if any of the major urine components have any effect on bacterial growth, AUM samples were prepared without urea, uric acid and creatinine, and bacterial growth in these samples were evaluated after 24h of incubation. Interestingly, AUM sample without urea displayed higher growth patterns when compared to control (AUM) in comparison with decreased bacterial growth seen in AUM samples without creatinine (Figure 2.4). Although not extensively researched, previous studies have indicated that urea has a bacteriostatic effect on many Gram-positive and Gram-negative bacteria (Bonin, 1949; Weinstein and McDonald, 1946; Chambers *et al.,* 1999).

Planktonic growth assays were performed to analyse the growth patterns in AUM of all uropathogens used in this study. All strains except *P. mirabilis* displayed similar growth patterns with a steady increase from 0h to 12h (Figure

2.5, Figure 2.7 and Figure 2.9). The highest growth rate was observed just after 12h of incubation. The increase in growth rates (0h to 12h) of *P. mirabilis* was not very consistent (Figure 2.9). Furthermore, similar growth rates were observed after 12h and 24h of incubation. Urease-producing organisms such as *P. mirabilis* can convert urea into ammonia and cause an increase in urine pH. This rise in pH can limit bacterial growth (Brooks and Keevil. 1997). The decrease in growth rates for all strains after 24h of incubation exhibits stationary and death phase (Paulton, 1991).

The interrelationship between planktonic and biofilm bacteria is quite complex. Planktonic bacteria are known to convert into biofilm bacteria via differential gene expression (Mikkelsen *et al.,* 2007). These sessile bacteria then form biofilms in many stages.

Biofilms can exist on both biotic and abiotic surfaces (Murugan *et al.,* 2016). The formation of a conditioning film is the first step in its formation and is crucial for bacterial surface attachment. Several features of the attachment surface (substrate) like surface hydrophobicity and roughness are influential in microbial adhesion (Lorite *et al.,* 2011). Apart from substrate characteristics, surface virulence factors such as pili and fimbrial adhesins also play a role in the initial surface attachment of microbes (Table 1.1). However, many bacteria don’t have direct mechanisms by which they can adhere to surfaces like those of catheters (Busscher and Weerkamp, 1987). In such cases, the formation of a conditioning film promotes bacterial attachment particularly to abiotic surfaces. It is well known that catheters are more prone to bacterial colonisation

due to the rapid formation of a conditioning film made up of proteins from body fluids (Stickler, 2014). An artificial bladder model was used to coat a urinary catheter with an AUM conditioning film. Bacteria were allowed to adhere to the conditioned PTFE coated latex catheters for a period of 3h. All uropathogens have exhibited greater affinity to catheters with a preformed conditioning layer (Figure 2.12). Adsorption of urine components onto the catheter surface forms a conditioning film, made up of various proteins present in the urine, like albumin, THP and alpha1-microglobulin (Tieszer, 1998). Previous studies on peritoneal catheters (Gorman *et al.,* 1997) and vascular catheters (Murga *et al.,* 2001) have highlighted the influence of conditioning film on bacterial adherence.

The next step of biofilm formation is the initial, reversible, surface attachment of planktonic bacteria followed by irreversible adsorption (Gottenbos *et al.,* 2002; Palmer *et al.,* 2007). A microtitre plate assay (Djordjevic *et al.,* 2002) was used to compare the ability of various uropathogens to form biofilms on the polystyrene surface of a 24-well plate. Viable bacteria from the biofilm were scraped and enumerated after various periods of incubation. *K. pneumoniae* (ESBL positive and negative strains) was observed to form biofilms at levels higher than the other uropathogens (Figure 2.7). Biofilms of all uropathogens except *E. coli* and *P. mirabilis* recruited high number of bacteria after 12h of incubation whereas for the latter two, high numbers were found in 24h and 6h biofilms respectively (Figure 2.9). In contrast to other uropathogens, the number of viable bacteria from *P. mirabilis* biofilms decreased after 6h of incubation (Figure 2.9). This can be attributed to the fact that *P. mirabilis* is a

urease-producing bacterium, which, in the presence of urine forms calcium and magnesium phosphate crystals which can interfere with their biofilm forming ability (Jones *et al.,* 2006).

Contact with biomaterials like urinary catheters are often linked to nosocomial infections (Murugan *et al.,* 2016). A catheter biofilm assay was used to compare the ability of uropathogens to form biofilms on silicone and latex catheters which are commonly used in the NHS (Pickard *et al.,* 2012). The findings from the present study (Figure 2.11) indicate that all uropathogens except *E. coli* (ESBL positive) formed better biofilms on silicone catheters compared with latex catheters. Between hydrogel and PTFE latex catheters, all uropathogens formed lesser biofilms on hydrogel coated catheters. One of the main purposes of hydrophilic-coating of catheters is to reduce microbial adherence. Although previous studies have reported conflicting findings on the anti-adherence ability of hydrogel coated catheters (Siddiq and Darouiche, 2012), our findings support the notion that these catheters decrease biofilm formation compared with non-hydrogel coated ones. Studies comparing colonisation and biofilm formation on latex and silicone catheters in terms of STC is very limited with conflicting results (Verma *et al.,* 2016).

Following irreversible attachment, bacteria undergo phenotypic changes and start the process of maturation, the bacteria become embedded and immobilised in EPS (Pace *et al.,* 2006). CV staining of the biofilm biomass has revealed that *K. pneumoniae* (ESBL positive and negative strains) biofilms have a higher biomass compared with other uropathogens (Figure 2.7 and

Figure 2.8). In terms of the incubation period, 48h *E. coli* biofilms had maximum biomass (Figure 2.5 and Figure 2.6) compared with 24h for *K. pneumoniae*. On the other hand, 24h, 48h and 72h *P. mirabilis* biofilms had similar amount of biomass (Figure 2.9 and Figure 2.10). Moreover, light microscopy images of uropathogenic biofilms (Figure 2.6 and Figure 2.8 and Figure 2.10) has revealed that 24h *K. pneumoniae* (ESBL positive and negative strains) biofilms are irregular with less spacing and a clumpy growth when compared to the other bacterial strains. A study conducted by Dzul *et al.*, (2011) has concluded that *K. pneumoniae* capsule primarily contributes to biofilm microstructures resulting in multicellular aggregates. Biofilm biomass consist of both bacteria and EPS (Butterfield *et al.,* 2002; Zhang *et al.,* 2013). EPS is mainly made up of proteins, polysaccharides, lipids and extracellular DNA (Jachlewski *et al.,* 2015). These EPS biopolymers are highly hydrated and form a matrix (Flemming *et al.,* 2007) which protects bacteria from a variety of environmental stresses by conferring resistance and promoting long-term persistence (Bales *et al.,* 2013). Amongst all the biopolymers, one of the major constituents in EPS is proteins (Jiao *et al.,* 2011). Nevertheless, polysaccharides are also important for biofilm structure and stability (Andersson *et al.,* 2011). Analysis of EPS extracted from 24h, 48h and 72h biofilms for carbohydrate and protein composition showed that uropathogenic biofilms use a higher proportion of protein in their microstructure (Figure 2.15 and Figure 2.16). The findings from the present study also indicate that 48h biofilms possessed higher amount of these biopolymers suggesting strengthening of their structure over longer incubation periods. However, these findings should be interpreted with caution since several studies have reported the complexity of analysing EPS

components particularly carbohydrates due to their unique structures and monomer diversity (Flemming *et al.,* 2007). SDS-PAGE analysis also revealed higher expression of proteins in biofilm bacteria compared with planktonic bacteria (Figure 2.17). This finding is consistent with previous studies wherein biofilm proteins different from the planktonic profile were identified (Sauer *et al.,* 2002; Mikkelsen *et al.,* 2007). Conversion of bacteria from planktonic to biofilm states result in the expression of a number of genes needed for cell surface proteins such as porins. These proteins regulate membrane transport in the complex biofilm structure (Garrett *et al.,* 2008).

The final stage is the detachment of bacteria and their dispersal into the environment to colonise new sites (Kaplan, 2010; Percival *et al.,* 2011). Detachment of bacteria from the biofilms completes the cycle (Trautner and Darouiche, 2004). When 24h, 48h and 72h biofilms were subjected to fed (AUM) and unfed (ds water) conditions for 24h, unfed biofilms dispersed less number of bacteria displaying a nutrient dependent response to cell dispersal (Figure 2.13). Dispersal under fed states was highest from 24h biofilms. Bacteria have devised mechanisms through which they can sense environmental changes and change their phenotype from biofilm cells to planktonic cells. Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is most commonly implicated in causing the shift between biofilm and planktonic states (Kostakioti *et al.,* 2013). Nutrition plays an important role in biofilm dispersal (Chao *et al.,* 2015). Under stress conditions such as nutrition depletion, some bacteria can reduce the levels of their intracellular c-di-GMP which results in biofilm dispersal (Chao *et al.,* 2014). However, dispersal in

some bacterial biofilms is activated under nutrient rich conditions (Uppuluri and Lopez-Ribot, 2016). The findings from the present study is consistent with the latter approach to nutrient availability.

On the question of biomass, all uropathogens except *K. pneumoniae* displayed significant ($p \leq 0.05$) differences between fed and unfed states (Figure 2.14). Under fed conditions, 48h and 72h biofilms possessed the highest amount of biomass. This finding suggests that older biofilms are more efficient in building EPS matrix. It is also known that nutrient availability can increase bacterial growth in biofilms (Garrett *et al.,* 2008) allowing the biofilms to retain more in their matrix (Donlan, 2002) which can cause an increase in their biomass (bacteria and EPS).

Apart from allowing biofilm formation, catheters act as an ideal bridge for bacteria to pass along from the contaminated external environment into the body (Jones *et al.,* 2004). A catheter bridge migration assay was performed to determine the ability of uropathogens to migrate along the three catheters used in this study. This assay revealed that all uropathogens can migrate along the three catheters with higher migration patterns seen in hydrogel coated latex catheters (Table 2.5). Previous studies have demonstrated that bacteria can easily migrate over hydrogel coated catheters (Siddiq and Darouiche, 2012). Amongst all the uropathogens, *P. mirabilis* has displayed the greatest ability to migrate across all the tested catheters. This can be linked to the ability of *P. mirabilis* to differentiate into swarmer cells (Figure 2.19) which are more

effective at moving over catheters (Sabbuba *et al.,* 2002). These findings have further strengthened the relationship between use of catheters and CAUTIs.

Bacterial motility is generally linked to their ability to colonise surfaces which in turn influences biofilm formation (Maya-Hoyos *et al.,* 2015). Bacterial motility assays were performed to expose any possible link between the motility of uropathogens used in this study and their ability to form biofilms. Only *K. pneumoniae* ESBL negative and *P. mirabilis* could swim across 0.3% LB agar whereas only the latter had the ability to swarm across 1.5% LB agar producing a bull's eye swarming ring (Figure 2.18 and Figure 2.19). When these results are compared with the findings from biofilm viability and biomass assays (Figure 2.7 and Figure 2.9), we can see that only *K. pneumoniae* had a high number of bacteria and biomass in their biofilms. *P. mirabilis* biofilms, even with their ability to swim and swarm had lesser viable bacteria and biomass compared with both strains of *K. pneumoniae* and ESBL negative strain of *E. coli*. This suggests that microbial motility is not the only factor that can influence the formation of biofilms and this relationship is diverse between different bacterial strains. (Ding and Wang, 2009). Bacteria have the ability to sense environmental cues and adapt to a surface by switching between the 'swim-or-stick' phases (Belas, 2013). Swimming and swarming require flagellar motility but in addition, the latter is dependent on the production and secretion of rhamnolipid surfactants to reduce friction between bacteria and its swarming surface (Deforet *et al.,* 2014). *E. coli* (DiLuzio *et al.,* 2005) and *P. mirabilis* (Belas *et al.,* 1998) are known to have the ability to swim and swarm. Although our findings confirm that *P. mirabilis* can swim and swarm on LB agar, we did

not find any pattern of motility for *E. coli* in the first 24h. On the other hand, *K. pneumoniae* is a non-motile pathogen (Guo *et al.,* 2012). However, our findings suggest that it can indeed swim. This finding is in agreement with a study conducted by Srinivasan and Rajamohan (2013) who also reported swimming motility in *K. pneumoniae* (NTUH-K2044 strain). In another interesting study, *K. pneumoniae* isolated from a patient with neonatal sepsis demonstrated swim-like motility and expression of a polar flagella (Carabarin-Lima *et al.,* 2016). These findings suggest an evolutionary pathway.

The current findings have revealed a number of features related to uropathogenic biofilm formation. Firstly, planktonic, biofilm viability and biomass, and nutrition assays have revealed that completion of the biofilm cycle takes 12h to 24h. Therefore, exposure of catheters to microbial colonisation in the first 24h is crucial and preventing this can reduce the incidence of CAUTIs particularly related to STC. Secondly, biofilm dispersal is related to nutrient availability. Dispersal often leads to colonisation of new areas causing recurrent infections. Limiting availability of crucial nutrients by using iron chelators for example, can prevent recurrence by inhibiting dispersal and enabling biofilm destruction. Thirdly, targeting over expression of proteins in biofilm bacteria may reduce buildup of EPS and therefore ABR and resistance to host immune responses. Finally, biofilm assays and migration assays have revealed that all uropathogens were able to form biofilms, and migrate across all tested catheters albeit at varying levels. Developing anti-biofilm and anti-adherence urinary catheters through coating mechanisms are some examples of approaches that can be used to tackle CAUTIs.

Catheter related biofilm mechanism is a very complex issue. Almost all medical implants are susceptible to colonisation by biofilm forming bacteria and this is the main contributing factor to recurrent infections. It is vital to fully understand the complex mechanism involved in biofilm formation and the role played by bacterial and environmental factors to deduce an effective anti-biofilm strategy. The results of the present study may provide useful insights for developing anti-biofilm approaches.

Apart from CAUTIs, rUTIs also confer some degree of resistance to uropathogens. Formation of IBCs are commonly implicated in the pathogenesis of rUTIs. The next chapter deals with the interaction of uropathogens and their cytotoxic effect on NHUC.

Chapter 3

Interaction of uropathogens with urothelial cells

3 Interaction of uropathogens with urothelial cells

3.1 Introduction

The urothelium lines the inner surface of the renal pelvis, ureters, bladder, upper urethra, and glandular ducts of the prostate. It forms a common boundary between the urinary space and the underlying tissues. It is a transitional epithelial tissue made up of three layers (basal, intermediate and umbrella cells). Apart from acting as a functional, high-resistance barrier, urothelial cells also play an important sensory role. They have the ability to detect changes in their extracellular environment and respond to these stimuli by expressing a variety of receptors and ion channels and secreting various factors such as ATP, nitric oxide, and acetylcholine (Birder and Andersson, 2013; Hickling *et al.,* 2015). The primary urothelial mediated response to infections is the exfoliation of superficial cells and urothelial regeneration. This response not only clears surface adhered and intracellular bacteria but can also promote dissemination of bacteria into deeper layers of the urothelium potentially benefiting both host and pathogen (Nagamatsu *et al.,* 2015).

It is widely recognised that a UTI typically starts with periurethral contamination, urethral colonisation and migration to the urinary bladder. In the bladder, complex host-pathogen interactions lead to successful colonisation or elimination (Flores-Mireles *et al.,* 2015). The former, results in the formation of biofilms and/or IBCs (Rosen *et al.,* 2007; Flores-Mireles *et al.,* 2015). The IBC pathogenic cycle is characterised by uropathogenic association, invasion and

survival within the urothelium (Rosen *et al.,* 2007). IBCs not only protect bacteria from immune response and antibiotic treatment but also release intracellular bacteria regularly into the surrounding environment to initiate new IBC cycles (Robino *et al.,* 2014). It has been suggested that the re-emergence of bacteria from the intracellular communities and subsequent colonisation of new cells might be the primary source of recurrent infections (Anderson *et al.,* 2004; Wright *et al.,* 2007).

These intracellular bacteria have the ability to induce host cell cytotoxicity by secreting a variety of virulence factors and by repressing host survival pathways (Lu *et al.,* 2013). Cellular cytotoxicity can lead to cell death via apoptosis and/or necrosis. Apoptosis is a programmed form of cell death used to eliminate cells that are not required, and to maintain normal cell populations. It can happen under both physiological and pathological conditions. Necrosis on the other hand, is accidental cell death, which is non-apoptotic in nature. It is a pathway of cell death related to cell injury and is always pathological (Robins and Cotran, 2015).

UPEC are implicated in the vast majority of UTIs. UPEC are known to trigger apoptosis by the secretion of haemolysins (Lu *et al.,* 2013), particularly a pore-forming toxin known as HlyA (Nagamatsu *et al.,* 2015). HlyA forms pores in cell membranes causing urothelial cell toxicity and eventually cell lysis. *K. pneumoniae* can induce apoptosis in human cells by forming membrane pores through a low molecular weight channel-forming bacteriocin known as microcin E492 (Hetz *et al.,* 2002) and by inducing cytotoxicity through their capsular

polysaccharides (Cano *et al.,* 2009). It has been suggested that mannose-resistant *Proteus*-like fimbria and flagella of *P. mirabilis* mediate cytotoxic effects on eukaryotic cells (Scavone *et al.,* 2015). *P. mirabilis* also produces haemolysin and *Proteus* toxic agglutinin, which are toxins implicated in tissue damage (Flores-Mireles *et al.,* 2015). Depending on the type of virulence factor, cell death can happen via different mechanisms.

In apoptosis, cell death can occur via the intrinsic or extrinsic pathway also known as signalling cascades. The intrinsic or mitochondrial pathway is caused by the imbalance of B-cell lymphoma-2 (Bcl-2) family proteins causing increased mitochondrial permeability, leakage and caspase activation whereas the extrinsic or death receptor pathway is caused by the engagement of plasma membrane death receptors on cells leading to the formation of 'death-including signalling complex' and finally caspase activation. However, in necrosis, the mechanism of cell death is associated with its specific pathology. In ischaemia-reperfusion injury, serine/threonine kinase receptor-interacting protein 1 (RIP1) causes ligand-receptor interaction and induces necrosis whereas in ischaemic injury ATP depletion plays a crucial role. Irrespective of the injury, elevated calcium levels and reactive oxygen species (ROS) are major players in inducing necrosis. Release of immunomodulatory factors, phagocytosis and significant immune response are also characteristic of necrosis (Robins and Cotran, 2015; Vanlangenakker *et al*., 2008; Guicciardi *et al*., 2003).

Apoptosis and necrosis also differ morphologically. The former is characterised by shrinkage of cells, nuclear changes such as pyknosis (nuclear shrinkage),

karyorrhexis (nuclear fragmentation) and karyolisis (fading of chromatin basophilia), intact/altered plasma membrane and no adjacent inflammation. Swelling of cells, chromatin condensation, disrupted plasma membrane, leakage of cellular contents due to enzymatic digestion and frequent adjacent inflammation are more characteristic of necrosis (Robins and Cotran, 2015).

These morphological markers are important in determining the stages of apoptotic cell death. It is well recognised that apoptosis and necrosis can co-exist in the same tissue or cell type (Lu *et al.,* 2013). Early apoptotic cells are characterised by an intact cell membrane with cell surface expression of phosphatidylserine (PS) to aid recognition by phagocytes. When the membrane becomes permeabilised, early apoptotic cells become late apoptotic cells, which can then progress on to secondary necrotic cells. Primary necrotic cells are also characterised by membrane permeability, however, this is due to direct exposure of viable cells to trauma such as chemical insults rather than a transition from apoptosis (Poon *et al.,* 2010).

Flow cytometry is traditionally used to differentiate dying cells. It is the preferred method for single cell analysis of multiple cellular attributes (Wlodkowic *et al.,* 2013). Flow cytometry measures many characteristics of apoptotic cells including changes in cell membrane, membrane permeability (cell and mitochondrial), caspase activation and DNA damage. It helps to quantify cells based on the stages of apoptotic cell death and it also provides valuable information about the pathway of cell death used (Allen and Davies, 2007). Several studies have employed the use of Annexin V/Propidium Iodide for

assessment of cell death. In early apoptotic cells, annexin V (Ca^{2+} dependent phospholipid-binding protein, which has high affnity for PS) binds to PS expressed on the outer leaflet of cell plasma membranes whereas in late apoptosis and necrosis, membranes of dead and damaged cells are permeable to propidium iodide (DNA stain). This differential staining is the primary mechanism of flow-cytometric detection of apoptosis and necrosis (Rieger *et al*., 2011).

Apart from flow cytometry, measurement of lactate dehydrogenase (LDH) is also a useful tool in investigating cellular cytotoxicity with regards to the necrotic pathway. LDH is a soluble cytoplasmic enzyme released into the extracellular space through damaged cell membranes. The assay employs the use of a tetrazolium salt which converts to a coloured formazan product in the presence of an electron acceptor using nicotinamide adenine dinucleotide (NADH) produced when LDH catalyses the oxidation of lactate to pyruvate. The formazan product can be colorimetrically measured to give an indirect estimate of the percentage of necrotic cells in a sample (Chan *et al.,* 2013).

In this study, mechanisms of host-pathogen interactions in UTIs were explored. The ability of uropathogens to associate, invade and survive within urothelial cells were assessed through interaction assays. Dispersal assays were also carried out to explore the ability of uropathogens to disseminate from IBCs formed within urothelial cells. Furthermore, the cytotoxic effect of intracellular bacteria and the pathway of cell death induced by uropathogens was

elucidated via flow cytometry and LDH assays were performed to differentiate between apoptotic and necrotic cytotoxicity.

3.2 Materials and methods

3.2.1 Cells

Telomerase Reverse Transcriptase-immortalised Normal Human Urothelial Cell (TERT-NHUC) line was kindly provided by Prof. Knowles, Cancer Research UK Clinical Centre, St. James's University Hospital, Leeds. The cell line was provided as a frozen sample.

3.2.1.1 Cell culture

The frozen samples were thawed and centrifuged (1000 x g, 1min) to remove the freezing medium (Appendix A). The cells were re-suspended in 5ml Keratinocyte growth medium 2 (KGM-2) [purchased from Promo Cell] with supplements (Appendix A) and passaged into sterile T25 flasks. The flasks were incubated at 37°C in humidified 95% air and 5% CO_2 incubator. The medium was changed at least three times a week. Once the flasks became confluent (~ 75%), the cells were ready to be seeded into 24-well plates or passaged into new T25 flasks.

3.2.1.2 Cell passage

The media from T25 flasks confluent with TERT-NHUC line were discarded and the cells were washed with PBS (~ 2ml). Accutase (0.5ml) was added and the flasks were incubated at 37°C for 5min to detach the cells after which the detached cells were resuspended in KGM-2 and centrifuged (1000 x g, 5min). The supernatants were discarded and the cells were resuspended in fresh

KGM-2 media and passaged into new T25 flasks and incubated at 37°C in humidified 95% air and 5% CO_2 incubator. For experimental assays, the cells were counted using a haemocytometer and the concentration was adjusted to yield approximately 5 x 10^5 cells per 0.5ml. The cells were seeded in 24-well plates (0.5ml per well) and incubated at 37°C in humidified 95% air and 5% CO_2 incubator. The plates were used for assays once the cells formed a confluent (~ 75%) monolayer.

3.2.2 Bacterial culture

All strains were cultured, inoculated and ODs were measured as described in chapter 2 (2.2.2).

3.2.3 Interaction assays

The percentage of bacteria interacted with TERT-NHUC line were calculated as follows:

Number of viable bacteria recovered (CFU/ml) / number of bacteria in initial inoculum x 100 (Alsam *et al.,* 2006; Yousuf *et al.,* 2013).

3.2.3.1 Association assays

To investigate the association of uropathogens with TERT-NHUC line, approximately 10^6 bacteria were added to each well in their log or exponential growth phase. The plates were incubated for 1h at at 37°C in humidified 95% air and 5% CO_2 incubator. Each well was washed once with PBS and the cells

were lysed using cell lytic (150 µl). Bacteria were quantified on CLED agar plates (Alsam *et al.,* 2006).

3.2.3.2 Invasion assays

Invasion of uropathogens with TERT-NHUC line was investigated as described above (3.2.3.1). However, instead of lysing the cells after association, Gentamicin (75 µg/ml, 1h, RT) was used to kill the extracellular bacteria for all uropathogens except *E. coli* ESBL positive (replaced by Meropenem 75 µg/ml, 1h, RT). After incubation, the supernatants were discarded and each well was washed once with PBS and the cells were lysed using cell lytic (150µl). Bacteria were quantified on CLED agar plates (Alsam *et al.,* 2006).

3.2.3.3 Survival assays

To investigate the ability of uropathogens to survive within TERT-NHUC line, instead of lysing the cells after the invasion assays (3.2.3.1), 500µl of KGM-2 was added to each well and the 24-well plate was incubated at 37°C in humidified 95% air and 5% CO_2 for 24h. After incubation, the supernatants were discarded and each well was washed once with PBS and the cells were lysed using cell lytic (150µl). Bacteria were quantified on CLED agar plates (Alsam *et al.,* 2006).

3.2.4 Cytotoxicity assays

3.2.4.1 Flow cytometry assays

Survival assays were carried out as described above (3.2.3.3). Post incubation, cells were harvested (100µl Accutase) and collected with their supernatants in

Eppendorf tubes and centrifuged (1000 x g, 5min). BioLegend's FITC Annexin V Apoptosis Detection Kit with Zombie NIR, was used as per manufacturer's instructions. Briefly, the cells were washed twice with 200µl of cold cell staining buffer (1000 x g, 5min) and resuspended in 100µl of Annexin V binding buffer containing 5µl of Annexin V FITC and 1µl of Zombie NIR. Samples were gently vortexed and incubated for 20min at RT in dark. Samples were then washed with cell staining buffer, centrifuged (1000 x g, 5min), and fixed with 450µl paraformaldehyde for 20min on ice. Post fixation, the samples were centrifuged (1000 x g, 5min), supernatants discarded and the pellets were washed once with cell staining buffer (1000 x g, 5min). The pellets were then resuspended in 400µl of Annexin V binding buffer. Cells were analysed using a flow cytometer [BD Accuri^{TM} C6] with each run restricted to 1000 moments.

3.2.4.2 LDH assays

Survival assays were carried out as described above (3.2.3.3). Post incubation, the supernatants from all wells (except high control wells) were collected in an eppendorf tube. Supernatant from lysed control cells (lysis buffer) and unlysed control cells were used for high and low control respectively (Alsam *et al.,* 2003). Cytotoxicity detection kit^{plus} [Roche] was used as per manufacturer's instructions. Briefly, the supernatants were centrifuged (10000 x g, 5min) and 50µl of each supernatant was loaded into a 96-well microtitre plate. Fifty microliters of reaction mixture was added to each well and the plate was incubated at RT for 15min. The reaction was stopped using the stop solution and the absorbance was quantified in a plate reader at 595nm [FLUOstar Omega].

3.2.5 Software and statistics

FlowJo software (v3.05470) was used to analyse apoptosis and necrosis data obtained from flow cytometry assays. Microsoft Excel (v15.30) was used for general data analysis and preparation of charts. Paired two-tailed *t* test was used for statistical analysis unless otherwise stated.

3.3 Results

3.3.1 Interaction of uropathogens with TERT-NHUC line

3.3.1.1 Interaction assays

Association assays were performed to determine the ability of uropathogens to associate with TERT-NHUC line. All bacterial strains were incubated in a 24-well microtitre plate seeded with TERT-NHUC line for 1h at 37°C in humidified 95% air and 5% CO_2.

As shown in Figure 3.1A, in comparison with the initial inoculum, all strains displayed significant ($p \leq 0.001$) ability to associate with urothelial cells. The most striking result to emerge from the data is that *E. coli* (~ 1.5%) displayed less association compared with *K. pneumoniae* (~ 8%) and *P. mirabilis* (~ 6%). There were no significant differences between the association patterns of ESBL positive and negative *E. coli* but the difference was significant ($p \leq 0.01$) for *K. pneumoniae*. In comparison with *E. coli* ESBL negative, *P. mirabilis* displayed a significantly ($p \leq 0.001$) higher ability to associate with TERT-NHUC line. Overall, *K. pneumoniae* ESBL positive strain displayed the highest ability (7.95%) to associate with TERT-NHUC line.

To assess the ability of uropathogens to invade TERT-NHUC line, invasion assays were performed. Uropathogens associated with TERT-NHUC line were treated with antibiotics to kill extracellular bacteria and promote invasion. The results obtained from the invasion assays are shown in Figure 3.1B. It is apparent from this figure that all bacterial strains were able to invade urothelial cells albeit at varying levels ($p \leq 0.001$ in comparison with the initial inoculum).

Following the trends seen in association assays, ESBL positive and negative strains of *E. coli* (~ 0.01%) displayed less invasion compared with the other strains. Between ESBL positive and negative strains of *K. pneumoniae*, the former exhibited ~ 2.4 fold higher ability to invade urothelial cells. The difference between the invasion patterns of ESBL positive and negative *E. coli* and *K. pneumoniae* is not significant, however, in comparison with *E. coli* ESBL negative, *P. mirabilis* displayed a significantly ($p \leq 0.05$) higher ability to invade TERT-NHUC line.

Finally, Intracellular survival assays were performed to determine the ability of uropathogens to survive and form IBCs within TERT-NHUC line. Urothelial cells invaded with bacteria were incubated at 37°C in humidified 95% air and 5% CO_2 for 24h. Data obtained (Figure 3.1C) from the survival assays reveal that all uropathogens were able to survive within urothelial cells. *E. coli* exhibited the lowest intracellular survival ability (< 0.1% for both ESBL positive and negative strains). The single most striking observation to emerge from the data is that *P. mirabilis* (165%) exhibited a higher intracellular survival ability than *K. pneumoniae* ESBL positive (31%). This is interesting because the latter displayed higher ability to associate with and invade TERT-NHUC line. Between ESBL positive and negative strains of *K. pneumoniae*, the former exhibited ~ 4.2 fold higher ability to survive within urothelial cells ($p<0.05$). Although the survival abilities of ESBL positive and negative *E. coli* are lower than the other uropathogens, *E. coli* ESBL negative strain (0.08%) exhibited a significantly ($p \leq 0.01$) higher survival ability in comparison with its ESBL negative counterpart (0.01%). In comparison with *E. coli* ESBL negative, *P.*

mirabilis (165%) displayed a significantly (p=0.001) higher ability to survive within TERT-NHUC line.

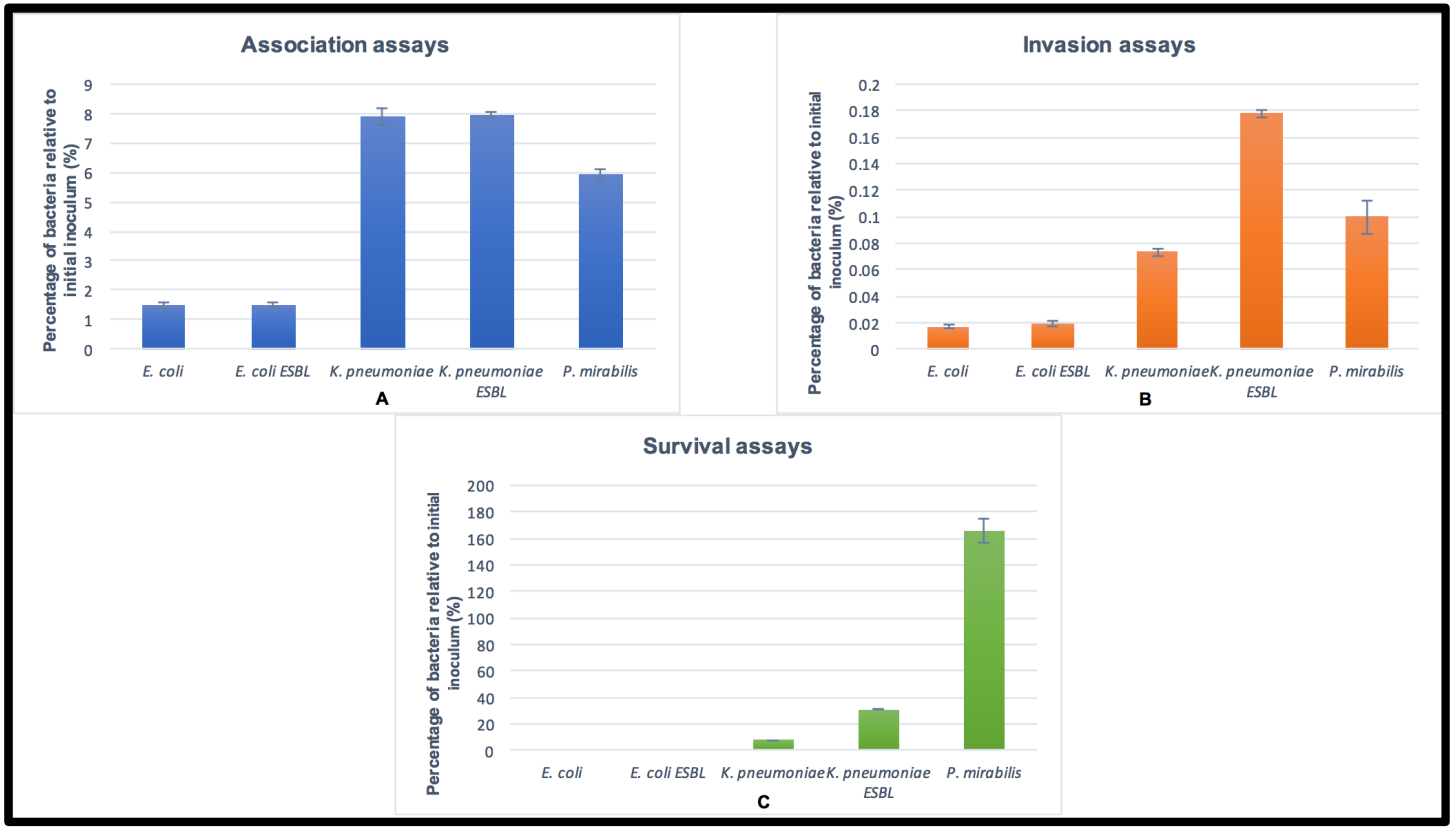

Figure 3.1: Interaction of uropathogens with TERT-NHUC line.

A – Association assay, the plate was incubated at 37°C for 1h; B – Invasion assay, extracellular bacteria were killed with antibiotics at RT for 45min ; C – Survival assay, the plate was incubated at 37°C for 24h . The percentages of bacteria relative to initial bacterial inoculum are shown. Results are obtained from at least three independent experiments. Error bars represent standard error. A and B significant at $p \leq 0.001$ (paired two-tailed *t* test).

3.3.1.2 Intracellular dispersal assays

The dispersal abilities of uropathogenic IBCs formed within TERT-NHUC line were investigated via intracellular dispersal assays. Urothelial cells invaded with bacteria were incubated at 37°C in humidified 95% air and 5% CO_2 for 24h and dispersed cells were recovered from the supernatant.

The findings (Figure 3.2) show that all uropathogenic IBCs were able to disperse bacteria over a 24h period at varying levels. ESBL positive strain of *E. coli* and *P. mirabilis* exhibited the lowest (0.0009 x 10^4 CFU/ml) and highest (9.65 x 10^4 CFU/ml) dispersal respectively. This difference is significant at $p \leq 0.05$. ESBL negative strain of *E. coli* and both strains of *K. pneumoniae* displayed similar dispersal abilities (6.8 to 7.2 x 10^4 CFU/ml). For the latter strain, its ESBL positive counterpart has dispersed bacteria at a slightly higher rate (7.2 x 10^4 CFU/ml compared with 6.8 x 10^4 CFU/ml for the ESBL negative strain). There were no significant differences between the dispersal patterns of ESBL positive and negative *K. pneumoniae* but the difference was significant ($p<0.001$) for *E. coli*.

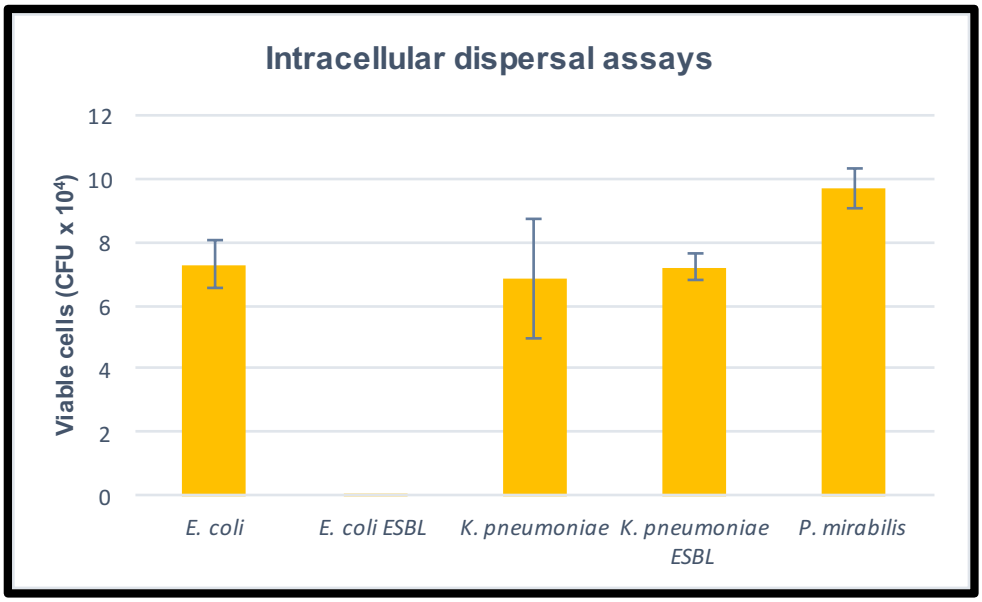

Figure 3.2: Dispersal of uropathogens from TERT-NHUC line.

The survival assay plate was incubated at 37°C for 24h and viable bacteria were obtained from the planktonic suspension. Results are obtained from at least three independent experiments. Error bars represent standard error.

3.3.2 Cytotoxicity assays

3.3.2.1 Flow cytometry assays

To determine the cytotoxic effect of uropathogenic IBCs on TERT-NHUC line and determine the pathway of cell death used, IBC cytotoxicity assays were analysed via flow cytometry.

Data from the flow cytometry images (Figure 3.5) were extrapolated into graphs for analysis. Simple sequential gating was applied to the images to get two parameter density plots.

As displayed in Figure 3.3, urothelial cells employed both apoptotic and necrotic pathways of cell death due to cytotoxicity induced by IBCs over a 24h period. Unviable cells were determined to be in their early apoptotic, late apoptotic/secondary necrotic and primary necrotic stages through differential staining. Early apoptotic cells were positive for Annexin-FITC and negative for Zombie NIR, late apoptotic/secondary necrotic cells were positive for both stains whereas primary necrotic cells were positive for Zombie NIR only. On the other hand, viable cells were negative for both stains. Flow cytometric analysis (Figure 3.3, Figure 3.4 and Figure 3.5) has revealed that all uropathogenic IBCs can induce cytotoxicity on urothelial cells with *E. coli* (ESBL positive and negative) inducing low cytotoxicity compared with *K. pneumoniae* (ESBL positive and negative) and *P. mirabilis*.

As shown via differential staining, cell populations that included early apoptotic cells (Figure 3.3) were highest for *E. coli* ESBL negative (15.36%) followed by

K. pneumoniae ESBL positive (13.81%), *K. pneumoniae* ESBL negative (11.8%), *P. mirabilis* (10.18%) and *E. coli* ESBL negative (8.5%). However, cells in the late apoptotic/secondary necrotic stages (Figure 3.3) were highest for *P. mirabilis* (37.56%) followed by *K. pneumoniae* ESBL positive (27.63), *K. pneumoniae* ESBL negative (24.83%), *E. coli* ESBL negative (22.1%) and *E. coli* ESBL positive (17.53%). *P. mirabilis (*5.84%) has also induced a high percentage of primary necrosis (Figure 3.3) on TERT-NHUC line followed by *K. pneumoniae* ESBL positive (3.79%), *K. pneumoniae* ESBL negative (3.48%), *E. coli* ESBL positive (2.64%) and *E. coli* ESBL negative (1.57%).

Although there are differences (Figure 3.4) in cytotoxicity induced by ESBL positive and negative strains of *E. coli* and *K. pneumoniae*, this difference is significant only for the former with regards to late apoptosis/secondary necrosis ($p \leq 0.05$). In comparison with *E. coli* ESBL negative (22.1%), *P. mirabilis* (37.56%) has exhibited a significant increase in its ability to induce late apoptosis/secondary necrosis ($p \leq 0.01$).

Interestingly, after 24h of incubation, amongst all uropathogens (Figure 3.4), *E. coli* ESBL negative induced the highest percentage of early apoptosis and lowest percentage of primary necrosis whereas *P. mirabilis* exhibited the opposite effect. Another interesting finding (Figure 3.4) is that ESBL positive strains of *E. coli* and *K. pneumoniae* induced a higher percentage of primary necrosis compared with their ESBL negative counterparts. However, this difference is not significant.

Overall (Figure 3.4), *P. mirabilis* (viable cells=46.43%) induced the highest cytotoxicity in comparison with *E. coli* ESBL positive (viable cells=71.3%) which induced relatively low levels of toxicity on TERT-NHUC line.

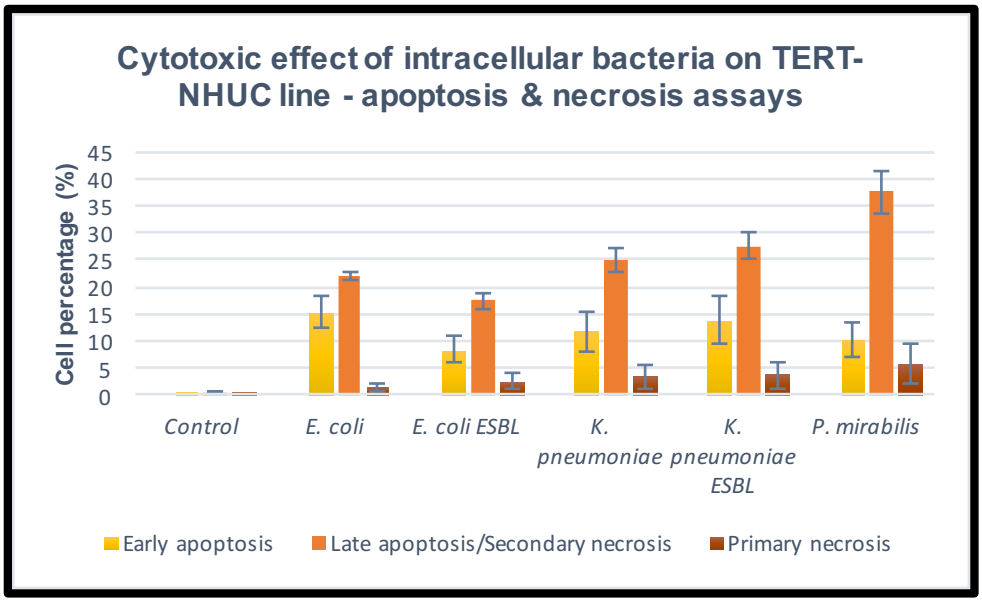

Figure 3.3: Cytotoxic effect of IBCs on TERT-NHUC line.

The percentage of early apoptosis, late apoptosis/secondary necrosis and primary necrosis were extrapolated from flow cytometry images. The total number of cells were restricted to 1000. Control cells without any IBCs are shown. Results are obtained from at least three independent experiments. Error bars represent standard error.

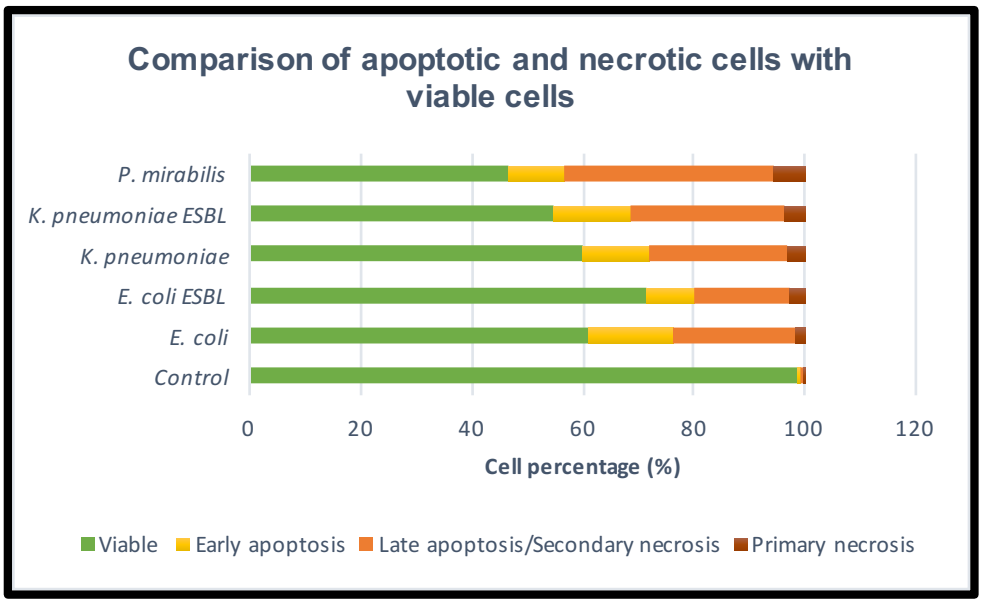

Figure 3.4: Comparison of viable, apoptotic and necrotic cells.

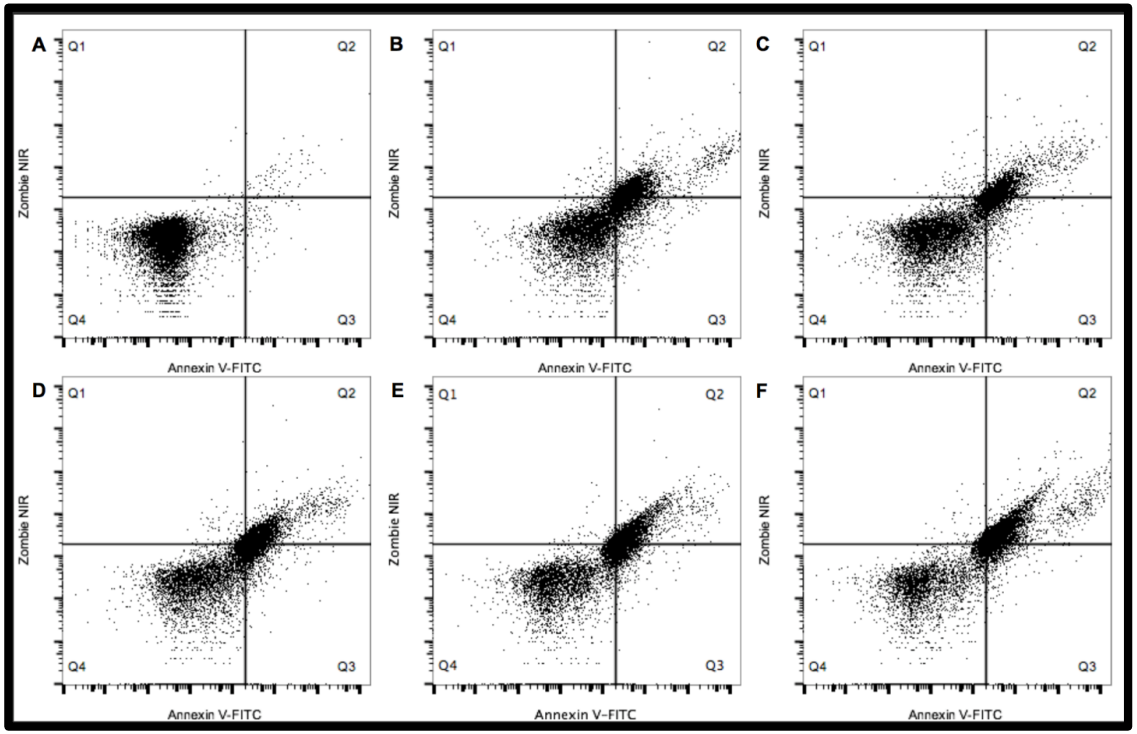

Figure 3.5: Flow cytometry analysis of IBC cytotoxicity.

Images showing viable, early apoptotic, late apoptotic/secondary necrotic and primary necrotic cells. Apoptosis and necrosis were induced on TERT-NHUC line by intracellular bacteria over 24h of incubation. In each panel, the lower left quadrant shows viable cells (Q4 - negative for both Zombie NIR and Annexin V), the lower right quadrant shows early apoptosis (Q3 - Annexin V positive cells), the upper right quadrant shows late apoptosis/secondary necrosis (Q2 - positive for both Zombie NIR and Annexin V) and the upper left quadrant shows primary necrosis (Q1 - Zombie NIR positive cells). A – cells without intracellular bacteria (control), B to F – cells with intracellular bacteria (B – *E. coli*, C – *E. coli* ESBL, D – *K. pneumoniae*, E – *K. pneumoniae ESBL*, F – *P. mirabilis*). Images were obtained from FlowJo (v3.05470) and are representative of results obtained from at least three independent experiments.

3.3.2.2 LDH assays

In order to further differentiate cytotoxicity induced by uropathogenic IBCs, LDH assays were performed to determine the percentage of necrosis (primary and secondary) induced on TERT-NHUC line. Necrotic effect of 24h IBCs on urothelial cells was indirectly measured based on the amount of LDH released from cells.

Figure 3.6 presents the necrotic effect of uropathogenic IBCs on TERT-NHUC line. A comparison of the results reveals that urothelial cells sustained higher necrosis from *P. mirabilis* IBCs (42.6%) compared with other strains ($p \leq 0.01$). Between ESBL negative and positive *E. coli* and *K. pneumoniae* IBCs, the former bacterium has induced lesser cytotoxicity (between 4.3% and 4.9% for *E. coli* and between 8.8% and 9.4% for *K. pneumoniae*). It is also interesting to note that ESBL positive strains induced higher necrosis compared with their ESBL negative counterparts, however, this difference is not significant. Overall, ESBL negative *E. coli* and *P. mirabilis* IBCs have induced lowest and highest necrosis respectively.

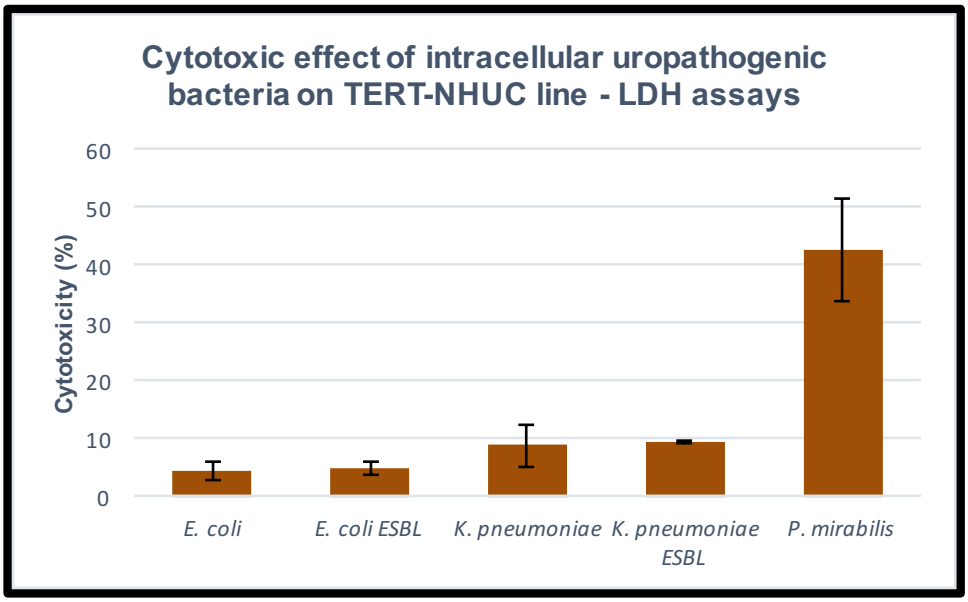

Figure 3.6: LDH assays - IBC induced cell cytotoxicity.

Uropathogens were allowed to survive within TERT-NHUC line for 24h and then the cells were assayed using standard protocol for LDH cytotoxicity detection kit. Percentage cytotoxicity relative to control cells (without IBCs) are shown. Results are obtained from at least three independent experiments. Error bars represent standard error.

3.3.3 Comparison of IBC formation and cytotoxicity

Table 3.1 compares the percentage of uropathogenic survival in TERT-NHUC line with the percentage of cytotoxicity. The uropathogens are listed in the order of decreasing survival ability and cytotoxicity. As evident in Table 3.1, it can be seen that for all uropathogens except *E. coli* (both strains) there is a link between the percentage of uropathogenic survival and the amount of late apoptosis, primary and secondary necrosis induced. For both strains of *E. coli*, the percentage of survival correlates with the amount of late apoptotic and secondary necrotic cells. Furthermore, both strains of *K. pneumoniae* have exhibited a link between their intracellular survival percentage and early apoptotic cells.

Table 3.1: Comparison of uropathogenic IBC formation (survival) and IBC induced cytotoxicity on TERT-NHUC line.

Data from survival assays (Figure 3.1C), flow cytometry images (Figure 3.5) and LDH assays (Figure 3.6) are extrapolated into the table. Uropathogens are placed in order of decreasing ability of intracellular survival and induction of cytotoxicity. The data is presented in percentage (%).

Survival	**Early apoptosis**	**Late apoptosis and/or Secondary necrosis**	**Primary necrosis**	**LDH (primary and secondary necrosis)**
P. mirabilis (165.4)	*E. coli* (15.3)	*P. mirabilis* (37.5)	*P. mirabilis* (5.8)	*P. mirabilis* (42.6)
K. pneumoniae ESBL (31)	*K. pneumoniae* ESBL (13.8)	*K. pneumoniae* ESBL (27.6)	*K. pneumoniae* ESBL (3.7)	*K. pneumoniae* ESBL (9.4)
K. pneumoniae (7.4)	*K. pneumoniae* (11.8)	*K. pneumoniae* (24.8)	*K. pneumoniae* (3.4)	*K. pneumoniae* (8.8)
E. coli (0.08)	*P. mirabilis* (10.1)	*E. coli* (22.1)	*E. coli* ESBL (2.6)	*E. coli* ESBL (4.9)
E. coli ESBL (0.01)	*E. coli* ESBL (8.5)	*E. coli* ESBL (17.5)	*E. coli* (1.5)	*E. coli* (4.3)

3.4 Discussion

This study set out with the aim of exploring the inter-relationship between uropathogens and TERT-NHUC cell line.

As discussed in section 3.1, the IBC cycle consists of association, invasion, survival and dispersal. IBC pathogenic cycle has been investigated in *in vitro* and *in vivo* studies but has not yet been investigated in humans (Rosen *et al.,* 2007). In a study conducted on urine samples collected from women with cystitis, Rosen *et al*., (2007) have found evidence of IBCs and filamentous bacteria in 18% and 41% of urine samples respectively, suggesting evidence of an IBC pathway in humans. They also inoculated into mice an *E. coli* isolate from an IBC positive urine sample, and found evidence of an IBC cycle in mice bladders. Several other studies have also documented the interaction of bacteria (mostly UPEC) with urothelial cells (Justice *et al.,* 2003; Rosen *et al.,* 2007; Garofalo *et al.,* 2007; Berry *et al.,* 2009; Robino *et al.,* 2013).

Results from the current study (Figure 3.1), indicate that all uropathogens are able to interact with TERT-NHUC line with ESBL positive and negative strains of *K. pneumoniae* and *P. mirabilis* displaying higher levels of interaction compared with ESBL positive and negative strains of *E. coli*.

Umbrella cells (superficial layer of urothelium) are covered with 2D crystalline plaques formed by a variety of uroplakins including UPIa, UPIb, UPII, and UPIIIa. UPEC uses type 1 fimbriae to adhere to UPIa triggering uptake by the umbrella cells (Schaffer *et al.,* 2016). From the results of the association and

invasion assays (Figure 3.1A & B), it is clear that both ESBL positive and negative strains of *E. coli* have displayed significant ($p<0.01$) ability to associate with and invade TERT-NHUC line. However, when it comes to intracellular survival, ESBL positive and negative strains of *E. coli* have displayed very low survival ability (~ 0.08% and 0.01% respectively) when compared with other strains (Figure 3.1C).

Rosen *et al*. (2008) conducted a study using a murine cystitis model to compare the IBC forming ability of *K. pneumoniae* with UPEC. They found that both strains were able to form IBCs, albeit *K. pneumoniae* to a lower extent. Although this is in contrast with our results (Figure 3.1), it should be noted that their results are from an *in vivo* study. Struve and Krogfelt (2003) have emphasised the importance of *K. pneumoniae* capsule on the colonisation of the urinary tract. They have also advised caution when interpreting results from *in vitro* studies due to a lack of correlation with *in vivo* studies. Data obtained from the current study provides evidence that both strains of *K. pneumoniae* are able to interact with TERT-NHUC line with the ESBL positive strain displaying higher levels of interaction (Figure 3.1).

Previously, Liu *et al*. (2015) have explored the mechanisms associated with *P. mirabilis* induced UTIs. RNA polymerase extracytoplasmic E (RpoE), is an alternative sigma factor, which is essential for cell envelope integrity in Gram-negative bacteria. They found that RpoE mutant strains had significantly lower fimbria expression, survival in macrophages, invasion ability, and colonisation in mice. Their results suggest a possible link between RpoE expression and *P.*

mirabilis virulence factors. It is interesting to note that urea (major component of urine) can induce expression of RpoE making the link even more probable. The results from the current study (Figure 3.1) show that *P. mirabilis* has the ability to interact with TERT-NHUC line and has exhibited higher intracellular survival in comparison with other strains. Although several studies have suggested that UPEC can form IBCs at a higher rate than *K. pneumoniae* and *P. mirabilis* (which is in contrast with our results), there are no *in vitro* studies that directly compare the IBC forming ability of the three strains. Furthermore, it is difficult to replicate UTI host-pathogen interaction *in vitro*. Therefore, the results from the interaction assays should be treated with caution and *in vivo* bladder studies are required to further elucidate the IBC forming ability of the three strains.

Nevertheless, the most striking observation to emerge from the invasion and survival assays (Figure 3.1B & C) from the current study is that *K. pneumoniae* and *P. mirabilis* possess higher interacting ability with NHUC when compared with *E. coli*. The interaction assays also reveals that ESBL positive K. pneumoniae has displayed a significantly higher ability to invade and survive within NHUC compared with its ESBL negative counterpart. UPEC is considered as the model organism for studying host-pathogen interaction in UTIs (Hannan et al., 2012). To this end, several studies (as discussed earlier) have demonstrated IBC formation by UPEC in urothelial cells. The present study, however, has shown a novel pattern of uropathogen interaction with bladder urothelial cells suggesting that UPEC is not the only microorganism

that can interact well with urothelial cells *in vitro* and that ESBL positive strains may possess a better interacting ability.

The dissemination of bacteria from their intracellular reservoirs is a clinically important stage of the IBC cycle, due to their relevance to rUTIs (Robino *et al.,* 2014). Data from the intracellular dispersal assays (Figure 3.2) reveals the ability of all uropathogenic IBCs to disperse bacteria from their intracellular niches over a period of 24h. IBCs of ESBL positive strain of *E. coli* have exhibited the lowest level (2.5 CFU/ml) of dispersal in contrast with *P. mirabilis* IBCs (9.65 x 10^4 CFU/ml). These results (Figure 3.2) further support the suggestion that the dissemination stage of the IBC cycle can play a very crucial role in initiating new IBC cycles through colonisation of new cells and thereby causing rUTIs.

Apart from forming IBCs, bacterial pathogens can also induce apoptosis and in some cases host cell necrosis (Kim *et al.,* 2010). Flow cytometry assays conducted in the present study reveals evidence of TERT-NHUC apoptosis and necrosis induced by IBCs of all strains (Figure 3.3, Figure 3.4, Figure 3.5 and Figure 3.6). A high percentage of late apoptosis/secondary necrosis and primary necrosis is seen in cells with *P. mirabilis* IBCs whereas a high percentage of early apoptosis is seen in cells with *E. coli* (ESBL negative) IBCs. This suggests that the latter bacterium takes a longer time to induce cytotoxicity. Mathoera *et al*. (2002) have suggested that, adhesion of crystals and/or bacteria to bladder cells and intracellular crystal formation are possible mechanisms for protection against bladder washout, which is necessary for the

maturation of *P. mirabilis* crystals into calculi. Interestingly, they found intracellular crystal formation and mitochondrial damage in *P. mirabilis* invaded ureter cell line (SV-HUC-1). Although the current study employs the use of TERT-NHUC line, a similar cytotoxic mechanism cannot be ruled out due to the high percentage of late apoptosis/secondary necrosis and primary necrosis seen in cells with *P. mirabilis* IBCs (Figure 3.3). Data from LDH assays (Figure 3.6) are in line with flow cytometry results, with *P. mirabilis* displaying high cellular cytotoxicity (primary and secondary necrosis ~ 43%). Looking at the comparison (Table 3.1) of the data obtained from survival, flow cytometry and LDH assays it is evident that the percentage of survival is linked to the amount of late apoptosis, primary and secondary necrosis for all uropathogens except both strains of *E. coli*. For the latter strain, the survival percentage correlates with the amount of late and/or secondary necrotic cells. This suggests that the presence of intracellular bacteria and their intracellular growth rate is one of the major causes of cell cytotoxicity. Overall, the results from the current study suggests that uropathogens not only have the ability to form IBCs in TERT-NHUC line but can also induce significant cytotoxicity in the process.

Monolayer cultures were used in the investigations of the present study. However, as described earlier (3.1), the human urothelium is made of 3 layers and the primary urothelial mediated response to infections and toxicity is the superficial cell exfoliation and regeneration of the urothelium (Nagamatsu *et al.,* 2015). Therefore, it is difficult to extrapolate these *in vitro* results to the urinary tract. Further studies which take these variables into account will need to be undertaken.

Nevertheless, the results from this chapter should provide the basis for further studies, particularly *in vivo*, to clarify the exact mechanism of host-pathogen interaction specifically for *K. pneumoniae* and *P. mirabilis*. In conclusion, these results provide preliminary insight into the complex mechanisms associated with the interactions of uropathogens with human urothelial cells.

In UTIs, uropathogens can form biofilms (Chapter 2) and IBCs (current chapter) as a protective mechanism. However, apart from biofilms, bacteria have also developed different tactics to resist phagocytosis and survive within other micro-organisms, particularly the ubiquitous protist *Acanthamoeba*. The next chapter aims to explore the inter-relationship between uropathogens and *A. castellanii* (T4).

Chapter 4

Interaction of uropathogens with *Acanthamoeba*

4 Interaction of uropathogens with *Acanthamoeba*

4.1 Introduction

Acanthamoeba is a free-living amoeba that exists in two forms: trophozoites or cysts. It is a ubiquitous protist that can be isolated from diverse environments including humans (1.4.3). Trophozoites can actively replicate and cause infection in humans. In unfavourable conditions such as extreme temperature, pH and osmolarity, *Acanthamoeba* undergoes encystation and turns into a double walled cyst, which is the dormant, resistant stage of its life cycle (Khan, 2009; Siddiqui and Khan, 2012).

In their trophozoite stage, *Acanthamoeba* actively feeds on bacteria, algae and fungi (Khan, 2009) through phagocytosis, a contact-dependent pathogenic mechanism (Alsam *et al.,* 2005a). However, the intracellular environment of *Acanthamoeba* can also protect some bacterial endosymbionts from adverse conditions such as the human immune response (Lovieno *et al.,* 2010). This symbiotic relationship with bacteria has enhanced its importance in medical research. Abd *et al.,* (2003) have shown that, the highly infectious, facultative pathogen *Francisella tularensis* has the ability to grow and survive intracellularly in *A. castellanii*. They found viable bacteria in intact trophozoites, excreted vesicles and cysts. Many other studies have been conducted to demonstrate the endosymbiotic relationship between *Acanthamoeba* and bacteria. *Vibrio cholera* (Abd *et al.,* 2004), *E. coli* (Alsam *et al.*, 2006) and neuropathogenic *E. coli* (Yousuf *et al.*, 2014) have all been able to survive and multiply within *Acanthamoeba*.

Acanthamoeba pathogenicity is also dependent on contact-independent mechanisms such as EPs, PLs and other indirect virulence factors (Alsam *et al.,* 2005b; Khan, 2009). EPs and PLs are either released as part of their normal physiological cycle (Siddiqui and Khan, 2012) or they are produced for survival (Iqbal *et al.,* 2014). Although these secretions are not fully characterised, several studies have highlighted their role in host pathology. Other studies have also investigated their antimicrobial potential (Khan *et al.,* 2000; Alfieri *et al.,* 2000; Harrison *et al.*, 2010).

Although *Acanthamoeba* is known to cause infections in humans (GAE, AK and DI) (CDC, 2013), the amoeba has been previously isolated from apparently healthy people (Cerva *et al.,* 1973; Mergeryan, 1991; Newsome *et al.,* 1992; Michel *et al.,* 1997). Santos *et al.,* (2009) conducted a study based on the hypothesis that uropathogenic bacteria can survive the effects of antimicrobial drugs, disinfectants and host immunity by potentially using *Acanthamoeba* for protection. They evaluated 63 urine samples collected from indwelling catheters of critically ill patients. In an interesting finding, 23% of these samples tested positive for the presence of *Acanthamoeba spp.* suggesting a possible role of *Acanthamoeba* in rUTIs. The findings from the above study has definitely paved the way for more research into the role of *Acanthamoeba* in HCAIs, particularly human UTIs.

A. castellanii (T4 genotype) is a pathogenic strain commonly implicated in human infections (Marciano-Cabral and Cabral, 2003). Hence, this strain was selected to explore the endosymbiotic relationship between uropathogens and

Acanthamoeba. In the present work, bacterial binding proteins were isolated from *Acanthamoeba* and interaction assays were carried out to assess the ability of uropathogens to associate with, invade and survive within the amoebae. Furthermore, *Acanthamoeba* conditioned medium (ACM) assays and co-cultivation assays were performed to explore the nature of amoeba-bacteria interactions and the ability of uropathogens to thrive and form biofilms in the presence of amoebae and their extracellular products.

4.2 Materials and methods

A. castellanii (T4 genotype) isolated from a patient diagnosed with AK used in this study was kindly provided by Dr. S Maciver, University of Edinburgh. The amoebae were grown and maintained in a T75 tissue culture flask in 10ml PYG (Appendix C).

4.2.1 Study of bacterial binding proteins involved in phagocytosis by *A. castellanii* (T4)

4.2.1.1 Formaldehyde fixation of bacteria

The bacteria were fixed using formaldehyde as described previously (Kessler, 1981) with few modifications. Briefly an overnight culture of bacteria was centrifuged at 9000 x g for 15min. The cells were washed in PBS at 3000 x g for 10min and the supernatant was discarded. The slurry was diluted to 90% PBS with 0.02% sodium azide. The bacterial cells were fixed with 1.5% formaldehyde for 90min at RT. The fixed cells were centrifuged (3000 x g, 10min), placed in a water bath (80°C, 5min) and then placed on ice. The cells were then washed twice in PBS with 0.02% azide and stored in PBS at -20°C.

4.2.1.2 Isolation of bacterial binding proteins

The fixed bacteria (4.2.1.1) were used for the co-sedimentation of bacterial binding proteins from *A. castellanii* (T4) to determine if any proteins can be isolated. Briefly, 50ml of *A. castellanii* (T4) culture was centrifuged (3000 x g, 10min) and the pellet was reconstituted in PBS with 2% Triton X100. The mixture was centrifuged (3000 x g, 5min) and the supernatant was incubated at RT for 15min with 100µl of fixed bacteria for each strain (5 uropathogens).

The cells were washed twice in PBS and then further washed with 100µl of radio immuno precipitation assay (RIPA) buffer. After washing, supernatants were collected and the proteins were precipitated by the addition of 0.6ml acetone and 0.6ml ethanol in a freezer for 30min. Following precipitation, the supernatants were centrifuged (3000 x g, 15min) and the pellets were dried at 37°C. The pellets were then reconstituted in 10µl of sample buffer and run on 12% SDS-PAGE as described in Appendix D.

4.2.2 Interaction assays

To investigate the interaction of bacteria with *A. castellanii* (T4), bacterial culture was grown and measured as described in section 2.2.2. Trophozoites from confluent flasks (~ 95%) were detached by placing the flasks on ice for 20min. The detached cells in PYG were seeded (~ 5 x 10^5 cells per 0.5ml) in a 24 well plate and incubated at 30°C overnight. The interaction assays were carried out as described in Figure 4.1. Gentamicin (75 µg/ml, 45min, RT) was used to kill the extracellular bacteria for all uropathogens except *E. coli* ESBL (replaced by Meropenem 75 µg/ml) (Alsam *et al.,* 2006). To determine the effect of urea, uric acid and creatinine on the interaction of bacteria with *A. castellanii* (T4)*,* AUM without the aforementioned components was used as the growth medium.

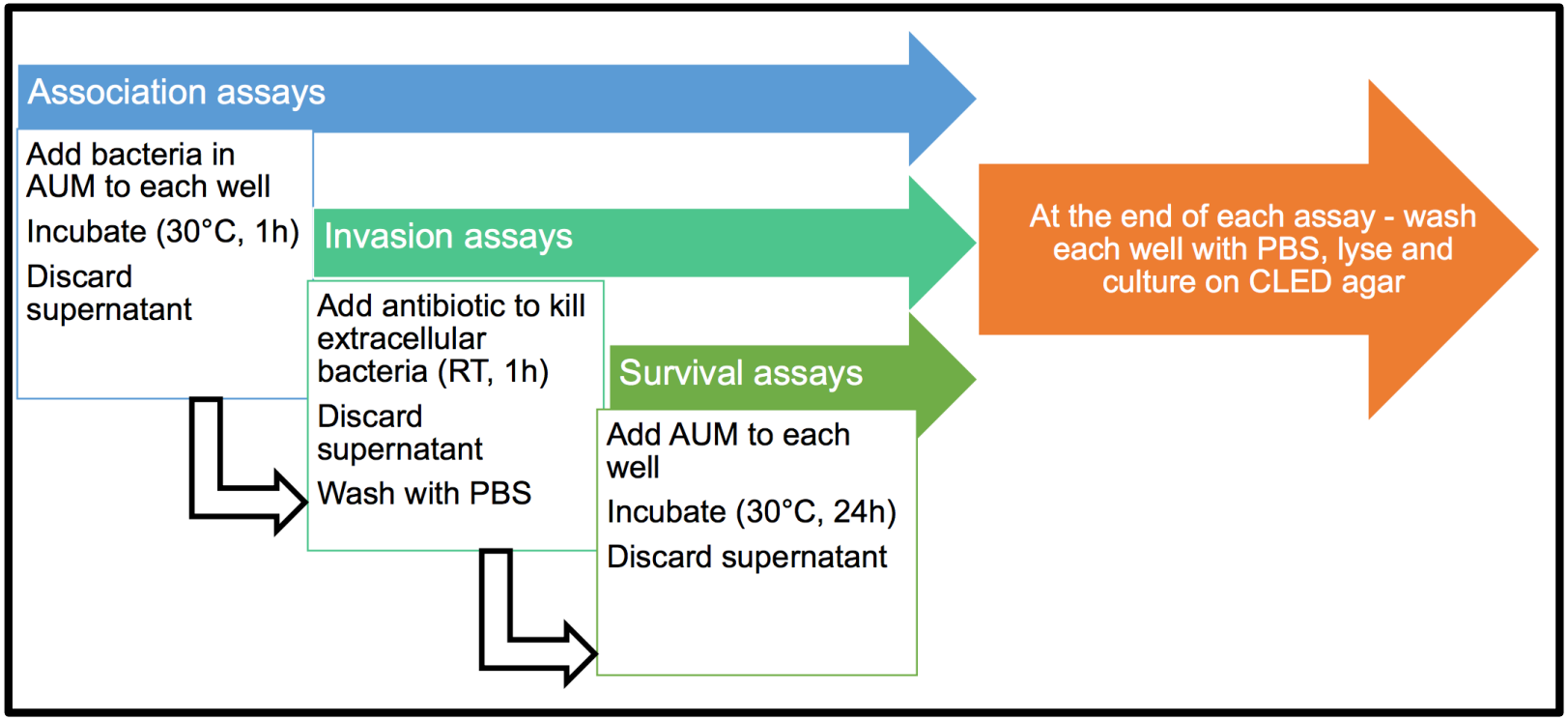

Figure 4.1: Flowchart showing the steps to assay the association, invasion and survival of bacteria within *A. castellanii* (T4).

Cell lytic was used to lyse the *amoebae.* Approximately 10^6 bacteria in their log or exponential growth phase were added to each well (Alsam *et al.,* 2006).

4.2.3 *A. castellanii* (T4) conditioned medium assays

PYG and AUM were conditioned with *A. castellanii* (T4) by incubating confluent cultures (~2 x 10^6 tropohozoites per ml) in T75 flasks for 48h. The media were collected, centrifuged (1000 x g, 5min) and the supernatants were filtered using 0.22µM filters (Iqbal *et al.,* 2014).

Assays were carried out as described in section 2.2.8.1 for planktonic bacterial growth, section 2.2.8.3 for biofilm biomass and section 2.2.9 for catheter biofilm formation. Conditioned medium in AUM and PYG were used as the growth medium whereas the control assays were carried out in AUM and PYG only. PTFE coated latex catheters were used for the catheter biofilm formation.

Based on the conditioned medium, the following abbreviations will be used in the rest of the chapter:

ACM-P: *A. castellanii* (T4) conditioned PYG

ACM-A: *A. castellanii* (T4) conditioned AUM

4.2.4 Co-cultivation assays

Amoebae cultures (~ 1 x 10^6 per ml) were grown in PYG in a T75 flask and 100µl of the culture was placed per well in a 24-well plate containing approximately 10^6 bacteria per well in their log or exponential growth phase. The final volume in each well was made up to 1ml by adding AUM or PYG. The plate was incubated at 37°C for 24h. Wells containing *amoeba* alone and bacteria alone were used as controls (Verhoeven *et al.,* 2010). After the incubation period, planktonic, biofilm and catheter biofilm assays were carried

out as described in section 2.2.8.1, 2.2.8.3 and 2.2.9. PTFE coated latex catheters were used for assaying catheter biofilm formation.

4.2.5 Software and statistics

Microsoft Excel (v15.30) was used for general data analysis and preparation of charts. Paired two-tailed *t* test was used for statistical analysis unless otherwise stated.

4.3 Results

4.3.1 Study of bacterial binding proteins involved in phagocytosis by *A. castellanii* (T4)

Bacterial binding proteins were isolated from *A. castellanii* (T4) using RIPA buffer and were analysed using SDS-PAGE, to determine specific proteins that promote or aid bacterial binding to *A. castellanii* (T4). Prior to isolation the bacterial cells were fixed using formaldehyde. Rectangles indicate specific proteins of interest which can be particularly seen for both strains of *E. coli* and *P. mirabilis* in the region of 30 – 40KDa.

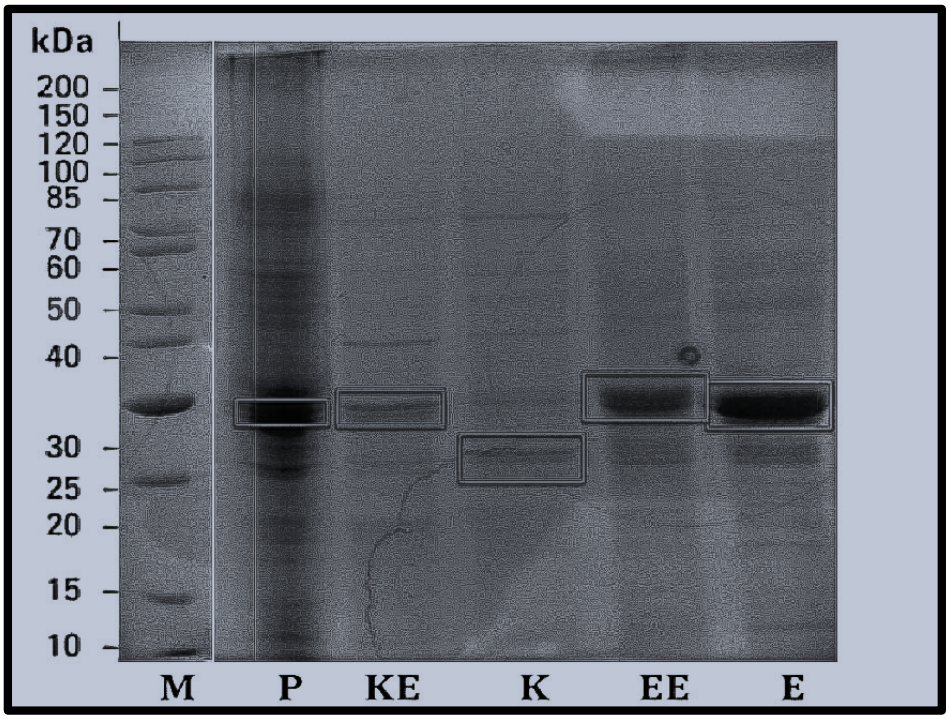

Figure 4.2: SDS-PAGE analysis of bacterial binding proteins isolated from *A. castellanii* (T4).

Proteins were eluted using RIPA buffer and the gel was stained using coomassie blue stain. M - Marker; P – *P. mirabilis*; KE – *K. pneumoniae* (ESBL positive); K – *K. pneumoniae* (ESBL negative); EE – *E. coli* (ESBL positive); E – *E. coli* (ESBL positive). Sample loaded: 7 µl. Rectangles indicate proteins of interest.

4.3.2 Interaction assays

4.3.2.1 Interaction of uropathogens with *A. castellanii* (T4)

Interaction assays were carried out, to investigate the ability of uropathogens to associate, invade and survive within *A. castellanii* (T4) in AUM as growth medium. The bacteria which interacted (association, invasion & survival) with the amoebae were calculated as follows:

Number of viable bacteria recovered (CFU/ml) / number of bacteria in initial inoculum x 100 (Alsam *et al.,* 2006; Yousuf *et al.,* 2013).

All the uropathogens have the ability to associate with *A. castellanii* (T4) (Figure 4.3A, with the highest percentage of association seen in ESBL positive strain of *K. pneumoniae* (5.33%) followed by its ESBL negative counterpart (4.63%), *E. coli* ESBL negative (4.09%), *P. mirabilis* (2.39%) and *E. coli* ESBL positive (1.15%). On the other hand (Figure 4.3B), *P. mirabilis* has exhibited the greatest ability to invade the amoebae, followed by *K. pneumoniae* (1.09% and 0.84% for ESBL positive and negative respectively) and *E. coli* (0.29% and 0.27% for ESBL positive and negative respectively). All uropathogens were able to survive (Figure 4.3C) within the amoebae albeit at varying levels. *P. mirabilis* has displayed the greatest survival ability at 10.89%. ESBL positive and negative strains of *K. pneumoniae* (0.98% and 0.72% respectively) and *E. coli* (0.0007% and 0.01% respectively) have exhibited low levels of survival. The data (association, invasion and survival) are significant at $p \leq 0.005$ (paired two-tailed *t* test).

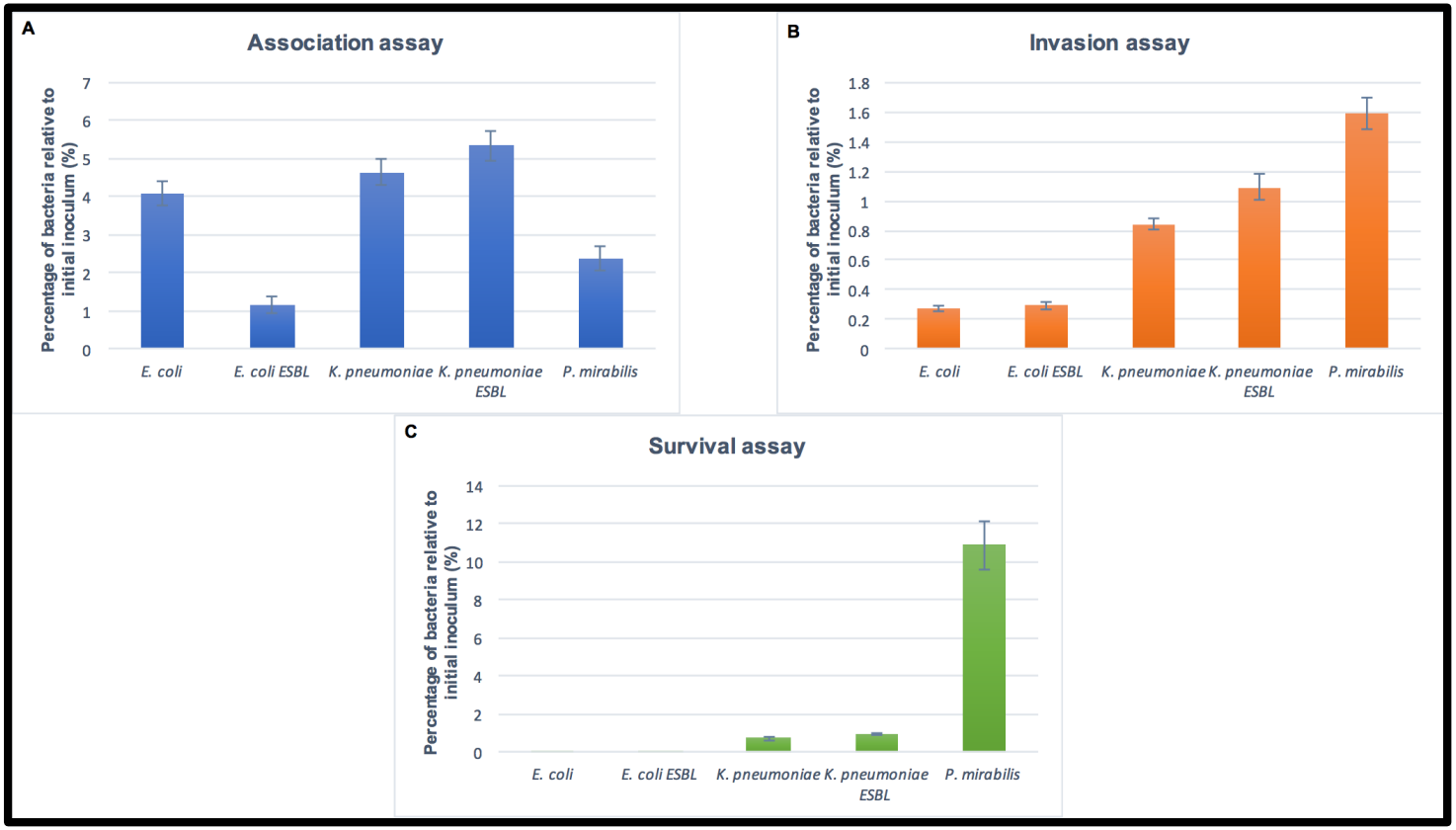

Figure 4.3: Interaction of uropathogens with *A. castellanii* (T4).

A – Association assay, the plate was incubated at 30°C for 1h; B – Invasion assay, extracellular bacteria were killed with antibiotics at RT for 45min; C – Survival assay, the plate was incubated at 30°C for 24h . The percentages of bacteria relative to the initial bacterial inoculum are shown. Results are obtained from at least three independent experiments. Error bars represent standard error. A, B & C significant at $p \leq 0.005$ (paired two-tailed *t* test).

4.3.2.2 Effects of urea, uric acid and creatinine on uropathogen-amoeba interaction

To determine the effects of urea, uric acid and creatinine on association of uropathogens with *A. castellanii* (T4), samples of AUM without the above mentioned components were prepared and bacterial interaction with *A. castellanii* (T4) was evaluated. AUM samples with all components were used as control.

From Figure 4.4, It is evident that samples of AUM without urea have increased bacterial interaction with *A. castellanii* (T4) compared with AUM samples without uric acid and creatinine. Similar trends are seen in all three (association, invasion & survival) assays. In urea negative AUM, the highest and lowest association (Figure 4.4A) is seen for *E. coli* (>200%) and ESBL negative *K. pneumoniae* (~100%). Similar trends are seen in invasion assays (Figure 4.4B). *P. mirabilis* (~140%) has displayed the greatest survival ability in urea negative AUM whereas the orther uropathogens have exhbited similar survival abilities (between 100 and 120%) (Figure 4.4C). Trends in association and invasion (uric acid and creatinine negative AUM) is different amongst the uropathogens tested. However, in the survival assays, all uropathogens have exhibited higher survival ability in uric acid negative AUM when compared with creatinine negative AUM.

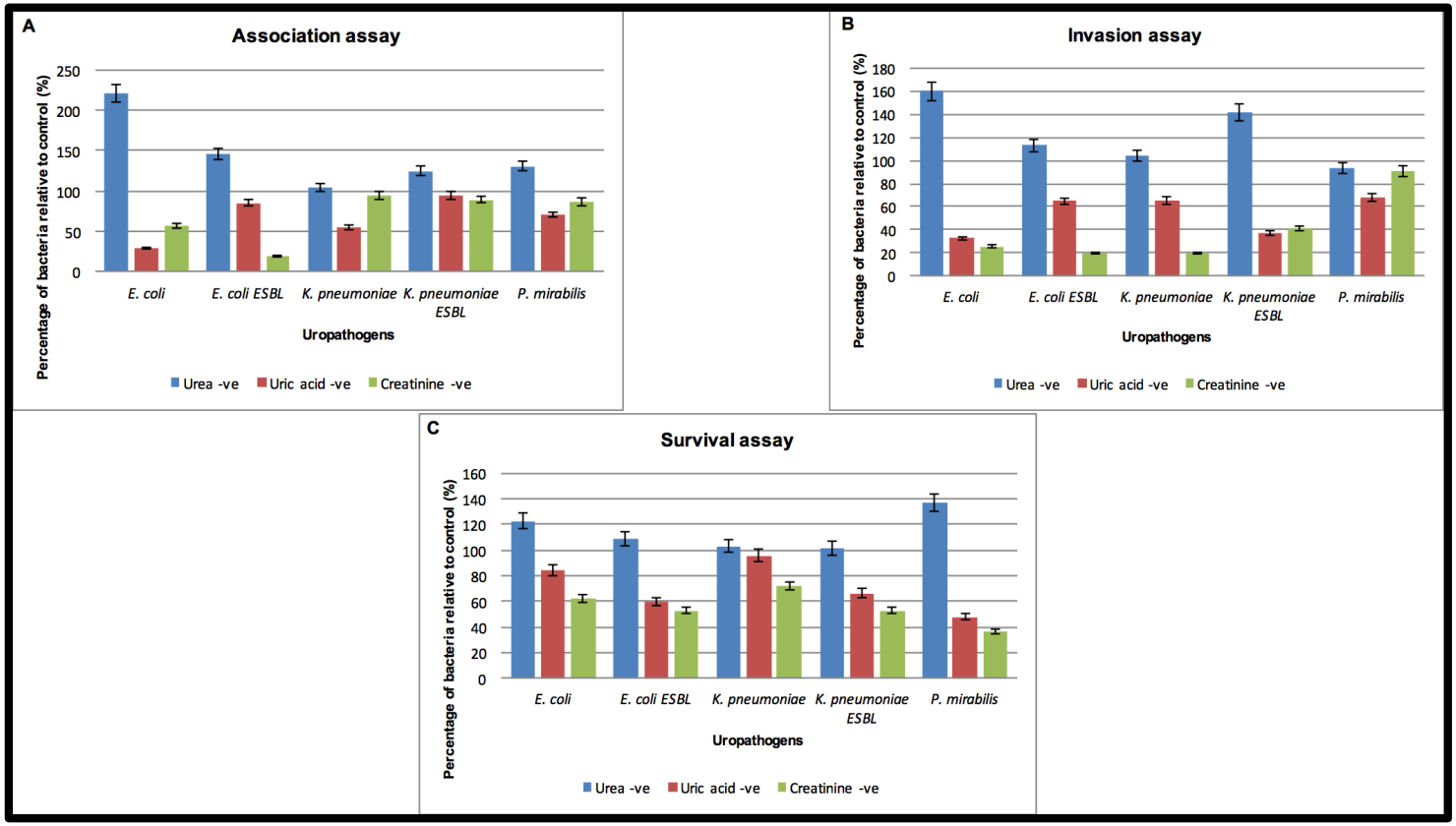

Figure 4.4: Interaction of uropathogens with *A. castellanii* (T4) in AUM samples without urea, uric acid and creatinine.

A – Association assay, the plate was incubated at 30°C for 1h; B – Invasion assay, extracellular bacteria were killed with antibiotics at RT for 45min; C – Survival assay, the plate was incubated at 30°C for 24h . The percentages of bacteria relative to that for the control (AUM) are shown. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at $p \leq 0.001$ (paired two-tailed *t* test).

4.3.3 *A. castellanii* (T4) conditioned medium assays

4.3.3.1 Planktonic bacterial growth

To determine the effect of ACM on planktonic bacterial growth, AUM and PYG were conditioned with *A. castellanii* (T4) for 48h. Uropathogens were then inoculated in the conditioned medium and incubated for 24h.

The results, as shown in Figure 4.5A, indicate that an increase in planktonic growth is seen in ACM-A (in comparison with unconditioned AUM) for all uropathogens with the highest increase seen for *E. coli* ESBL positive strain and the lowest for *P. mirabilis*. These results are significant at the $p \leq 0.01$ level (paired two-tailed *t* test).

Interestingly, there is very minimal difference between growth patterns in PYG and ACM-P for both strains of *E. coli*. However, a large increase in cell numbers is seen when both strains of *K. pneumoniae* and *P. mirabilis* were grown in ACM-P (Figure 4.5B).

Comparing planktonic bacterial growth in ACM-A and ACM-P (Figure 4.5C), all uropathogens have grown at a better rate in the latter medium with very minimal difference seen in both strains of *E. coli* but a significantly ($p \leq 0.05$, two-tailed paired *t* test) higher difference seen in both strains of *K. pneumoniae* and *P. mirabilis*. Overall, planktonic cell growth is notably higher in PYG than AUM for both control and conditioned medium assays.

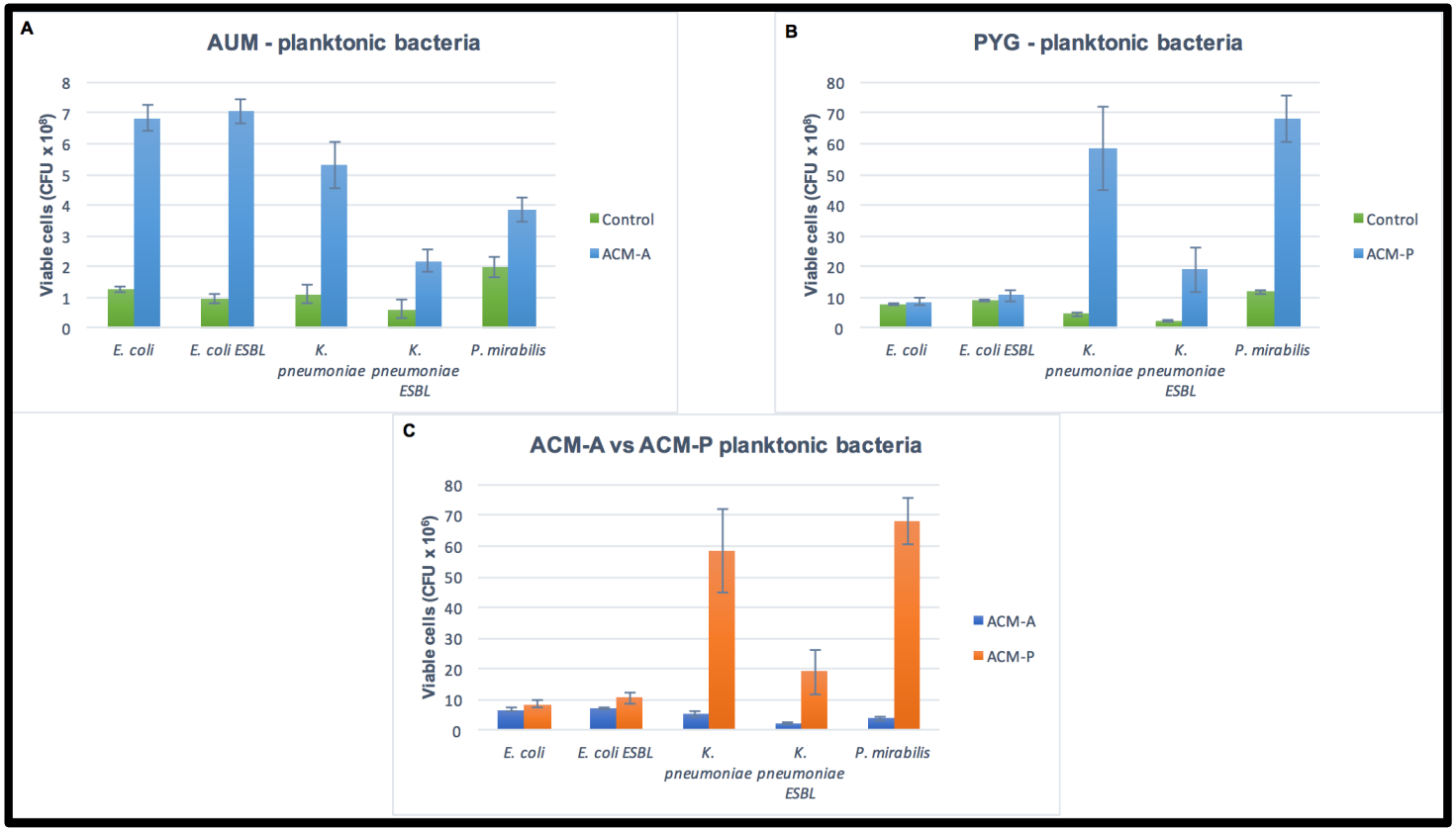

Figure 4.5: Uropathogenic planktonic cell growth in ACM.

Viable cells were enumerated after 24h of incubation at 37°C. Unconditioned AUM and PYG were used as control. A – Viable bacteria from planktonic bacterial suspension of ACM-A; B - Viable bacteria from planktonic bacterial suspension of ACM-P; C – Comparison of planktonic bacterial growth in ACM-A and ACM-P. Results are obtained from at least three independent experiments. Error bars represent standard error. A significant at $p \leq 0.01$ and C significant at $p \leq 0.05$ (paired two-tailed *t* test).

4.3.3.2 Biofilm biomass

Biofilm biomass (which consists of bacteria and EPS - 4.2.3) formation in ACM-A and ACM-P were compared to analyse the effect of *A. castellanii* (T4) extracellular products on biofilm formation. Biofilm formation was assayed using the CV staining technique and unconditioned AUM and PYG were used as control.

From the data in Figure 4.6A & B, it is apparent that there is a significant reduction ($p \leq 0.05$, paired two-tailed *t* test) in biofilm biomass formation for all uropathogens in both ACM-A and ACM-P. The greatest reduction is seen in *K. pneumoniae* ESBL negative strain (63%) when artificial urine was conditioned with *A. castellanii* (T4). On the other hand, with ACM-P, *P. mirabilis* (37.49%) displayed the greatest reduction in biofilm biomass formation.

Overall, biofilm biomass formation (control assays) is notably higher in PYG than AUM for all uropathogens except *K. pneumoniae* ESBL positive strain which seems to prefer AUM to a slightly greater proportion (Figure 4.6A & B). However, in the conditioned medium assays, both strains of *K. pneumoniae* seem to possess significantly ($p \leq 0.01$, paired two-tailed *t* test) better biofilm biomass in AUM (1.4 and 0.76 for ESBL negative and positive strains respectively) than PYG (1.2 and 0.3 for ESBL negative and positive strains respectively) (Figure 4.6C) which is in contrast with the other uropathogens.

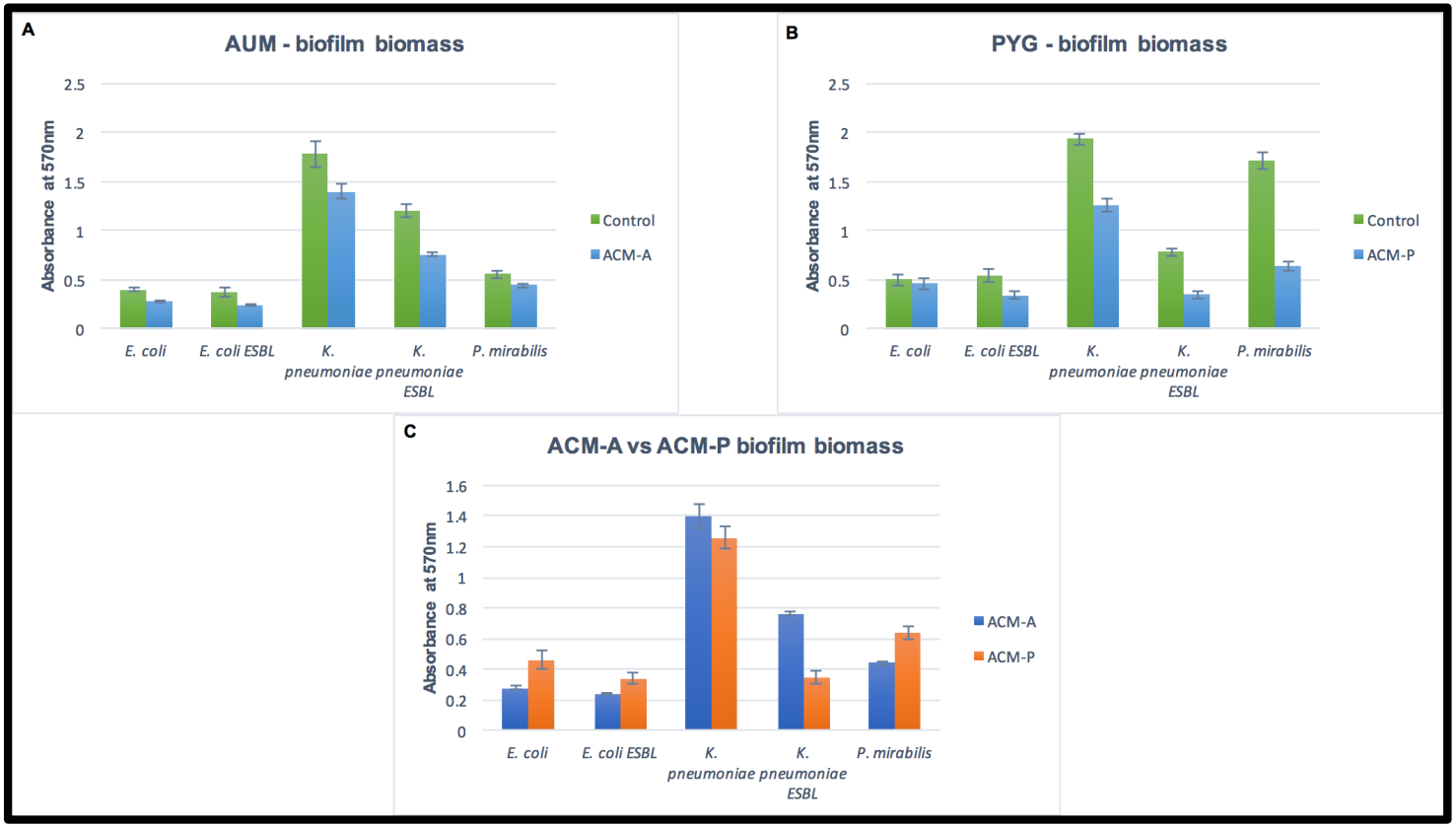

Figure 4.6: Uropathogenic biofilm biomass formed in ACM.

CV absorbance values of the biofilm biomass (bacteria and EPS after 24h of incubation at 37°C) measured spectroscopically at 570nm are shown. Unconditioned AUM and PYG were used as control. A – Biofilm formation in ACM-A; B - Biofilm formation in ACM-P; C – Comparison of biofilm formation in ACM-A and ACM-P. Results are obtained from at least three independent experiments. Error bars represent standard error. A and B significant at $p \leq 0.05$ and C significant at $p \leq 0.01$ (paired two-tailed *t* test).

4.3.3.3 Biofilm architecture

To study the differences in the architecture of biofilms formed in AUM and PYG (control groups) and ACM-A and ACM-P (experimental groups), CV stained biofilms were imaged using a light microscope. The results (Figure 4.7 and Figure 4.8) have revealed the diversity in biofilm architecture formed in different culture mediums.

Although there was a decrease in biofilm biomass (Figure 4.6) when ACM-A was used, all uropathogens seem to form clumpy aggregates when grown in ACM-A (Figure 4.7B, D, F, H & J). Both strains of *K. pneumoniae* have formed characteristic mushroom like structures with hollow voids in between bacterial aggregates (Figure 4.7F & H). There is no discernible difference in the structure of AUM and ACM-A biofilms of the other uropathogens (Figure 4.7).

In PYG, *K. pneumoniae* ESBL negative strain and *P. mirabilis* have formed specific surface structures forming close links with neighboring bacteria in a particularly sophisticated pattern (Figure 4.8E & I). The latter, however, forms smaller hollow voids (Figure 4.8I). Interestingly, both strains have lost their ability to form these specific surface structures in ACM-P (Figure 4.8F & H) albeit to a lower extent for *K. pneumoniae* ESBL negative strain. Although there is a noticeable difference in PYG and ACM-P biofilms of *K. pneumoniae* ESBL negative and *P. mirabilis*, in terms of their struggle to form chains in a specific pattern, the bacterial cells are still able to form micro colonies that are linked to each other. Similar to ACM-A, clumpy bacterial aggregates are seen for all uropathogens in ACM-P (Figure 4.8 B, D, F, H & J). The ESBL negative strain

of *E. coli* has displayed an evenly distributed biofilm surface coverage in PYG with only a slight reduction in ACM-P when compared with its ESBL positive counterpart, which has micro colonies that are grouped together and more distant to each other (Figure 4.8 A, B, C & D). Overall, these results indicate that uropathogens form structurally different biofilms in ACM.

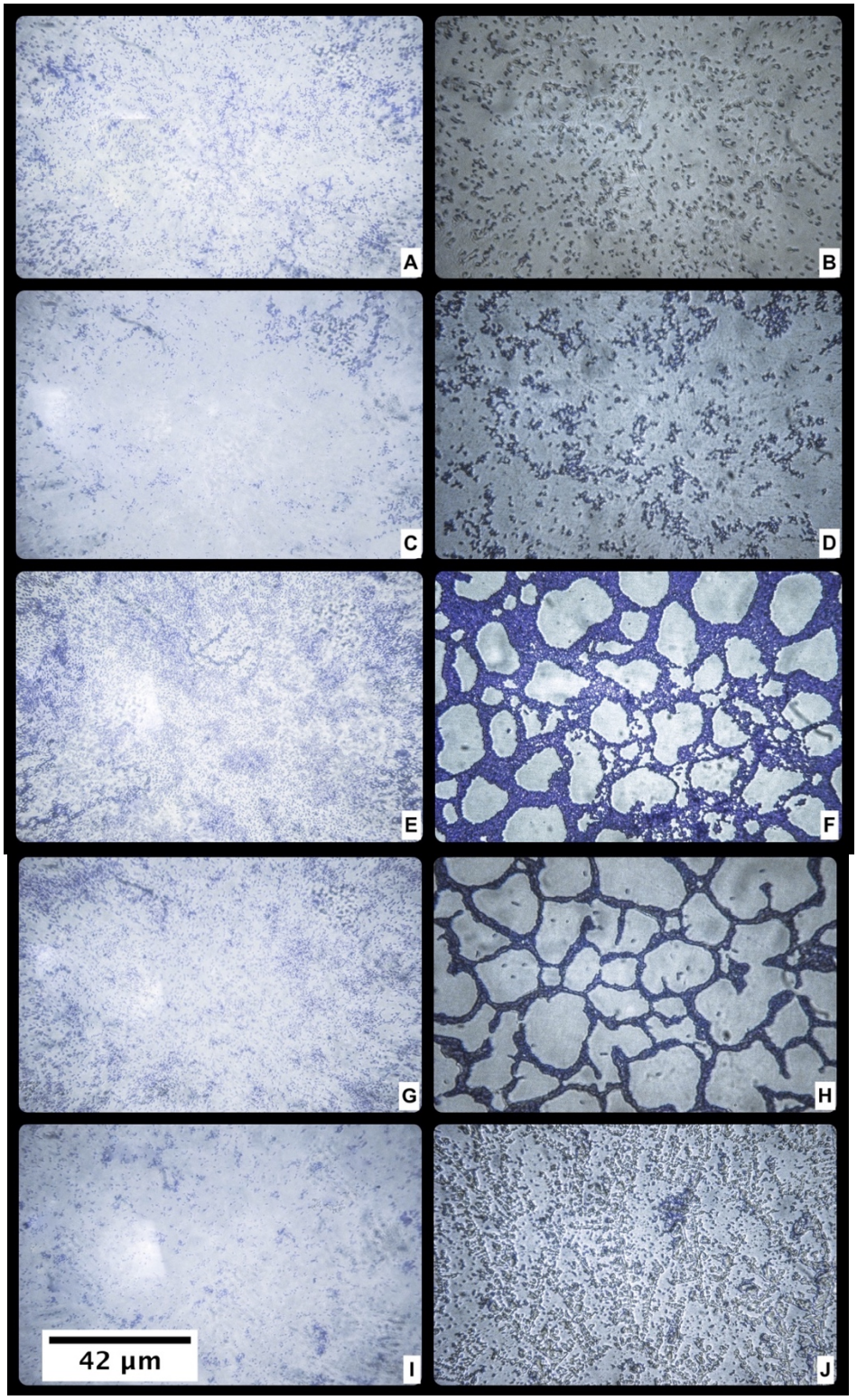

Figure 4.7: CV stain showing uropathogenic biofilm biomass (bacteria with EPS) formation.

Biofilm biomass in AUM (A, C, E, G & I) and ACM-A (B, D, F, H & J) stained with 1% CV. A&B – *E. coli* (ESBL negative); C&D – *E. coli* (ESBL positive); E&F – *K. pneumoniae* (ESBL negative) G&H – *K. pneumoniae* (ESBL positive); I&J – *P. mirabilis*. The figure is representative of images obtained from a bright field microscope (scale bar: 42µm).

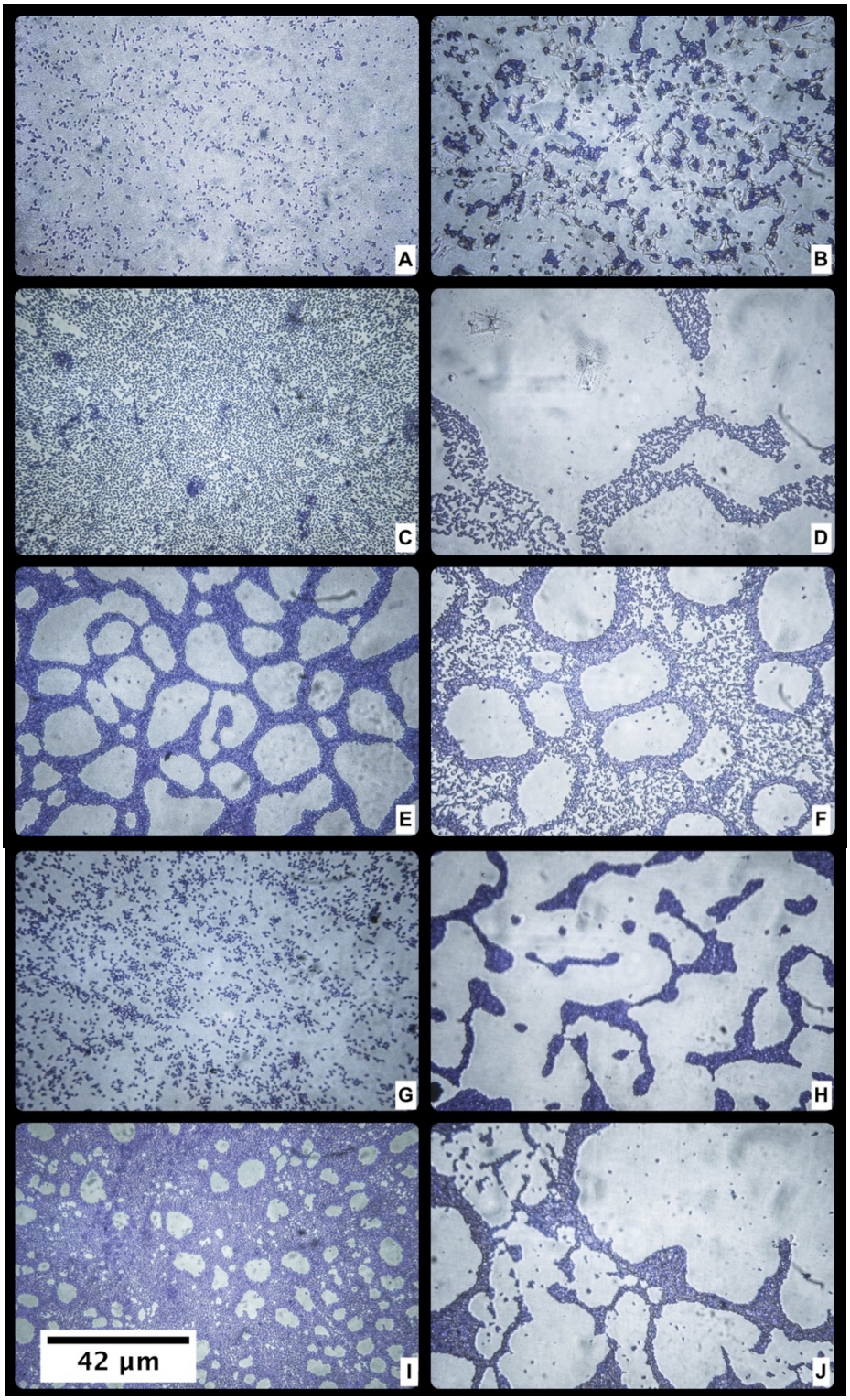

Figure 4.8: CV stain showing uropathogenic biofilm biomass (bacteria with EPS) formation.

Biofilm biomass in PYG (A, C, E, G & I) and ACM-P (B, D, F, H & J) stained with 1% CV. A&B – *E. coli* (ESBL negative); C&D – *E. coli* (ESBL positive); E&F – *K. pneumoniae* (ESBL negative) G&H – *K. pneumoniae* (ESBL positive); I&J – *P. mirabilis*. The figure is representative of images obtained from a bright field microscope (scale bar: 42µm).

4.3.3.4 Catheter biofilm formation

To investigate the effect of ACM on catheter biofilm formation, PTFE coated latex catheters (Ch 12) were used to assay the biofilm formation. Catheters were incubated in AUM, PYG and conditioned medium for 24h.

The data in Figure 4.9A indicates that in comparison with the control (AUM), higher number of viable cells were recovered from *P. mirabilis* catheter biofilms when ACM-A was used as the growth medium (0.9 to 1.5 x 10^6 CFU/ml respectively). On the other hand, in comparison with PYG control, a higher number of viable cells were recovered from *E. coli* ESBL positive catheter biofilms when ACM-P was used as the growth medium (0.9 to 1.1 x 10^6 CFU/ml respectively) (Figure 4.9B).

When comparing biofilm formation in ACM-A and ACM-P, it is evident that all uropathogens formed better catheter biofilms in ACM-A except for *P. mirabilis* which preferred ACM-P (01.1 to 5.21 x 10^6 CFU/ml respectively) (Figure 4.9C).

Although the difference between biofilm formation in control and conditioned medium assays is not significant, the data reveals that uropathogens can form biofilms in the presence of *A. castellanii* (T4) extracellular products (Figure 4.9).

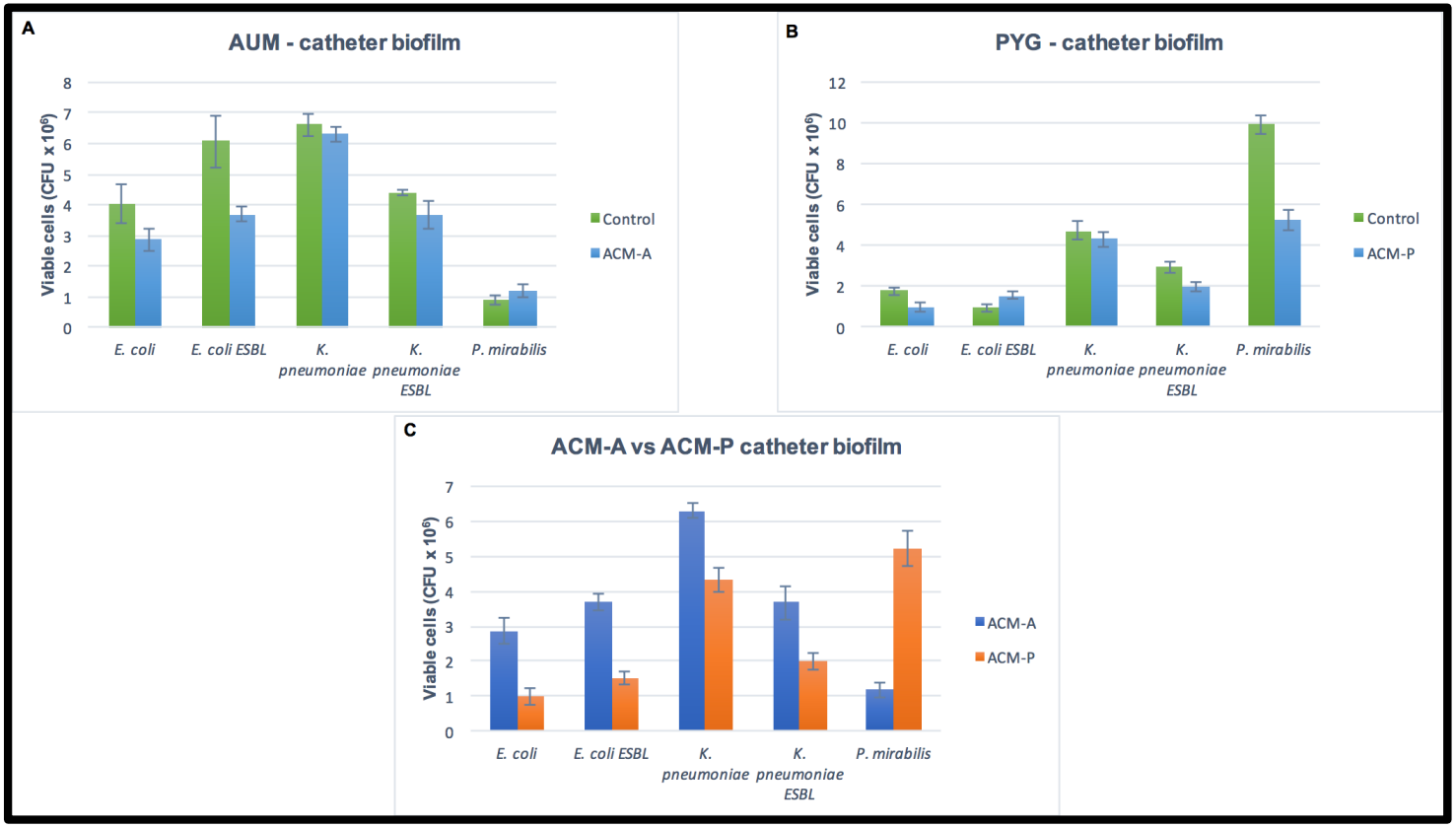

Figure 4.9: Uropathogenic catheter biofilm formation in ACM.

Viable cells were enumerated after 24h of incubation at 37°C. Unconditioned AUM and PYG were used as control. A – Viable bacteria from catheter biofilms grown in ACM-A; B - Viable bacteria from catheter biofilms grown in ACM-P; C – Comparison of catheter biofilm formation in ACM-A and ACM-P. Results are obtained from at least three independent experiments. Error bars represent standard error.

4.3.4 Co-cultivation assays

4.3.4.1 Planktonic bacterial growth

A co-cultivation assay was performed to investigate the planktonic growth of uropathogens in the presence of *A. castellanii* (T4). The organisms were co-cultured for 24h and the resulting planktonic bacterial growth was assayed. Bacteria cultured alone were used as control.

When AUM was used as the growth medium, all uropathogens except for *K. pneumoniae* ESBL negative strain have displayed nearly similar planktonic growth patterns when cultured individually or co-cultivated with the amoebae. The latter has exhibited approximately 40% decrease in planktonic growth in the presence of amoebae. Overall, uropathogens have grown at a better rate when cultured individually (Figure 4.10A). However, in PYG all uropathogens have displayed increased planktonic growth when co-cultivated with *A. castellanii* (T4) (Figure 4.10B). This finding is significant at $p \leq 0.05$ (paired two-tailed *t* test).

Comparing AUM and PYG co-cultivated (in the presence if *A. castellanii* (T4)) growth patterns, it is evident (Figure 4.10C) that both strains of *E. coli* have displayed a decreased growth rate (59.9% and 61.7% for ESBL negative and positive strain respectively) in PYG whereas both strains of *K. pneumoniae* (173.1% and 315.4% for ESBL negative and positive strain respectively), and *P. mirabilis* (930%) have exhibited better growth rates in PYG.

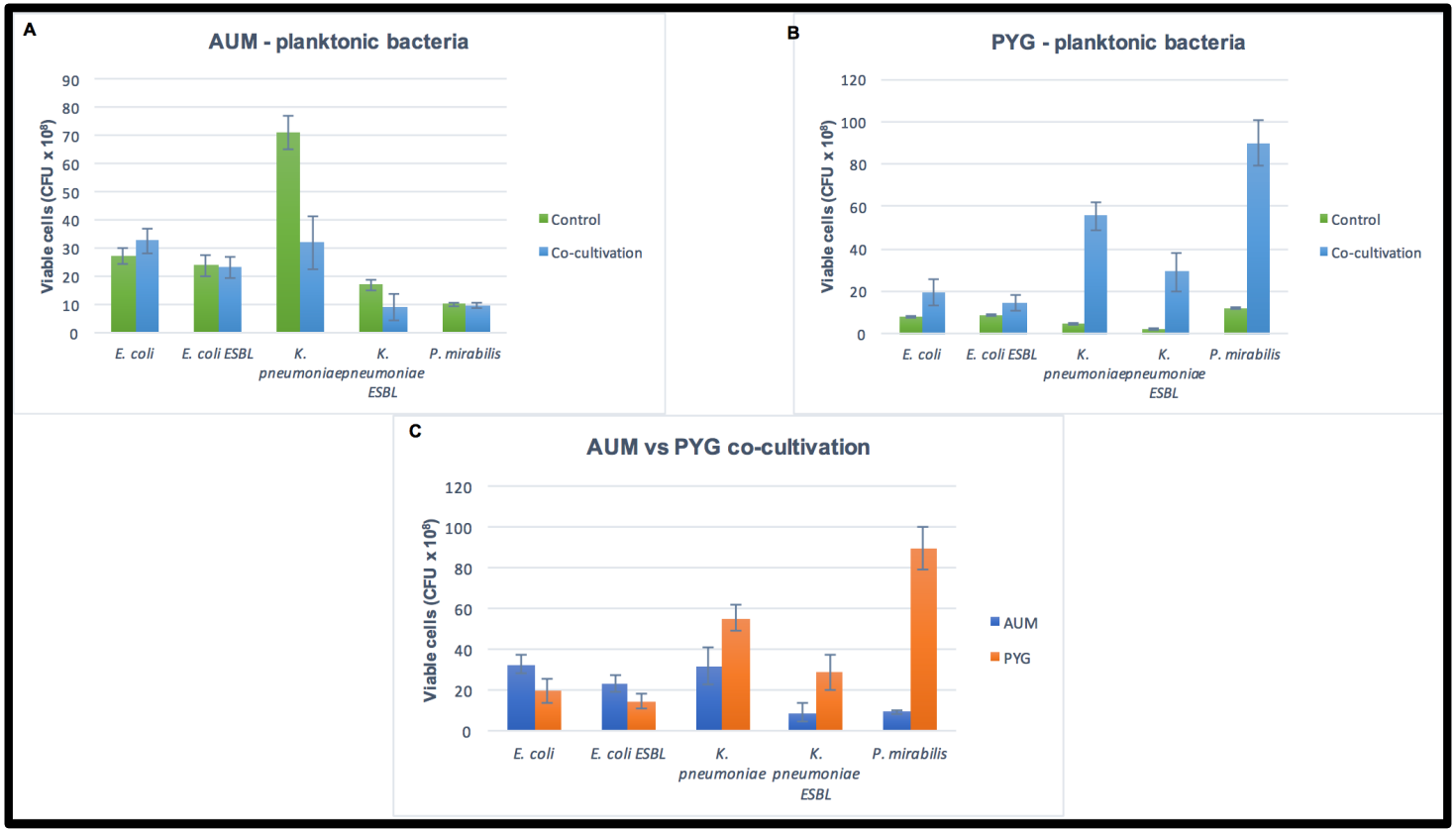

Figure 4.10: Uropathogenic planktonic cell growth in AUM and PYG co-cultivated with *A. castellanii* (T4).

Viable bacteria were enumerated after 24h of incubation at 37°C. Bacteria without co-cultivation were used as control. A – Viable bacteria from planktonic bacterial suspension in AUM; B - Viable bacteria from planktonic bacterial suspension in PYG; C – Comparison of planktonic bacterial growth in AUM and PYG co-cultivated with *A. castellanii* (T4). Results are obtained from at least three independent experiments. Error bars represent standard error. B significant at $p \leq 0.05$ (paired two-tailed *t* test).

4.3.4.2 Biofilm biomass

In order to indirectly assess the effect of co-cultivation (bacteria and *A. castellanii* (T4)), CV absorbance values of the surface adhered amoebae and bacteria cultured individually were combined and then compared with co-cultured absorbance values (Figure 4.11A & C). It should be noted that the amoebae have a strong ability to absorb CV (absorbance of 1.302 at OD_{570}) (Figure 4.12).

Looking at Figure 4.11 it is apparent that there is a significant ($p \leq 0.001$, paired two-tailed *t* test) difference between the two sets of data (A & C). Combined CV absorbance values are significantly higher when the amoebae and bacteria were cultured separately. The greatest difference is seen in ESBL positive and negative strains of *E. coli* for both AUM and PYG whereas a greater difference was seen in *K. pneumoniae* ESBL positive strain and *P. mirabilis* for PYG.

Assuming that uropathogens and *A. castellanii* (T4) display similar surface adherence ability when co-cultivated, then;
Absorbance values of uropathogens (cultivated individually) + absorbance values of *A. castellanii* (T4) (cultivated individually) = Absorbance values of uropathogens and *A. castellanii* (T4) (co-cultivated).

However, Figure 4.11A and C displays a marked decrease in the absorbance values when the microorganisms were co-cultivated compared with their combined biomass values (when they were cultured individually). This could mean that there was either a decrease in bacterial adherence or amoebic

adherence when the two organisms were co-cultured. To distinguish between these two possibilities, absorbance values of bacteria alone and bacteria co-cultured with *A. castellanii* (T4) were analysed (Figure 4.11B & D). From this analysis, it can be concluded that there is no significant difference in the biofilm biomass between bacteria cultured individually and bacteria cultured with amoebae. Therefore, indicating that the decrease is indeed in amoebic adherence.

These results suggest that *A. castellanii* (T4) was outcompeted by the uropathogens in the competition for space and that all uropathogens were able to form biofilms in the presence of live amoebae.

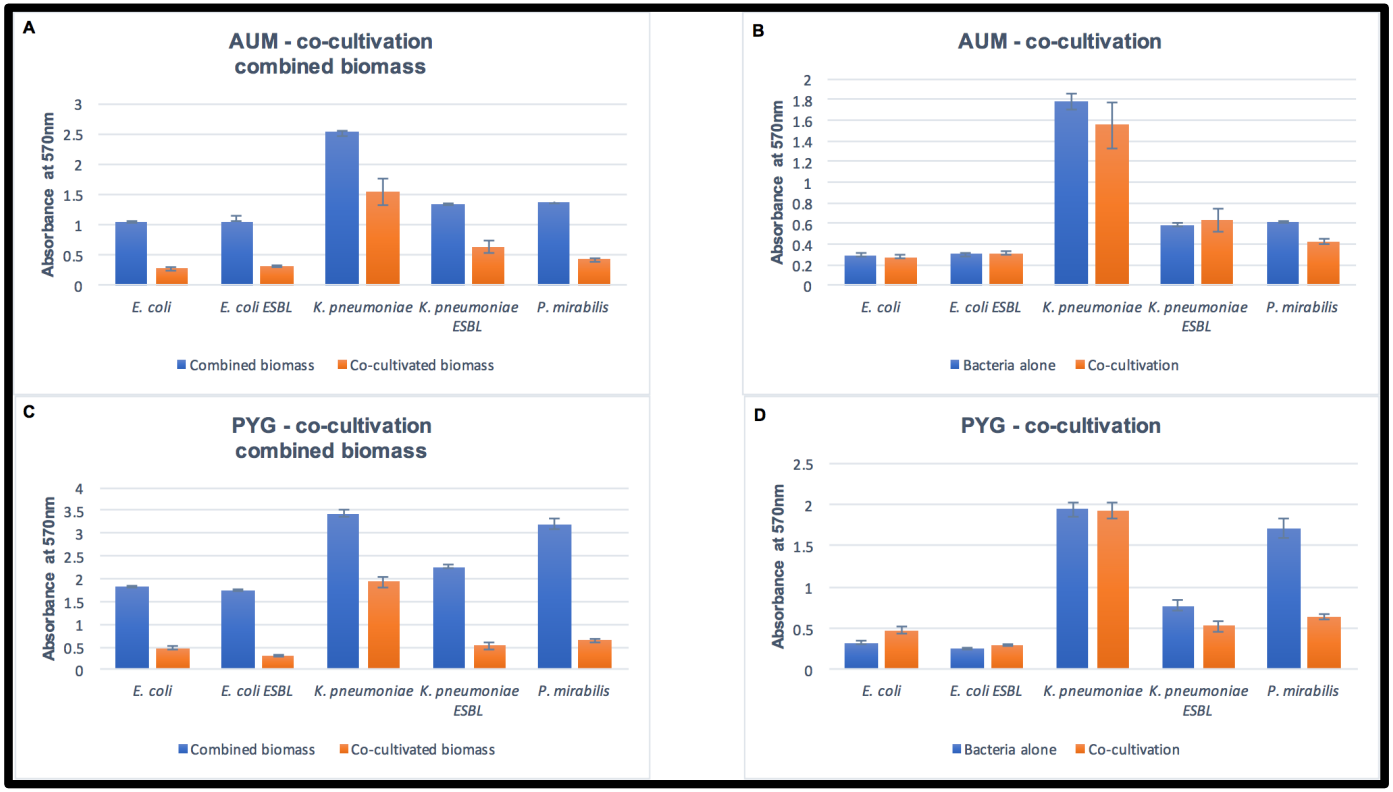

Figure 4.11: Uropathogenic biofilm biomass in in AUM and PYG co-cultivated with *A. castellanii* (T4).

Bacteria alone and amoebae alone were used as control. CV absorbance values of the biofilm biomass (bacteria with EPS) measured spectroscopically at 570nm are shown. A&B – Biofilm formation in AUM; C&D - Biofilm formation in PYG. Results are obtained from at least three independent experiments. Error bars represent standard error. A and C significant at p≤0.001 (paired two-tailed *t* test). Combined biomass is the sum of absorbance values of amoebae cultured on its own and bacteria cultured on its own. Co-cultivated biomass is the absorbance values of bacteria and amoebae cultivated together.

4.3.4.3 Biofilm architecture

Light microscopy imaging has further strengthened our previous (Figure 4.11) conclusion that *A. castellanii* (T4) was outcompeted by the uropathogens in the competition for space. Figure 4.12 shows *A. castellanii* (T4) cultured on its own, while Figure 4.13 and Figure 4.14 show bacteria co-cultured with live amoebae in AUM and PYG respectively. Clearly, there is a decrease in the number of adhered amoebae when co-cultured with bacteria indicating competition for space.

Both (AUM & PYG) sets of images (Figure 4.13 and Figure 4.14) reveal the presence of bacteria around surface adhered amoebae indicating possible bacterial preference for amoebic grazing.

Interestingly, the images (Figure 4.14 E, G & I) also reveal that, amoebae when co-cultured with *K. pneumoniae* (ESBL positive and negative) and *P. mirabilis* in PYG do not form part of the biofilm but instead tend to protrude into the hollow voids of the micro colonies while still maintaining a faint connection with the rest of the biofilm matrix. Furthermore, comparing these images with images obtained when bacteria were cultured alone in PYG (Figure 4.14), it is evident that the structural integrity of *K. pneumoniae* (ESBL positive strain) and *P. mirabilis* biofilm is affected in the presence of *A. castellanii* (T4).

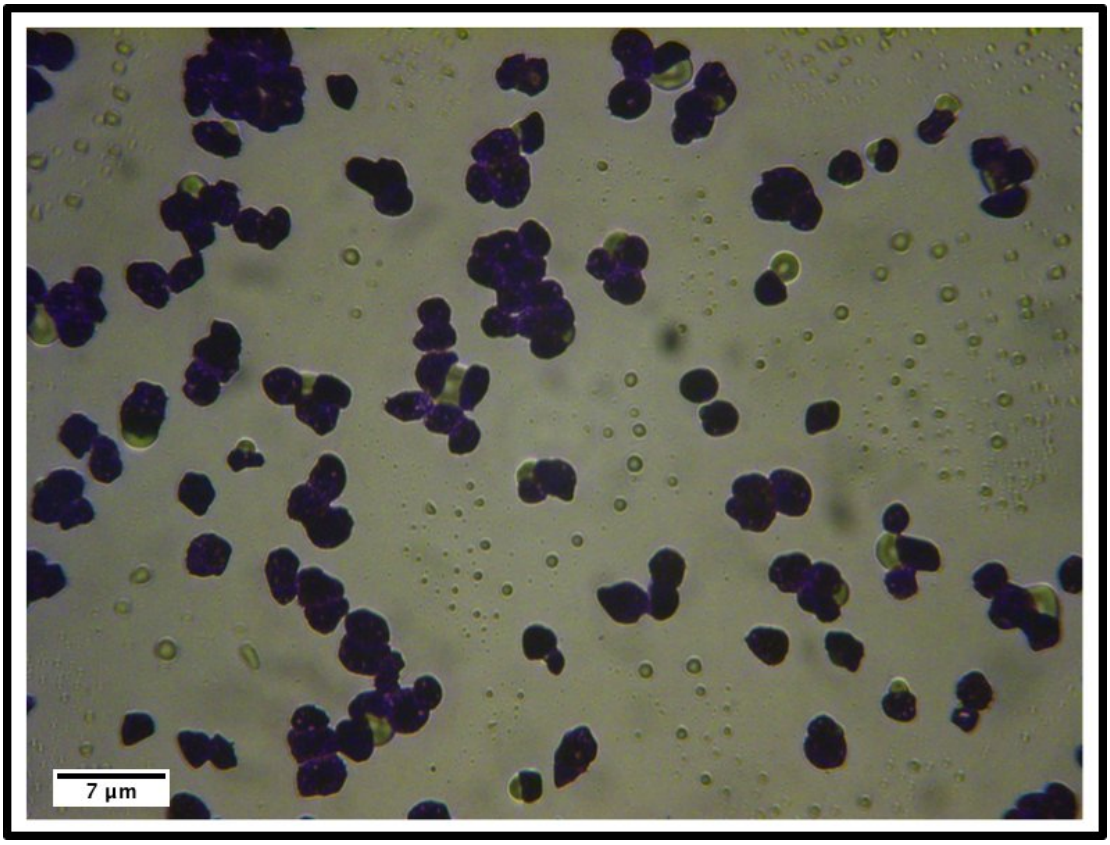

Figure 4.12: CV stain showing *A. castellanii* (T4) when grown on its own.

Amoebae were stained with 1% CV after 24h of incubation at 37°C. The figure is representative of images obtained from a bright field microscope (scale bar: 7µm).

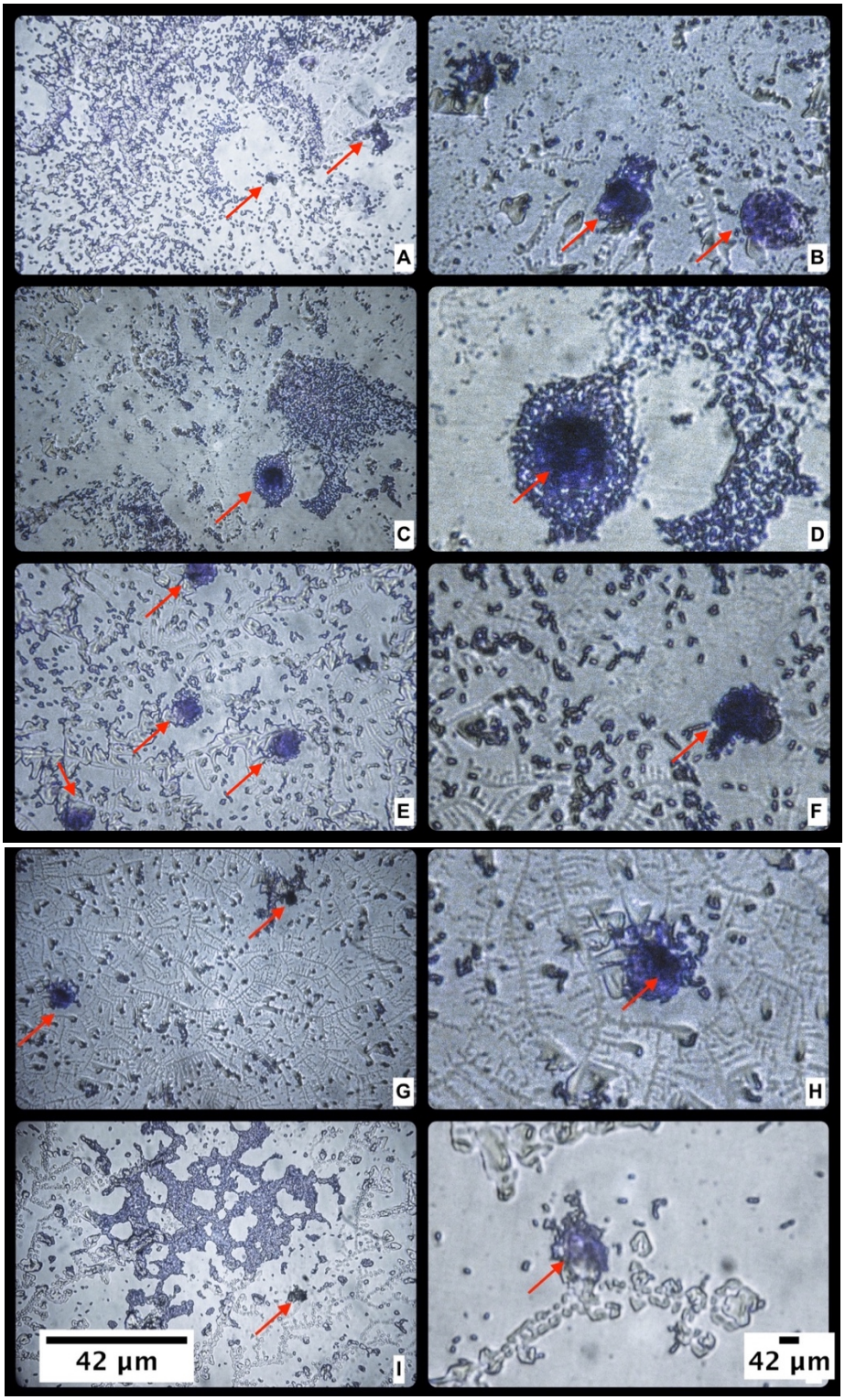

Figure 4.13: CV stain showing uropathogenic biofilm biomass (bacteria with EPS) formation in AUM.

Biofilm biomass in AUM co-cultivated with *A. castellanii* (T4). A&B – *E. coli* (ESBL negative); C&D – *E. coli* (ESBL positive); E&F – *K. pneumoniae* (ESBL negative) G&H – *K. pneumoniae* (ESBL positive); I&J – *P. mirabilis*. Red arrow indicates amoeba. The figure is representative of images obtained from a bright field microscope (scale bar: 42µm).

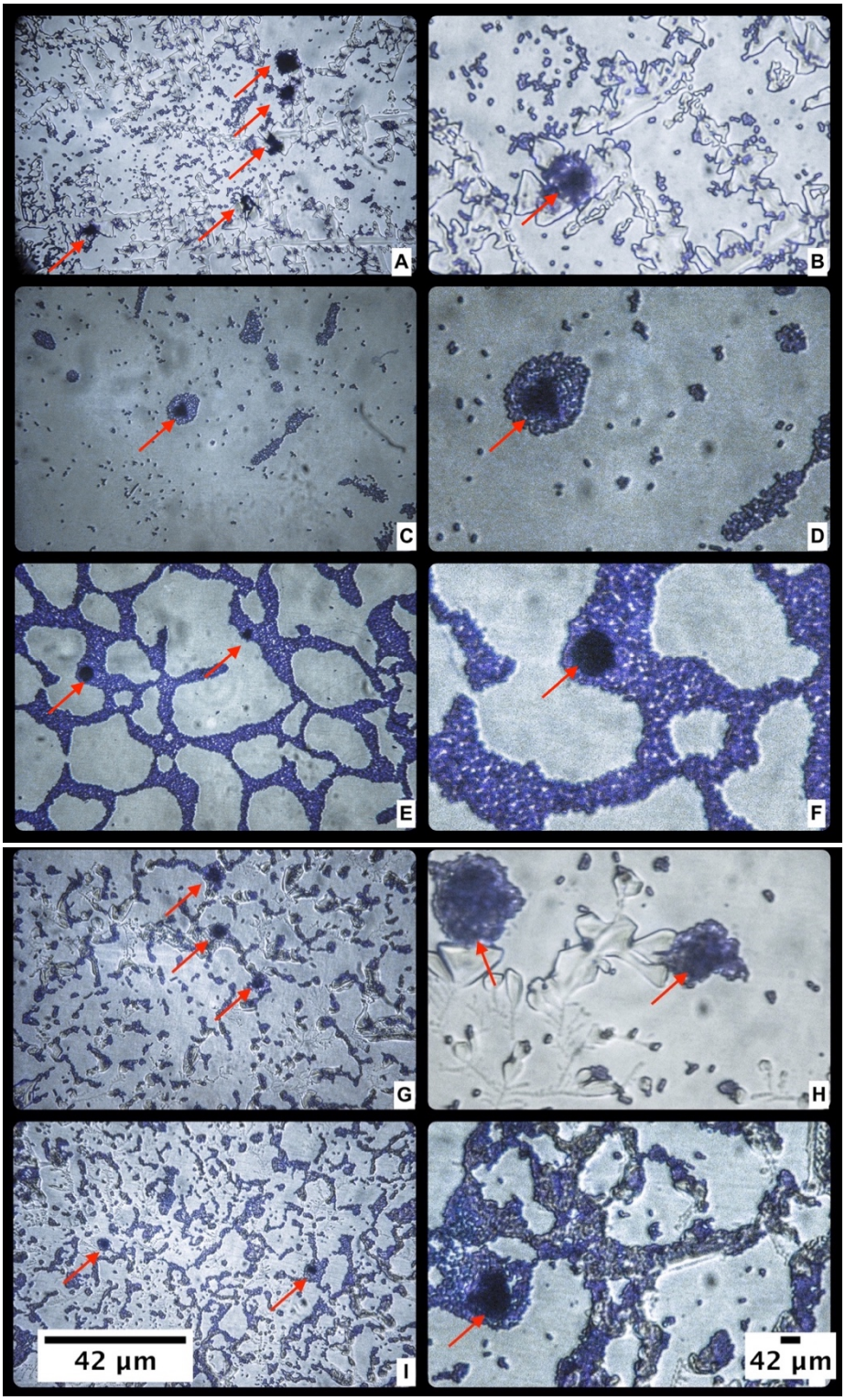

Figure 4.14: CV stain showing uropathogenic biofilm biomass (bacteria with EPS) formation in PYG.

Biofilm biomass in PYG co-cultivated with *A. castellanii* (T4). A&B – *E. coli* (ESBL negative); C&D – *E. coli* (ESBL positive); E&F – *K. pneumoniae* (ESBL negative) G&H – *K. pneumoniae* (ESBL positive); I&J – *P. mirabilis*. Red arrow indicates amoeba. The figure is representative of images obtained from a bright field microscope (scale bar: 42µm).

4.3.4.4 Catheter biofilm formation

To investigate the effect of *A. castellanii* (T4) on catheter biofilm formation, PTFE coated latex catheters (Ch 12) were used to assay the biofilm formation. Catheters were incubated in AUM and PYG with and without (control) amoebae.

From the data in Figure 4.15A we can see that, all uropathogens except *P. mirabilis* (0.9 x 10^6 CFU/ml when cultured on its own compared with 0.4 x 10^6 CFU/ml when co-cultivated with amoebae) form better biofilms when cultured on their own in AUM. In PYG (Figure 4.15B), although the difference between the two groups (cultured on its own and co-cultivated with amoebae) is minimal for all tested uropathogens, it is still significant at $p \leq 0.05$ (paired two-tailed t test). When comparing co-cultivated data (Figure 4.15C) in AUM and PYG, it is evident that all strains except *K. pneumoniae* ESBL negative strain (2 x 10^6 CFU/ml when co-cultivated in AUM and 2.8 x 10^6 CFU/ml when co-cultivated in PYG) and *P. mirabilis* (4.5 x 10^6 CFU/ml when co-cultivated in AUM and 9.2 x 10^6 CFU/ml when co-cultivated in PYG) form better biofilms in AUM than PYG. This difference, is however, not significant.

Overall, the data reveal that uropathogens form lesser biofilms on catheters when co-cultivated with *A. castellanii* (T4), possibly indicating their preference for amoebic grazing over biofilm formation.

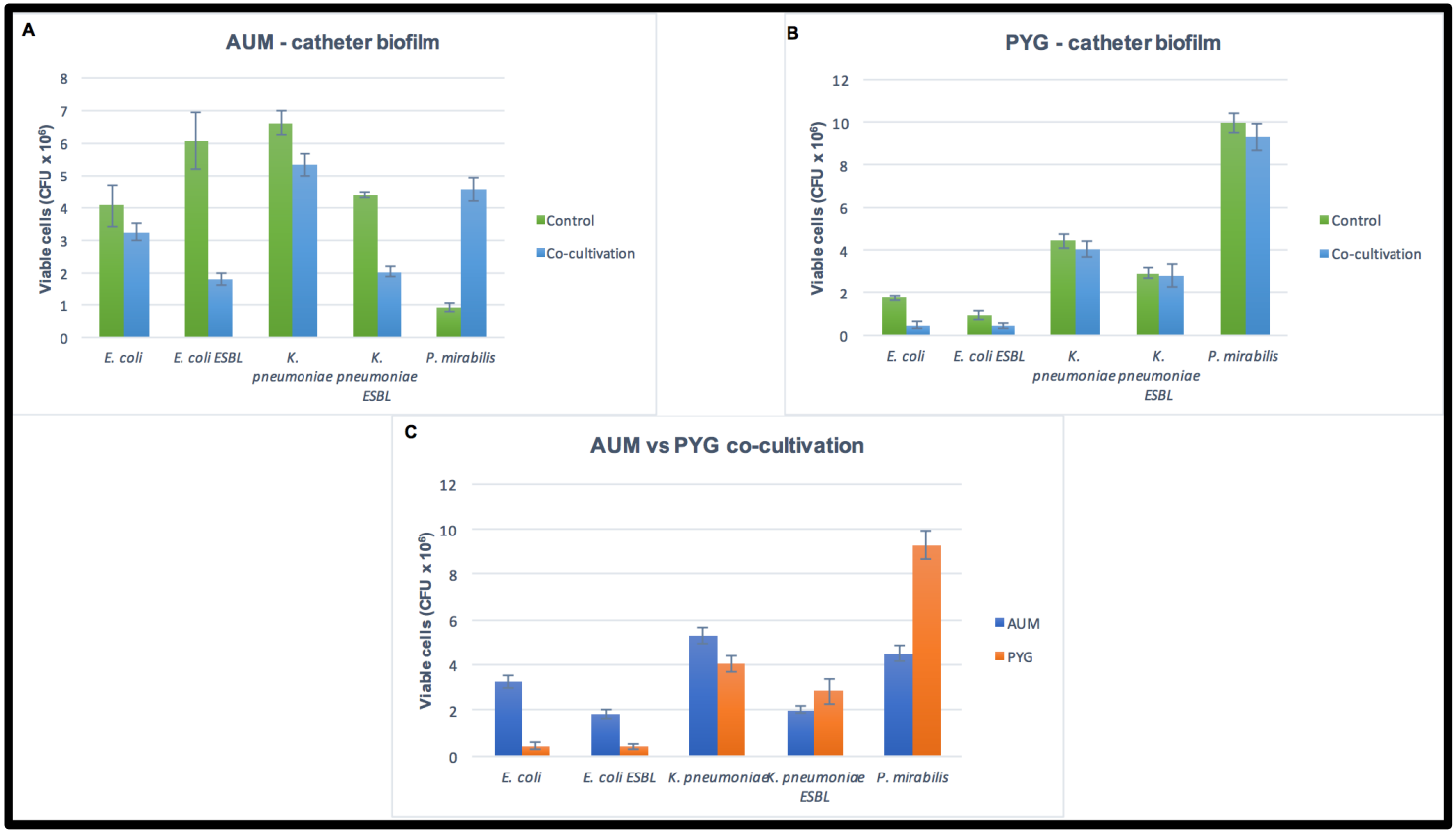

Figure 4.15: Uropathogenic catheter biofilm formation in AUM and PYG co-cultivated with *A. castellanii* (T4).

Viable bacteria from biofilms were enumerated after 24h of incubation at 37°C. Bacteria without co-cultivation were used as control. A – Viable bacteria from catheter biofilms grown in AUM; B - Viable bacteria from catheter biofilms grown in PYG; C – Comparison of catheter biofilm formation in AUM and PYG co-cultivated with *A. castellanii* (T4). Results are obtained from at least three independent experiments. Error bars represent standard error. B significant at $p \leq 0.05$ (paired two-tailed *t* test).

4.4 Discussion

This study set out with the aim of exploring the interrelationship between uropathogens and *A. castellanii* (T4). It is widely recognised that *Acanthamoeba* is pathogenic to humans, mainly causing GAE, AK and DI (4.1). Studies conducted over the past decade (Spanakos *et al.,* 2006; Ozkoc *et al.,* 2008; Di Cave *et al.,* 2014) have demonstrated the presence of *Acanthamoeba* in ocular clinical samples. Interestingly, *Acanthamoeba* has also been found in brain tissue (Yagi and Schuster, 2007), cerebrospinal fluid samples (Sheng *et al.,* 2009), sputum samples (Bradbury *et al.,* 2014) and in nasal swabs (Cruz and Rivera, 2014). In 2009, Santos *et al.* found *Acanthamoeba spp* in urine samples collected from indwelling catheters of critically ill patients indicating the co-localisation of uropathogens and amoebae in infected urine. These findings raise intriguing questions about the nature and extent of *Acanthamoeba* pathogenicity.

Acanthamoeba – bacteria interactions are complex, varied and depend on the density and species of bacteria. It can result in amoebic destruction, bacterial destruction or the development of a symbiotic relationship (Marciano-Cabral and Cabral, 2003). To assess the nature of this relationship, interaction assays were performed. The current findings indicate that all uropathogens are able to associate, invade and survive within *A. castellanii* (T4) albeit to different degrees. Both strains of *K. pneumoniae* have exhibited a higher capability for associating with *A. castellanii* (T4) compared with the other uropathogens (Figure 4.3A). A similar trend can be seen in the invasion assays (Figure 4.3B). However, *P. mirabilis* seems to have outcompeted the other uropathogens in

their ability to survive and multiply within the amoebae (Figure 4.3C). According to Khan (2009) a variety of bacteria including *E. coli* and *K. pneumoniae* were more resistant to free chlorine in the presence of *Acanthamoeba* suggesting an increase in virulence. Other studies have also reported similar findings (Literature review 1.4.8). The interaction assay results from the present study, supports the hypothesis that uropathogens possibly use *Acanthamoeba* to survive harsh conditions and cause recurrent infections in the urinary tract.

Alsam *et al.,* (2006) have found that *E. coli* outer membrane protein A (OmpA) is crucial for successful interaction with *Acanthamoeba*. The molecular mass of this protein was found to be 35.177KDa (UNIPROT, 2014). A protein of interest (30 – 40KDa) can be seen for *E. coli* (ESBL positive and negative) in the SDS-PAGE analysis of bacterial binding proteins isolated from *A. castellanii* (T4) (Figure 4.2) suggesting the possible isolation of OmpA. Similarly, proteins of interest for *K. pneumoniae* (ESBL positive and negative) and *P. mirabilis* can be further analysed and identified using techniques such as 3D gel and western blot. Although incompletely understood, it is evident that bacteria use specific proteins to interact with *Acanthamoeba*.

In order to determine if any of the major urine components have any effect on uropathogen-amoeba interaction, AUM samples were prepared without urea, uric acid and creatinine, and interaction assays were carried out. Interestingly, AUM sample without urea has displayed higher interaction patterns with *A. castellanii* (T4) when compared with control (AUM). Although not extensively researched, previous studies have indicated that urea has a bacteriostatic

effect on many Gram-positive and Gram-negative bacteria (Bonin, 1949; Weinstein and McDonald, 1946).

Acanthamoeba can release a variety of extracellular factors like proteases and LPs into their environment, either as a protective mechanism or as part of their normal physiological process. These released factors can potentially have a significant effect on other microorganisms living in the same environment. Conditioned medium assays were performed in nutrient rich PYG and nutrient poor urine. The current study found that the biomass of uropathogenic biofilms is slightly decreased in ACM for all tested uropathogens (Figure 4.6 A & B). Biofilm light microscopy has revealed defective biofilm architecture in ACM-P compared with minimal changes in ACM-A (Figure 4.7 and Figure 4.8). This finding supports previous research, which links extracellular *Acanthamoeba* proteases to degradation of the extracellular matrix (Sissons *et al.,* 2006). However, biofilms of both strains of *K. pneumoniae* possess better structural organisation in ACM-A compared with unconditioned AUM (Figure 4.7). Another important finding was that ACM significantly ($p \leq 0.001$, paired two-tailed *t* test) increased planktonic growth for all uropathogens (Figure 4.5A & B) suggesting the presence of amoebae released factors that promote bacterial growth. These findings are in accord with previous studies conducted by Verhoeven *et al.* (2010). The exact composition of ACM is unknown but in a recent study, Iqbal *et al.,* (2014) has indicated the presence of active components in the range of 5 to 10 kDa. This study also found that all uropathogens can form catheter biofilms in ACM (Figure 4.9A, B & C). Interestingly, *P. mirabilis* can form better catheter biofilms in ACM-A than AUM

(Figure 4.9A). This can be attributed to the fact that *P. mirabilis* is a urease-producing bacterium, which, in the presence of urine forms calcium and magnesium phosphate crystals (Jones *et al.,* 2006) thereby reducing biofilm biomass. However, artificial urine conditioned by amoeba has increased the biofilm forming ability of *P. mirabilis* despite the presence of urea. Overall, these findings suggest that uropathogens have the ability to survive in an environment that was inhabited by *A. castellanii* (T4) and can use it as a niche to grow and multiply. Therefore, indicating that ACM is not bactericidal to the uropathogens employed in this study.

Since *Acanthamoeba* is a ubiquitous protist, it is highly likely that they can co-exist with bacteria in the same environment. On the question of co-cultivation of bacteria and amoeba, this study found that all uropathogens exhibited similar biofilm biomass when cultured individually or co-cultured with live amoebae (Figure 4.11B & D). However, the most important finding was that *A. castellanii* (T4) displayed lesser surface adherence when co-cultured with bacteria (Figure 4.11A & C). Although this finding is in agreement with those obtained by Bottone *et al.* (1992) and Verhoeven *et al.* (2010), it should be noted that very few co-culture studies have been conducted. A possible explanation for decreased amoebic adherence could be competition for space from an overwhelming bacterial population. If this is the case, when co-cultured, uropathogens have displayed the ability to outcompete amoebae in surface adherence. There are, however, several possible explanations including decreased preferential adherence of *Acanthamoeba* in the presence of bacteria and anti-amoebic activity. These results therefore need to be

interpreted with caution. There is abundant room for further progress in determining the exact nature of interaction. It will be interesting to investigate the number of floating amoebae in the media surrounding the biofilms and to check for the presence of viable bacteria within these floating amoebae.

Light microscopy images (Figure 4.13 and Figure 4.14) of co-cultured biofilms reveal reduced numbers of amoebae, primarily located in hollow voids at the edges of the micro colonies. Amoebae trophozoites are known to adhere preferentially to different surfaces (de Moraes and Alfieri, 2008), however, in the present study, uropathogens seem to have outcompeted amoebae in surface adherence. These images also display protozoan grazing activity, which is consistent with previous studies (Weitere *et al.,* 2005 and Verhoeven *et al.*, 2010). Planktonic bacterial growth assays have revealed similar growth patterns (except for *K. pneumoniae*) when cultured individually or co-cultured in AUM (Figure 4.10A). In PYG, however, all uropathogens seem to have a better growth rate when co-cultivated with *Acanthamoeba* (Figure 4.10B). On PTFE coated latex catheters, with the exception of ESBL positive *E. coli*, all uropathogens have displayed similar biofilm forming ability when cultured individually or co-cultured (Figure 4.15). Overall, these findings suggest that the ability of uropathogens to form biofilms is not diminished in the presence of *A. castellanii* (T4) but in some cases, they can form better biofilms.

In conclusion, the findings from this study primarily suggest that uropathogens can survive with *A. castellanii* (T4) and indeed grow at a faster rate in the presence of amoebic extracellular factors suggesting that the presence of

Acanthamoeba in an infected bladder can potentially increase the rate of infection. Although there hasn't been any significant effect on biofilms, the fact that uropathogens can form biofilms in the presence of *A. castellanii* (T4), survive and multiply within them and in cohabitation indicates a higher risk of colonising new surfaces. Therefore, it is reasonable to hypothesise that *Acanthamoeba* may play a crucial role in causing rUTIs.

The next chapter deals with the investigation of the presence of *Acanthamoeba* in urine samples collected from patients suspected of UTIs.

Chapter 5

Investigation of the presence of *Acanthamoeba* in urine samples

5 Investigation of the presence of *Acanthamoeba* in urine samples

5.1 Introduction

Acanthamoeba are free living eukaryotic protist microorganisms distributed worldwide. They have been isolated from a variety of environments including natural water sources, soil, public water supplies, swimming pools, air conditioning units, contact lenses, hospital units, medical equipment and the human body (1.4.3).

Several species of *Acanthamoeba* including genotypes T1, T2a, T3, T4, T5, T6, T10, T11, T12 and T15 are known to cause diseases in humans (Siddiqui and Khan, 2012). *Acanthamoeba* can gain entry into the human body through the eyes, nasal passages and ulcerated skin (CDC, 2013). When it enters the eye, it can cause severe infection of the cornea known as AK. Although AK is more common in contact lens users, it has been reported in non-contact lens users as well (Lorenzo-Morales *et al*., 2015). *Acanthamoeba* can also cause GAE when it enters the nasal passages or ulcerated skin and disseminates into blood. It is a life threatening infection of the CNS with a high mortality rate (over 50%) and is particularly seen in immunocompromised hosts (Zamora *et al*., 2014). It can also cause DI or skin lesions in individuals with compromised immune systems. Disseminated or cutaneous *Acanthamoeba* infections are characterised by granulomatous infiltration of the skin and other extracerebral organs. It is a rare type of infection and has progressed to GAE in many cases (Aichelburg *et al*., 2008).

Severe life threatening CNS infection in humans is primarily caused via the haematogenous route of entry. It is not very clear how the amoebae circulating in blood can cross the BBB and gain entry into the CNS, however, it has been suggested that an increase in the permeability and apoptosis of human brain microvascular endothelial cells (HBMEC) caused by factors released by the amoebae such as serine proteases and host cell determinants such as host immune mediated responses can lead to disruptions in the BBB and allow the parasite to enter the brain (Khan, 2008). *In vitro* studies have revealed the ability of *Acanthamoeba* to increase the permeability of the BBB, to bind to HBMEC and cause cytotoxic effects (Alsam *et al*., 2005b; Alsam *et al*., 2003). It is also possible for *Acanthamoeba* to enter the brain via the blood-cerebrospinal fluid barrier (Khan and Siddiqui, 2011).

Apart from the well-established routes of entry, *Acanthamoeba* has been isolated from human faeces (Zaman *et al*., 1998) suggesting a possible oral route of entry and passage of the parasite via the digestive tract. In an isolated study conducted by Sadaka and Emam (2001), mouse stools were tested positive for *Acanthamoeba* after oral amoebic inoculation.

Although *Acanthamoeba* has been found in human urine (Santos *et al*., 2009), the mechanism of entry into the urinary system is poorly understood. Similar to crossing the BBB, *Acanthamoeba* has to either cross the glomerulus in nephrons or directly enter the urinary bladder. The various possible routes of entry into urine for *Acanthamoeba* are displayed in Figure 5.1.

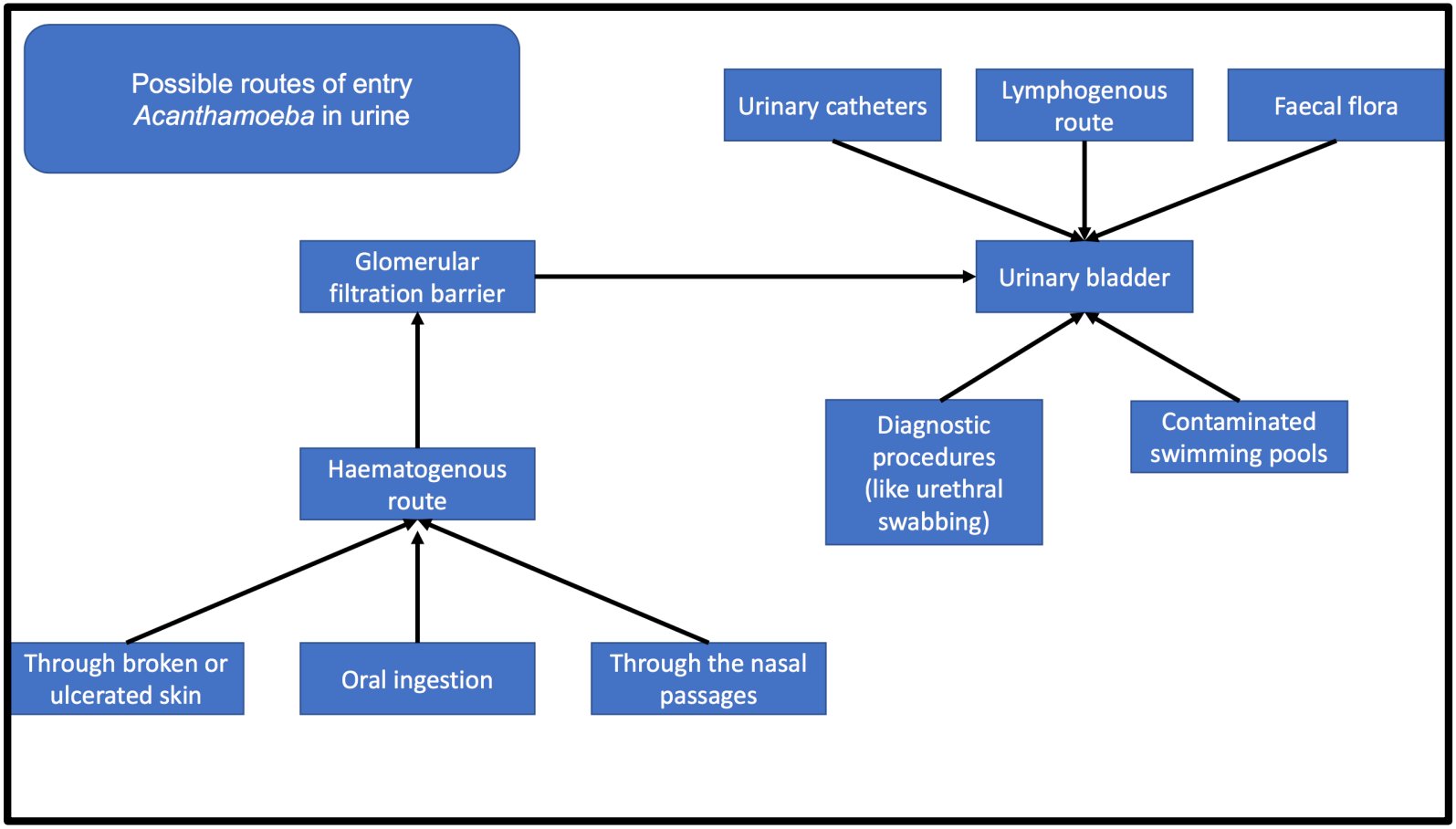

Figure 5.1: Possible routes of entry into urine for *Acanthamoeba*.

For *Acanthamoeba* to be present in urine, it has to either enter the bladder directly or cross the glomerular filtration barrier. This figure displays the various possible routes of entry into the urinary bladder.

Santos (2009) and his team reported that 23% of urine samples collected from indwelling catheters of critically ill patients tested positive for the presence of *Acanthamoeba spp*. This result coupled with our own results (4.3) and results from several studies (1.4.8) that have shown the symbiotic relationship between *Acanthamoeba* and bacteria support our hypothesis that uropathogenic bacteria can potentially survive the effects of antimicrobial drugs and host immunity by using *Acanthamoeba* as a protective tool and thereby cause rUTIs.

Since very few studies (Santos *et al.*, 2009; Bradbury *et al*., 2014) have been conducted to investigate the presence of *Acanthamoeba* in urine, this study aims to test urine samples collected in CHUFT from patients suspected with UTIs for *Acanthamoeba* and conduct genomic analysis on any positive samples by targeting the 16s rRNA gene.

5.2 Materials and methods

All chemicals were purchased from Sigma unless otherwise stated.

5.2.1 Ethical approval

Applications for ethical approval for this study were submitted and approved in three stages (Appendix F).

1. Research and Enterprise Office, University of Essex
2. Integrated Research Application System, Yorkshire & The Humber – Sheffield Research Ethics Committee (Reference: 16/YH/0266)
3. Research and Development, CHUFT

5.2.2 Collection and processing of urine samples

A total of 200 urine samples were collected from the Microbiology Department, CHUFT over a period of four weeks. Samples from male and female patients were randomly selected and anonymised by assigning a unique identification number. Approximately 10ml of urine from each sample was transferred to 15ml sterile tubes and centrifuged (3000 x g, 15min). The supernatants were discarded and the pellets were treated with 2ml of 0.25M sulphuric acid for 20min, centrifuged and washed with 2ml of PBS (2000 x g, 5min). Sulphuric acid treatment was used to decontaminate specimens heavily contaminated with Gram-negative bacteria (Public Health England, 2016).

5.2.3 *Acanthamoeba* culture

5.2.3.1 Xenic culture

An overnight culture of *E. coli* K12 strain was placed in a water bath at 95°C for 20min. The stock of *E. coli* was stored at 4°C and refreshed every two weeks. Two ml of the stock was added on to non-nutrient agar (NNA) plates (Appendix C) and the culture was allowed to soak into the agar at RT for 20min. Excess culture fluid was removed, the plates were dried and the pellets from the processed urine samples (5.2.2) were inoculated onto the centre of the agar plates. The plates were incubated at 30°C and were observed daily for the presence of amoebae. Small pieces of agar from plates which were positive for amoebic growth (cysts/trophozoites) were cut and placed (inverted) on fresh NNA plates lawned with *E. coli*. The plates were incubated at 30°C and were observed daily for the growth of cysts or trophozoites (Khan and Paget, 2002).

5.2.3.2 Axenic culture

Pieces of agar from NNA plates which were positive for amoebic growth were cut using a sterile scalpel and transferred into a T25 flask containing 5-7ml of PYG with and without antibiotics (Penicillin/Streptomycin 100U/ml and 0.1mg/ml respectively) (Hughes and Kilvington, 2001). The flasks were incubated at 30°C and observed daily for amoebic growth (cysts/trophozoites).

Flasks which were contaminated with bacteria were placed on ice for 20min to dettach surface adherent amoebae, the medium was collected and centrifuged (2000 x g, 5min). The pellets were transferred to fresh T25 flasks containing

PYG and antibiotics. This step was repeated (1-2 times per week) until the medium was free of bacterial contamination.

Flasks which were contaminated with fungi were placed on ice for 20min to dettach surface adherent amoebae. The medium was carefully collected and treated with 0.5M sodium hydroxide for 30min (Public Health England, 2016). Post treatment, the medium was washed in PBS (2000 x g, 5min) and the pellets were transferred to fresh T25 flasks containing PYG and antibiotics.

5.2.4 Genomic analysis

5.2.4.1 DNA extraction

Confluent flasks (~ 30 – 50% of cysts/trophozoites) were placed on ice for 20min to dettach surface adherent amoebae, the medium was collected and centrifuged (2000 x g, 5min). DNA was extracted with Chelex resin. Two hundred µl of the Chelex solution (10% [wt/vol]) in 0.1% Triton X-100 and 10mM Tris buffer [pH 8.0]) was added to the pellets and the mixture was vortexed (10s), centrifuged (10000 x g, 10s) and heated in a water bath at 95°C for 20min. The mixture was centrifuged (10000 x g, 20s) again and the supernatant (2µl) was used as a substrate for PCR (Lovieno *et al*., 2011).

5.2.4.2 Polymerase chain reaction

Specific primers (Table 5.1) were used for PCR amplification. PCR was performed in 50µl reaction mixtures (buffer 10X containing 5µl (KCl, Tris-$MgCl_2$), 3µl $MgCl_2$, 1µl 10mM dNTPs, 1µl FP16 primer, 1µl RP16 primer, 1µl DNA, 0.5µl Taq polymerase, and 38µl ds water). The PCR cycle profile was set

up as listed in Table 5.2. The total PCR product with sample loading buffer (5:1) was run on safe view DNA-stained 0.5% agarose gel at 150v for 15min. Finally, the PCR product was detected in the gel under UV light (Yu *et al*., 1999).

Table 5.1: Gene and primer sequences

Target gene	Primer (nucleotide sequence)	Product length (bp)	Reference
16s rRNA	F 5-TTATATTGACTTGTACAGGTGCT-3	180	Yu *et al*., 1999
	R 5-CATAATGATTTGACTTCTTCTCCT-3		

Table 5.2: PCR cycling setup

16s rRNA	Temperature (°C)	Time (min)
Initial denaturation	94	10
Denaturation	94	1
Annealing	55	0.5
Extension	72	1
Final extension	72	10
No of cycles	35	

5.2.4.3 Purification of PCR product

The PCR product was purified using the QIAquick PCR purification kit using a microcentrifuge (QIAGEN Ltd., Crawley, UK) following manufacturer's instructions. Briefly, 5µl of the PCR product was mixed with 25µl of buffer PB. The mixture was further mixed with 10µl of 3M sodium acetate (pH 5.0) and when the colour of the mixture turned yellow, it was applied to a QIAquick column and centrifuged (17900 x g, 60s). The flow-through was discarded and the product was washed with 750µl of buffer PE (17900 x g, 60s). The flow-through was again discarded and QIAquick column was centrifuged (17900 x g, 60s). The QIAquick column was then placed in a clean 1.5ml microcentrifuge tube and DNA was eluted by adding 30µl elution buffer to the centre of the QIAquick membrane. The column was allowed to stand at RT for 1min and centrifuged (17900 x g, 60s).

5.2.4.4 Gene sequencing

Purified PCR products were commercially sequenced using Sanger Sequencing Services (Source Bioscience, Birmingham, UK). Forward primer was used to obtain the sequences.

5.2.4.5 BLAST search and phylogenetic analysis

Sequences were identified using the nucleotide Basic Alignment Search Tool (n-BLAST). Nucleotide collection database (GenBank, EMBL, DDBJ, PDB, RefSeq sequences) was used and the program selection was optimised for highly similar sequences (megablast). Sequences were analysed on the phylogeny.fr platform (Dreeper *et al*., 2008; Dreeper *et al*., 2009). MUSCLE

(v3.8.31) with default settings and configured for highest accuracy was used to align the sequences. Poorly aligned sequences and gaps were removed with Gblocks (v0.91b) using the following parameters –

- minimum length of a block after gap cleaning: 10
- no gap positions were allowed in the final alignment
- all segments with contiguous non-conserved positions bigger than 8 were rejected
- minimum number of sequences for a flank position: 85%

Finally, the phylogenetic tree was reconstructed via PhyML program (v3.1/3.0 aLRT) using the maximum likelihood method. The tree was graphically represented and edited with TreeDyn (v198.3).

5.3 Results

Out of the 200 samples analysed for the presence of *Acanthamoeba*, 21 samples tested positive yielding a prevalence percentage of 10.5. Samples were anonymised with a unique identification number and details for the positive samples were collected from the Microbiology department, CHUFT. The population pyramid (Figure 5.2) reveals non-specific age related prevalence of *Acanthamoeba* in men and women. The highest prevalence in men is seen in the age groups of 45-49, whereas in women, it is over the age of 85. Samples collected from men aged 40 and above tested positive for the amoebae whereas in women, samples were positive for a wider age group. n-BLAST revealed the identification of two different species of *Acanthamoeba* (Table 5.3). Out of the 21 positive samples, 19 were *Acanthamoeba sp*. (unclassified) and 2 were *A. castellanii* (Figure 5.3). The *A. castellanii* positive urine samples were collected from women in the age group of 80-84 (Table 5.3).

DNA extracted from positive samples was successfully amplified (Figure 5.4) for the partial sequence of the 16s rRNA gene (mitochondrial subunit) and sequences were commercially obtained from SourceBioscience, Birmingham, UK. Sequence alignment was performed with MUSCLE (v3.8.31) using default settings and configured for highest accuracy. The phylogenetic tree (Figure 5.5) was reconstructed via PhyML program (v3.1/3.0 aLRT) using the maximum likelihood method. The tree was graphically represented and edited with TreeDyn (v198.3).

The two *A. castellanii* positive samples (59.28.10 & 34.28.10) are clustered closely with the reference sequence for *A. castellanii* (AF479550.1) whereas all the other samples, except for 17.21.10 and 57.28.10 are closely clustered with the reference sequence for *Acanthamoeba sp*. unclassified (AB795716.1).

Table 5.3: List of urine samples tested positive for *Acanthamoeba*.

Organisms was identified using the n-BLAST search and accession numbers were extracted from GenBank at NCBI. Sample details were collected from the Microbiology department, CHUFT. UIN – unique identification number, CD – clinical diagnosis.

	UIN	Organism	Accession number	Sample details		
				Age	Sex	CD
1	17.21.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	75	M	UTI?
2	12.21.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	19	F	UTI?
3	02.28.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	93	F	UTI?
4	14.21.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	87	F	UTI?
5	29.21.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	82	M	UTI?
6	04.28.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	58	F	UTI?
7	01.21.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	28	F	UTI?
8	59.28.10	*A. castellanii* strain CDC V014 16S small subunit ribosomal RNA gene, partial sequence	AF479550.1	82	F	UTI?
9	15.28.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	85	F	UTI?
10	28.21.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	49	M	UTI?
11	57.28.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	51	M	UTI?
12	34.28.10	*A. castellanii* strain CDC V014 16S small subunit ribosomal RNA gene, partial sequence	AF479550.1	85	F	UTI?
13	51.28.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	82	M	UTI?
14	23.04.11	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	8	F	UTI?
15	54.04.11	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	54	M	UTI?
16	30.21.10	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	40	M	UTI?
17	01.04.11	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	19	F	UTI?
18	14.04.11	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	60	F	UTI?
19	60.04.11	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	63	M	UTI?
20	32.11.11	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	48	M	UTI?
21	39.11.11	*Acanthamoeba* sp. JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1	79	M	UTI?

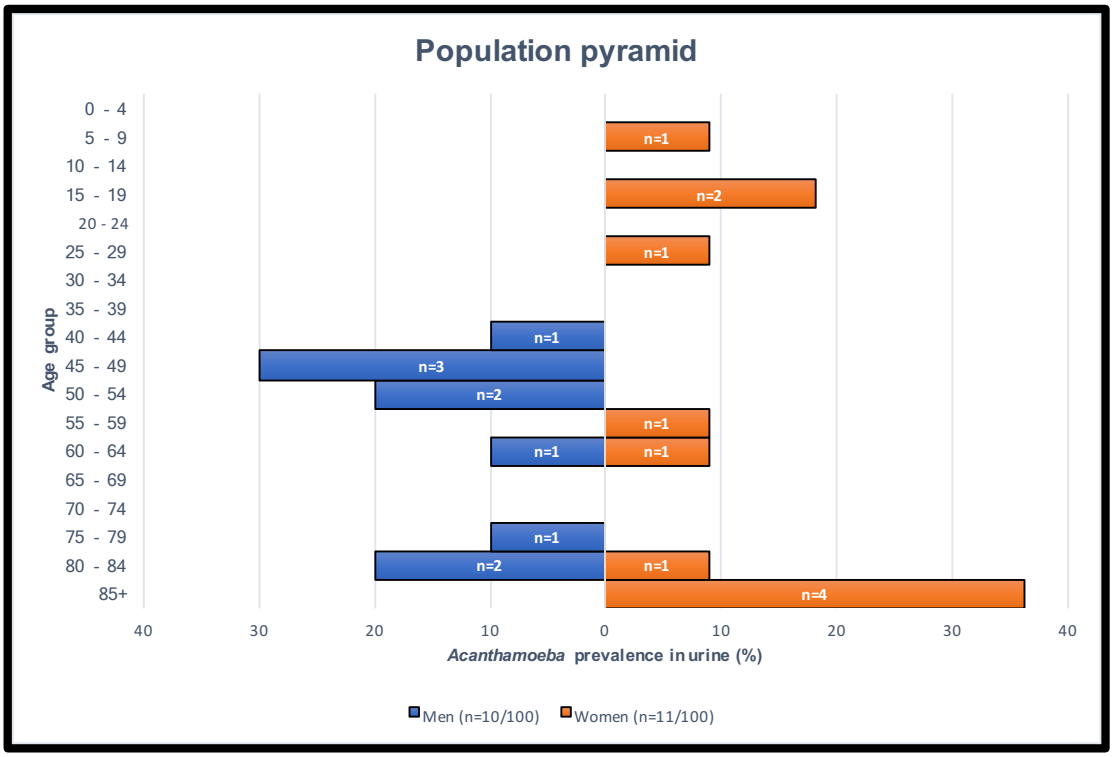

Figure 5.2: Population pyramid displaying the prevalence of *Acanthamoeba* in urine of men and women for different age groups.

Prevalence percentage (%) was calculated for the total number of positive samples for men and women as individual groups.

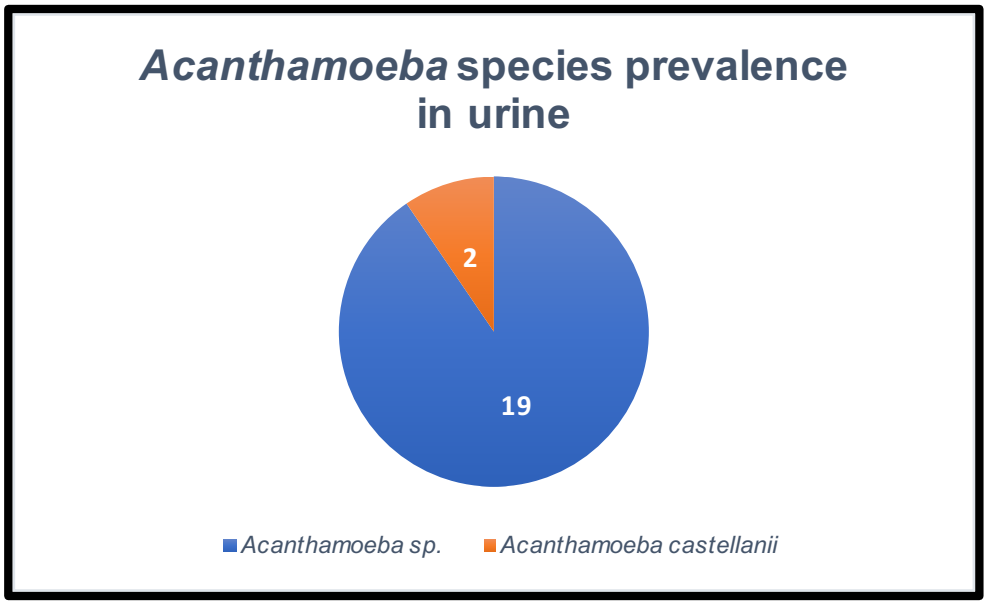

Figure 5.3: Pie chart displaying the prevalence of different species in *Acanthamoeba* positive urine samples.

Total number of positive samples, n=21.

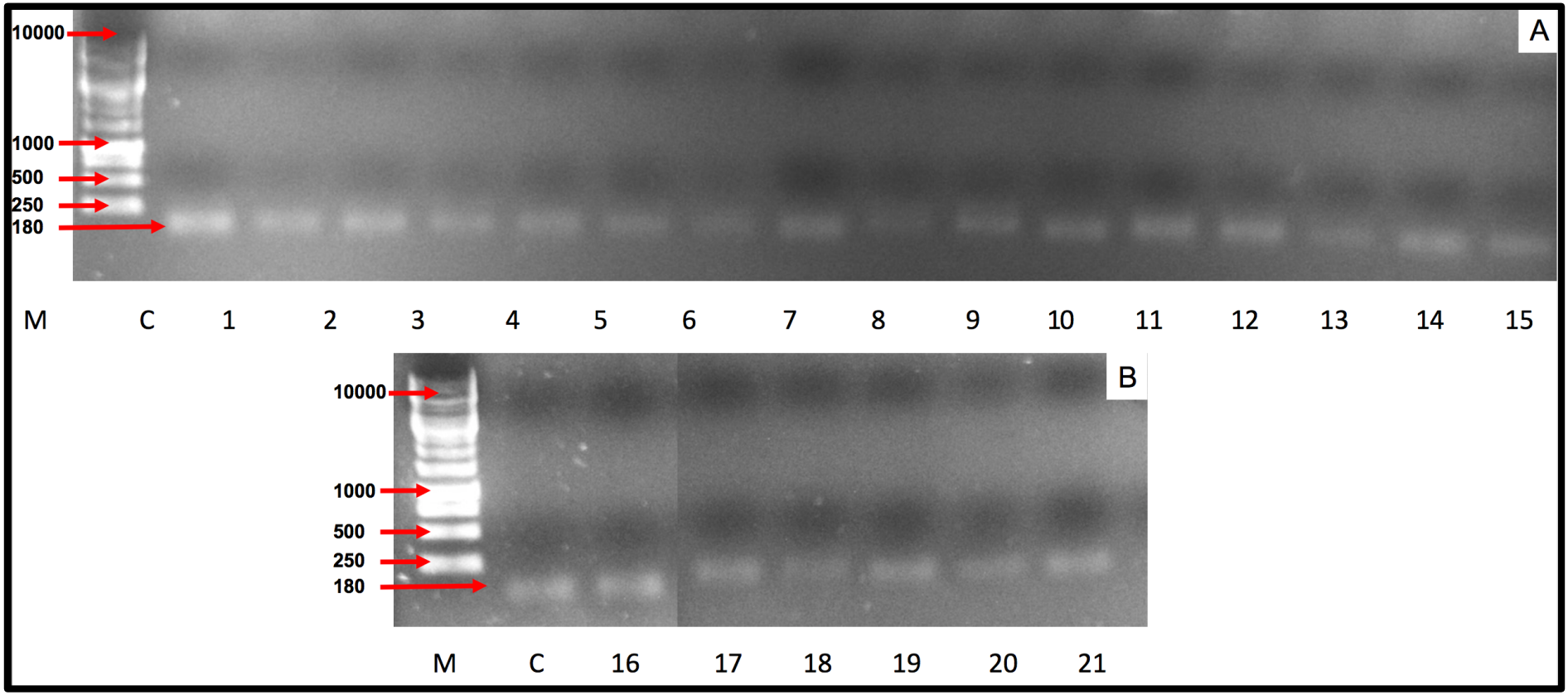

Figure 5.4: PCR products amplified using the FP16 and RP16 primer.

Bands are observed in all lanes with an expected size of 180bp (16s rRNA). M – 1kbp DNA ladder, C – *A. castellanii* (T4 - control), 1 to 21 – positive urine samples (see Table 5.3 for unique identification numbers).

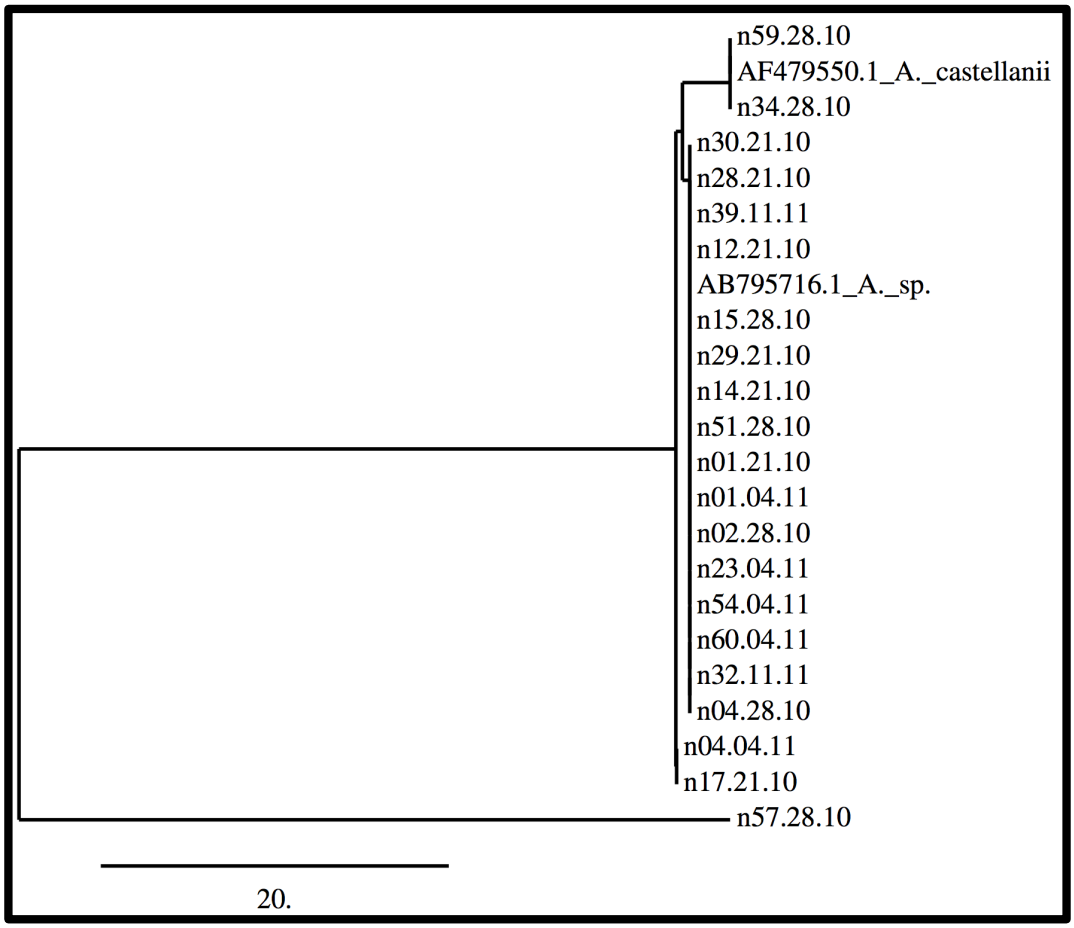

Figure 5.5: 16s rRNA phylogenetic tree for the positive samples.

Two reference sequences (AB795716.1 – *Acanthamoeba sp.* unclassified & AF479550.1 – *Acanthamoeba castellanii*) were used. The phylogenetic tree was reconstructed via PhyML program (v3.1/3.0 aLRT) using the maximum likelihood method. The tree was graphically represented and edited with TreeDyn (v198.3).

5.4 Discussion

A total of 200 samples collected from patients suspected of UTIs were tested for the presence of *Acanthamoeba* using the gold standard method of culture on NNA plates (Lorenzo-Morales *et al*., 2015). Positive NNA plates were axenically cultured in PYG and DNA extracted from cysts/trophozoites were used for genomic analysis.

Several studies have employed the use of primers JDP1/JDP2 which targets the ~450 bp fragment of *Acanthamoeba* 18S rRNA gene (Schroeder *et al*., 2001) and primers FP16/RP16 which targets the mitochondrial small subunit rRNA gene (Yu *et al*., 1999). This study employed the use of 16s rRNA primers. The advantages of using the mitochondrial small subunit ribosomal RNA gene is that the sequences are shorter, more consistent in length, higher proportion of bases are alignable for sequence comparisons and they do not have any of the complications caused by multiple alleles or introns which are more common in the nuclear ribosomal RNA gene (Ledee *et al*., 2003).

Of the total samples analysed, 10.5% (n=21) of urine samples tested positive for *Acanthamoeba*. n-BLAST search (Table 5.3) revealed that the amoebae from nineteen samples (90.4%) were positive for *Acanthamoeba sp*. (unclassified *Acanthamoeba*) and the other two samples (9.6%) were positive for *A. castellanii* (Figure 5.3). Interestingly, the two positive *A. castellanii* samples were isolated from female patients over the age of eighty (Table 5.3) suggesting a possible link with weakened immune systems and other age related physiological and anatomical changes. *A. castellanii* is known to cause

GAE and AK in immunocompromised patients (Khan, 2006). From the population pyramid (Figure 5.2), it is quite clear that the prevalence of *Acanthamoeba* in urine is not specific to any age group. However, it is interesting to note that a higher percentage of urine samples collected from female patients tested positive for the protozoa in the age group of 85+. With regards to the clinical diagnosis, all patients were suspected for UTIs (Table 5.3) tending to support the hypothesis that *Acanthamoeba* plays a role in UTIs.

The presence of *Acanthamoeba* in public water supplies, may be consistent with the idea of an oral route of entry for the parasite. However, its eventual entry into blood remains poorly understood. With regards to the urinary system, *Acanthamoeba* can enter the urinary bladder using various routes of entry as displayed in Figure 5.1. In terms of clinical implications, the haematogenous route (indirect route) of entry and passing through the glomerulus is of primary interest.

The glomerulus is the filtering unit of the kidney. The filtration barrier is composed of three layers. The proximal layer is made up of fenestrated endothelial cells with a pore size of 70 to 100nm in diameter. The middle layer is the glomerular basement membrane (GBM) which is a complex meshwork of extracellular proteins. This layer is very thin (250 to 400nm) and restricts the passage of plasma proteins. The visceral layer is composed of epithelial cells and podocytes, with the latter forming filtration slits (~ 40nm) spanned by slit diaphragms that restrict passage of molecules based on their size (Pollack *et al*., 2014; Suh and Miner, 2013; Deen, 2004). Together, these layers known as

the glomerular filtration barrier (GFB), allow the continuous filtration of the blood plasma.

Under physiological conditions, the GFB prevents the passage of proteins, RBCs and other large molecules. In proteinuria, due to changes in glomerular permeability, proteins can be detected in urine. It has been reported that an increase in the glomerular pore size is directly related to urinary albumin excretion (Wiwanitkit, 2009). In microscopic glomerular haematuria, the exact mechanism of how red blood cells (RBCs), which are 100-fold bigger (6.2 to 8.2 μm) than the glomerular pores can pass through the GFB remains unclear. However, it has been suggested that inflammatory or chemotactic signals released from the damaged GFB may promote the passage of RBCs (Yuste *et al*., 2015). It is therefore, reasonable to hypothesise that *Acanthamoeba* may possibly use a similar mechanism to pass through the GFB and reach the urinary bladder.

The extracellular matrix in the GBM mainly consists of laminin, type IV collagen, nidogen, and heparan sulfate proteoglycan. These transmembrane proteins are vital in maintaining the GFB (Miner, 2012). Several studies have reported the ability of *Acanthamoeba* to preferentially bind to laminin and type IV collagen (Gordon *et al*., 1993; Hong *et al*., 2004; Rocha-Azevedo *et al*., 2009; Clarke *et al*., 2013). Again, it is reasonable to hypothesise that interactions of *Acanthamoeba* with the proteins in the extracellular matrix of GBM may allow their passage into the, otherwise impenetrable GFB.

It is well known that *Acanthamoeba* produces a variety of proteases (1.4.6.2) that are vital for their pathogenic activity, particularly proteolysis and induction of apoptosis (Homan & Bowers, 1984; Alizadeh *et al*., 1994). If *Acanthamoeba* does use the haematogenous or oral route of entry to pass the GFB and enter the bladder, its clinical implications can be profound.

This is the first study in the UK to show the presence of *Acanthamoeba* in urine samples. Further analysis of the patient history, particularly their complete urine profile is required to fully explore the exact impact of *Acanthamoeba* on the urinary system. Nevertheless, the presence of *Acanthamoeba* in urine supports our hypothesis that uropathogenic bacteria can establish a symbiotic relationship with the amoebae, evade host defence mechanisms, increase their virulence factors and cause rUTIs.

The next chapter explores the antimicrobial efficacy of CPC; a QAC primarily used to treat oral bacterial infections. Several studies have investigated their use in oral care and the poultry industry but not UTIs.

Chapter 6

Evaluation of cetylypyridinium chloride as an antimicrobial agent

6 Evaluation of cetylypyridinium chloride as an antimicrobial agent

6.1 Introduction

UTIs are one of the most common HCAIs worldwide, especially affecting women and children (Lüthje and Brauner, 2016). rUTIs and CAUTIs have compounded the overall burden of UTIs (Barber *et al*., 2013; Saint *et al*., 2009).

IBCs formed inside bladder urothelial cells are implicated in rUTIs (Rosen *et al*., 2007; Robino *et al*., 2013; Glover *et al*., 2014; Scott *et al*., 2015). These IBCs not only allow the bacteria to circumvent the host immune response (Anderson *et al*., 2004) but also offer them resistance to antibiotics (Hannan *et al*., 2012). On the other hand, CAUTIs are a result of biofilm formation on urinary catheters (Trautner and Darouiche, 2004). It is well known that greater antibiotic resistance is exhibited by bacteria found within biofilms (Costerton *et al*., 1995; Stewart and Costerton, 2001). Several mechanisms confer greater resistance to biofilms than planktonic bacteria. These mechanisms include limited antibiotic diffusion through the biofilm EPS, inactivation of the antibiotic, expression of efflux pumps, transmission of resistance genes and formation of highly tolerant persister cells (Soto, 2014).

A decrease in the development of novel antibiotics and an increase in microbial resistance patterns are causing a global threat of antimicrobial resistance (McArthur *et al*., 2013). There is a constant need to develop new and effective

antimicrobial drugs. To this effect, many researchers are considering the possibility of using alternative, non-antibiotic products against bacteria.

More HCAI's are related to the use of urinary catheters than any other medical device. An estimated 450,000 people in the UK use catheters on a long-term basis (Kokare *et al*., 2009). Maughan *et al*. (2010) have found that a staggering 79% of patients on LTC have had at least one complication over two years, out of whom, 62% had a CAUTI. Uropathogens are known to readily form biofilms on catheters. Although several catheter related studies have been conducted over the past forty-five years, none of them have resulted in the production of a potent, long-term antimicrobial catheter (Singha *et al*., 2016). Catheter coating, use of nanoparticles, bacteriophages, liposomes, enzyme inhibitors, iontophoresis, bacterial interference, anti-adhesion agents and low-energy surface acoustic waves are some of the new trends in anti-biofilm treatment (Soto, 2014).

QACs are cationic surfactants used as ingredients in a variety of household and industrial products. Structurally, they contain at least one hydrophobic hydrocarbon chain, a positive nitrogen atom and short chain alkyl groups (Zhang *et al*., 2015). Many of the synthesised QACs possess significant antibacterial, antifungal and antiviral abilities. Preparation of antimicrobial biomaterials by impregnating these QACs into polymers is an increasingly promising area of research (Jiao *et al*., 2017). CPC is a cationic surfactant belonging to the group of QACs. It is currently utilised as an antimicrobial agent in oral hygiene products such as mouthwashes, toothpastes, lozenges and

dental sprays (Hwang *et al*., 2013). These compounds have a positively charged head containing central nitrogen and various alkyl or 'R' groups and a long, uncharged hydrocarbon chain (Talaro and Talaro, 1993). CPC has both hydrophilic and hydrophobic regions. Bacterial cell surfaces carry a net negative charge under physiologic conditions due to the presence of negatively charged molecules on their surface. Positively charged hydrophilic regions of CPC interact with the negatively charged molecules on the bacterial cell surface thereby allowing the hydrophobic regions to interact with the bacterial cell membrane causing leakage of cell components, growth inhibition and cell lysis (FDA, 2003).

Most studies investigating the antimicrobial potential of CPC have only focused on oral care (Hwang *et al*., 2013) and poultry processing (Li *et al*., 1997; Arritt *et al*., 2002; Beers *et al*., 2006). Considering its potent antimicrobial activity and its cost effectiveness, CPC can be investigated for its potential use in treating UTI's, particularly CAUTIs.

This chapter has been divided into four parts. The first part deals with the antimicrobial effect of CPC on planktonic bacteria and biofilms. Secondly, cytotoxicity assays were performed to explore the cytotoxic effects of CPC on *Acanthamoeba* and TERT-NHUC line. This is followed by antimicrobial intracellular amoebic and urothelial cell survival assays. The last part evaluates the anti-biofilm activity of CPC impregnated urinary catheters.

6.2 Materials and methods

Silicone and PTFE coated latex catheters (Ch 12) were kindly provided by Dr. Tony Elston, CHUFT. Catheters were aseptically cut into approximately 1cm pieces, sterilised in 70% ethanol and air-dried. The catheter pieces were stored in sterile petri dishes at RT.

6.2.1 Bacterial culture

All strains were cultured, inoculated and ODs were measured as described in chapter 2 (2.2.2).

6.2.2 Antimicrobial assays

A stock solution (1mg/ml) of CPC was made in ds water and stored at RT. Various concentrations of CPC were prepared from the stock solution for use in the experiment.

6.2.2.1 Planktonic assays

Planktonic assays were carried out to investigate the effect of CPC on planktonic bacteria. Uropathogens (~10^6 CFU/ml) were inoculated in AUM with and without CPC (1, 2, 3, 4 & 5µg/ml) in a 24-well plate and the plate was incubated at 37°C for 24h in an incubator. After the incubation period, the suspension in each well was diluted and cultured on CLED agar and the CFUs were counted after 18h of incubation at 37°C.

6.2.2.2 Biofilm viability assays

The effect of CPC on bacterial biofilms was investigated via biofilm viability assays. After the incubation period (6.2.2.1), the suspension from each well was discarded, wells were washed twice with 500µl PBS and the biofilm was scraped using a cell scraper and enumerated on CLED agar.

6.2.3 Cytotoxicity assays

CPC 3µg/ml was the minimal bactericidal concentration for uropathogens, therefore a range of concentrations starting from 3µg/ml were used for the cytotoxicity assays.

6.2.3.1 Anti-amoebic assays

To determine the cytotoxic effect of CPC on *A. castellanii* (T4), 24-well plates were seeded with amoebae as described in chapter 4 (4.2.2). Each well was washed once with PBS and supplemented with fresh AUM (control) and AUM with CPC (3, 5 & 10µg/ml) and the plates were incubated for 24h at 30°C in an incubator. After incubation, amoebae viability was determined by adding 0.1% trypan blue and the number of live (non-stained) and dead (stained) amoebae were enumerated using a haemocytometer.

6.2.3.2 LDH assays

To determine the cytotoxic effect of CPC on TERT-NHUC line, 24-well plates were seeded with TERT-NHUC line as described in chapter 3 (3.2.1.1). Each well was washed once with PBS and supplemented with fresh KGM-2 (control) and KGM-2 with CPC (3, 5 & 10µg/ml). The plates were incubated for 24h at

37°C in humidified 95% air and 5% CO_2 incubator. After the incubation period, LDH assays were performed as described in chapter 3 (3.2.4.2).

6.2.4 Intracellular survival assays

Intracellular survival assays of uropathogens in *Acanthamoeba* and TERT-NHUC line were performed as described in section (3.2.3.3 and 4.2.2). However, after killing the extracellular bacteria, each well was washed with PBS and fresh media (control) or media with CPC (3, 5 & 10µg/ml) were added and the plates were incubated for 24h. For *Acanthamoeba*, AUM was used as the medium and the incubation temperature was 30°C whereas for TERT-NHUC lines, KGM-2 and 37°C in humidified 95% air and 5% CO_2 were used as the medium and incubation conditions respectively. After incubation, each well was washed once with PBS and the cells were lysed using cell lytic (150 µl). Bacteria were quantified on CLED agar plates (Alsam *et al*., 2006).

6.2.5 Catheter impregnation

6.2.5.1 Catheter coating

Chloroform was used as an organic solvent and a penetrating agent to promote penetration of the antimicrobial agent into the material of the catheter. CPC rapidly dissolves in chloroform at RT. Catheter pieces (1cm) were dipped into the antimicrobial catheter coating solution (1, 2, 3 & 4mg/ml) and placed in a water bath (45°C) for 1h. Post incubation, the catheter pieces were aseptically removed and allowed to dry overnight (RT). Control catheter pieces (without CPC) were prepared in the same way (Darouchie and Raad, 1997). Based on

the impregnation process, the following terms are used in the rest of the chapter:

Uncoated catheters: Catheters that were not subject to the impregnation process.

Coated catheters without CPC: Catheters that were subject to the impregnation process but the catheter coating solution did not contain any CPC.

Coated catheters with CPC: Catheters that were subject to the impregnation process and the catheter coating solution contained various concentrations of CPC.

6.2.5.2 Anti-biofilm assays

Uncoated and coated catheters with and without CPC were washed once in 70% ethanol and air-dried. Bacterial biofilms were grown on the catheter by placing each piece in a 24-well plate containing approximately 10^6 bacteria per well in their log or exponential growth phase. The final volume in each well was made up to 1ml by adding AUM. The plate was incubated for 24h at 37°C in an incubator. After the incubation period the catheter pieces were washed once with PBS, sonicated in an ultrasonic bath at 38.5 KHz for 5min and vortexed for 30s. The suspension was diluted and cultured on CLED agar and the CFUs were counted after 18h of incubation at 37°C (Kadurugamuwa *et al*., 2003).

6.2.6 Microbial challenge test

To test the anti-biofilm efficacy of CPC coated catheters on repeated bacterial challenge (fresh inoculum every 24h), anti-biofilm assays were performed as described above (6.2.5.2). However, after the incubation period, the catheter pieces were washed once with PBS and placed in a new 24-well plate with a fresh inoculum of bacteria (~10^6) in AUM, and incubated for a further 24h at 37°C in an incubator. After the second incubation period, bacterial biofilm was enumerated on CLED agar plates as described above (6.2.5.2). The bacterial challenge test is continued until the drug coated catheters allow formation of biofilms.

6.2.7 CPC elution assays

To determine if CPC is released from the coated catheters over a 24h period, anti-biofilm assays were performed as described above (6.2.5.2). However,

after the incubation period, the planktonic suspension in each well was diluted and cultured on CLED agar and the CFUs were counted after 18h of incubation at 37°C.

6.2.8 HPLC analysis

To determine the rate of drug elution from CPC coated catheters over a 24h period, High performance liquid chromatography (HPLC) analysis was performed. HPLC long gradient technique was used.

Uncoated and coated catheters with and without CPC were placed in 1ml of ds water and incubated for 24h at 37°C. After the incubation period, the catheter pieces were removed and the ds water suspensions were tested for the presence of CPC. The samples were analysed on a HP1050 HPLC system equipped with an autosampler, a quaternary pump and a Diode-Array detector. A Zorbax SB C-18 2.1mm x 10cm column was employed. The flow rate was 0.2mL/min and the eluents were monitored at wavelengths between 210-280nm. A linear gradient of mobile phase B (acetonitrile containing 0.1% trifluoroacetic acid) over mobile phase A (0.1% trifluoroacetic acid in water) from 0-60% B in 60 minutes was performed, followed by column re-equilibration step. Data were collected and analysed using ChemStation software.

6.2.9 Further drug release analysis

Further drug release studies were employed to support the results obtained from HPLC analysis. Coated catheters with and without CPC were placed in 1ml of ds water and incubated for 24h at 37°C in an incubator. After the

incubation period, the drug eluted catheter pieces and the ds water suspensions were separated.

Drug eluted catheter pieces (from above) were placed in AUM containing approximately 10^6 bacteria per well in their log or exponential growth phase and bacterial biofilms were grown and enumerated as described in section 6.2.5.2. Non-eluted CPC (3mg/ml) catheter piece was used as control.

Bacteria (~10^6 bacteria per well in their log or exponential growth phase) were added to the ds water suspensions (eluant from above) in a 24-well plate and the plates were incubated for 24h at 37°C in an incubator. After the incubation period, the suspension in each well was diluted and cultured on CLED agar and the CFUs were counted after 18h of incubation at 37°C. Uropathogens grown in fresh ds water were used as control.

6.2.10 Software and statistics

HPLC chromatograms were created suing ChemStation software. Microsoft Excel (v15.30) was used for general data analysis and preparation of charts. Paired two-tailed *t* test was used for statistical analysis.

6.3 Results

6.3.1 Antimicrobial assays

6.3.1.1 Planktonic assays

Antimicrobial planktonic assays were performed to determine the antimicrobial effect of CPC on uropathogens. Five different concentrations of CPC were tested for their antimicrobial efficacy over a period of 24h. Paired two-tailed *t* tests were used to analyse the relationship between the control (bacterial growth AUM only) and experimental groups (bacterial growth in AUM with various concentrations of CPC).

From the graph below (Figure 6.1) we can see that CPC has displayed potent antimicrobial effects at very low concentrations. At a concentration of 1µg/ml, CPC has significantly ($p \leq 0.0001$) reduced planktonic bacterial growth to less than 60% whereas a further 50% decrease ($p \leq 0.001$) was seen at a concentration of 2µg/ml. Although there was a very low percentage of planktonic bacterial growth (0.0001 to 0.0002%), CPC (3µg/ml) can be considered as the optimal bactericidal concentration ($p \leq 0.01$). At 4 & 5µg/ml, CPC has completely killed all uropathogens (Figure 6.1). In the presence of 1µg/ml of CPC, the ESBL positive strains of *E. coli* (52%) and *K. pneumoniae* (36%) have a higher percentage of survival compared with their ESBL negative counterparts (45% and 35% respectively). However, this difference is not significant. Amongst the tested uropathogens, in the presence of 1µg/ml of CPC, *P. mirabilis* (58%) had the highest survival rate followed by *E. coli* ($ESBL^-$ 45% & $ESBL^+$ 52%) and *K. pneumoniae* ($ESBL^-$ 35% & $ESBL^+$ 36%).

In order to determine the time taken by CPC (3µg/ml) to kill uropathogens, 12 and 24h antimicrobial assays were performed. As evident in Figure 6.2, after 12h of incubation with CPC (3µg/ml) all uropathogens have exhibited survival rates albeit to a very low percentage. The bactericidal effect of CPC (3µg/ml) after 12h of incubation is significant at $p \leq 0.001$.

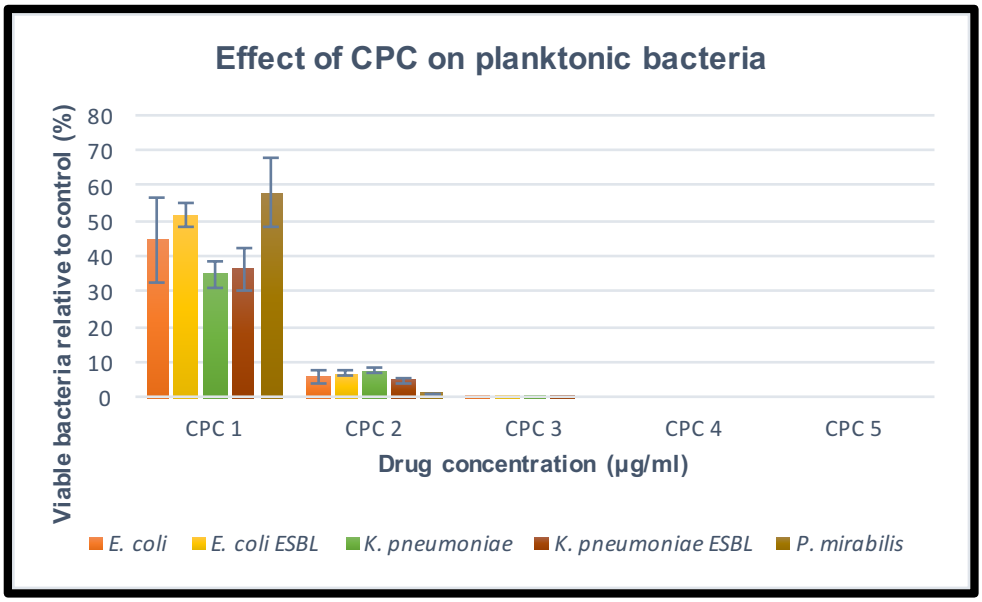

Figure 6.1: Bactericidal effect of CPC on uropathogens (planktonic growth).

Viable bacteria were enumerated after 24h of incubation at 37°C. Percentages of viable bacteria relative to control (planktonic growth in AUM only) are shown. CPC 1 - 1µg/ml; CPC 2 - 2µg/ml; CPC 3 - 3µg/ml; CPC 4 - 4µg/ml; CPC 5 - 5µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. CPC 1 and CPC 2 significant at p≤0.0001 and p≤0.01 respectively (paired two-tailed *t* test).

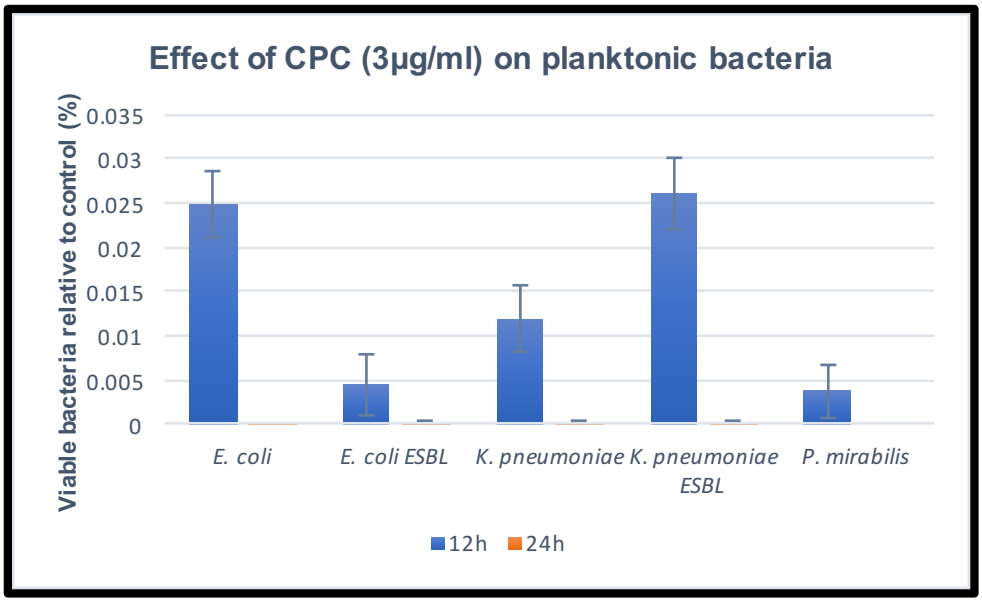

Figure 6.2: Bactericidal effect of CPC (3µg/ml) on uropathogens (planktonic growth).

Viable bacteria were enumerated after 12h & 24h of incubation at 37°C. Percentages of viable bacteria relative to control (planktonic growth in AUM only) are shown. Results are obtained from at least three independent experiments. Error bars represent standard error. 12h and 24h significant at p≤0.001 and p≤0.01 respectively (paired two-tailed *t* test).

6.3.1.2 Anti-biofilm assays

Anti-biofilm assays were performed to determine the antimicrobial effect of CPC on uropathogenic biofilm formation. Uropathogens were inoculated in AUM with CPC and allowed to form biofilms for 24h. Three different concentrations of CPC were tested for their anti-biofilm efficacy over a period of 24h. Comparisons between the control and experimental groups were made using two-tailed paired T-tests.

Figure 6.3 presents the experimental data on biofilm viability. As evident, in all uropathogenic biofilms, the percentage of viable bacteria scraped from the biofilms was very low in the presence of CPC (3µg/ml). The highest percentage of viable bacteria was scraped from *K. pneumoniae* ESBL positive biofilm (~3%) and the lowest from *E. coli* ESBL positive biofilm (~0.004%). The anti-biofilm effect of CPC (3µg/ml) after 24h of incubation was significant at $p \leq 0.001$. At a concentration of 4µg/ml and 5µg/ml, CPC has completely eradicated uropathogenic biofilms over a 24h period.

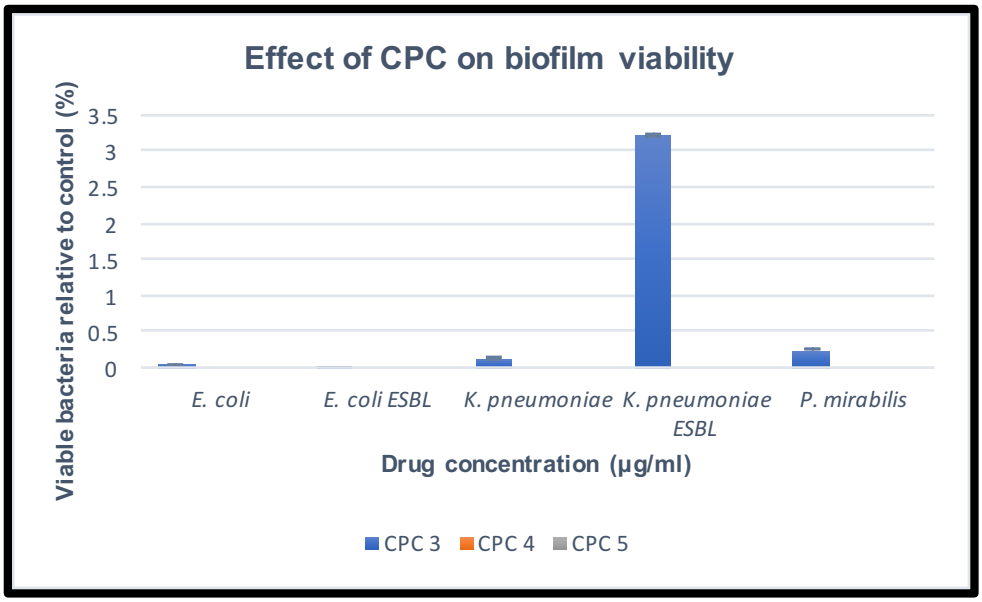

Figure 6.3: Anti-biofilm effect of CPC on 24h uropathogenic biofilms.

Percentages of viable bacteria scraped from the biofilms relative to control (biofilms formed in AUM only) are shown. CPC 3 - 3µg/ml; CPC 4 - 4µg/ml; CPC 5 - 5µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. CPC 3 significant at $p \leq 0.0001$ (paired two-tailed *t* test).

6.3.2 Cytotoxicity assays

Trypan blue viability assays were performed to determine the cytotoxic effect of CPC on *A. castellanii* (T4) over a 24h period. The cytotoxicity was measured based on differential staining; dead cells take up the trypan blue stain. As evident in Figure 6.4, CPC has induced very low levels of cytotoxicity on amoebae. The data reveal that cytotoxicity is dose dependent. Although the percentage of cytotoxicity is low for all tested concentrations of CPC, these results are statistically not significant. It should also be noted that higher concentrations of CPC (>15µg/ml) caused the amoebae to dettach from the surface and turn to cysts.

LDH assays were performed to determine the percentage of necrosis (primary and secondary) induced on TERT-NHUC line by CPC. Necrotic effect of various concentrations of CPC on urothelial cells was indirectly measured based on the amount of LDH released from cells. Figure 6.5 presents the necrotic effect of CPC on TERT-NHUC line. As evident in the figure, there is a positive correlation between cytotoxicity and CPC concentration. The minimum bactericidal concentration (3µg/ml) of CPC has induced 25% cytotoxicity on TERT-NHUC line. This result is significant at $p \leq 0.01$ level (paired two-tailed *t* test). The higher concentrations (5 & 10µg/ml) of CPC have also induced significant cytotoxicity ($p < 0.01$).

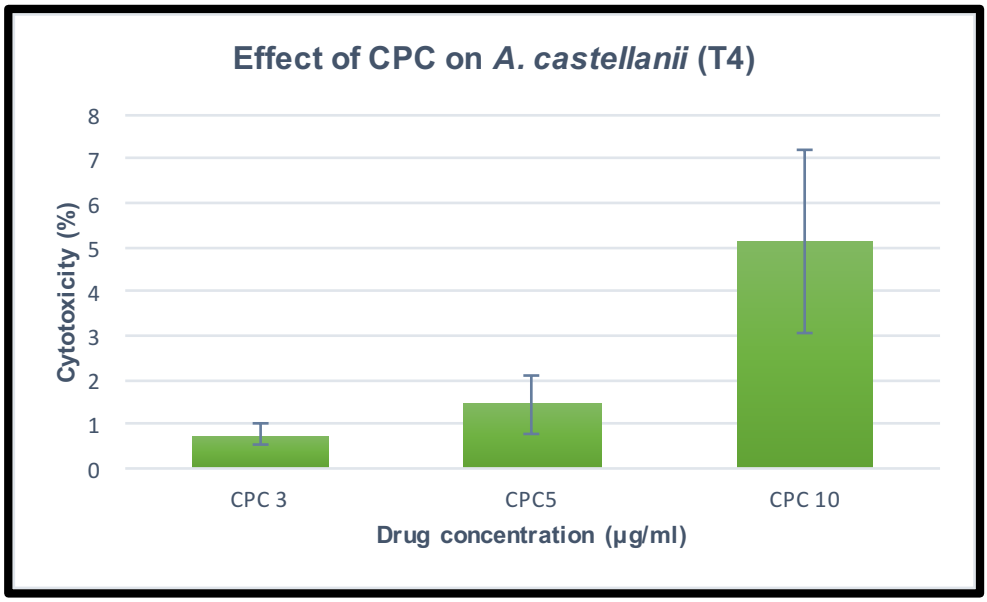

Figure 6.4: CPC induced cytotoxicity on *A. castellanii* (T4).

Amoebae were incubated with CPC for 24h and cytotoxicity was assayed using trypan blue. Percentage cytotoxicity relative to control cells are shown. CPC 3 - 3µg/ml; CPC 5 - 5µg/ml; CPC 10 - 10µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error.

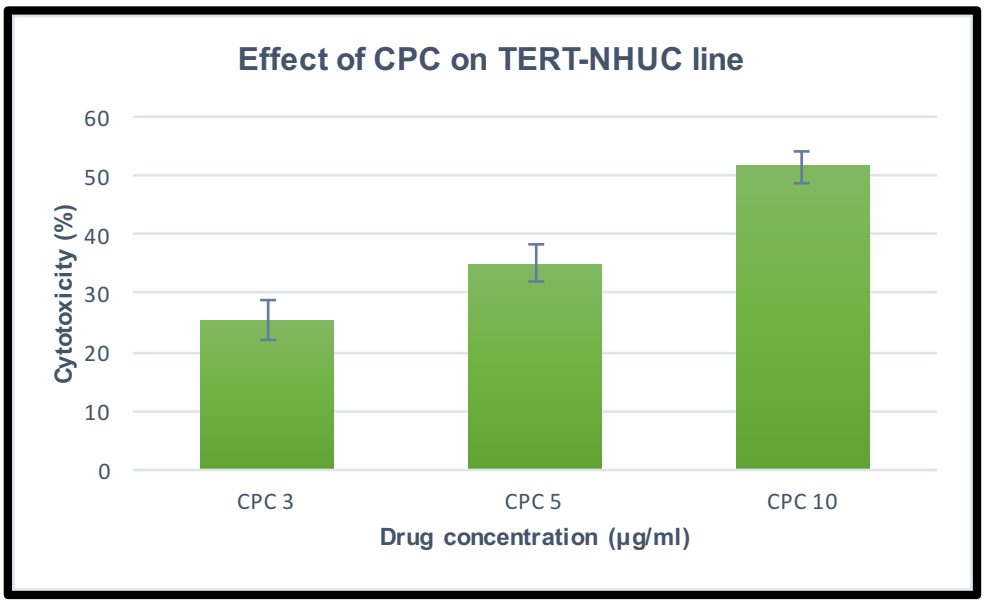

Figure 6.5: CPC induced urothelial cell cytotoxicity.

TERT-NHUC line were incubated with CPC for 24h and the cells were then assayed using standard protocol for LDH cytotoxicity detection kit. Percentage cytotoxicity relative to control cells are shown. CPC 3 - 3µg/ml; CPC 5 - 5µg/ml; CPC 10 - 10µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. CPC3, CPC 5 & 10 significant at $p \leq 0.01$ (paired two-tailed *t* test).

6.3.3 Antimicrobial intracellular survival assays

6.3.3.1 Uropathogens in *A. castellanii* (T4)

Amoeba survival assays were performed to determine the ability of CPC to kill intracellular bacteria. The bacteria survived within *A. castellanii* (T4) were calculated as follows: number of viable bacteria recovered (CFU/ml) / number of bacteria in initial inoculum x 100 (Alsam *et al*., 2006; Yousuf *et al*., 2013). Figure 6.6 presents the intercorrelations among the three different concentrations of CPC tested against the survival of uropathogens in *A. castellanii* (T4). Comparisons between the groups were made using paired two-tailed *t* tests. As evident in the figure, ESBL positive and negative strains of *E. coli* have displayed very low levels of survival compared with other uropathogens. The single most striking observation to emerge from the data comparison is that in the presence of CPC (3µg/ml), all uropathogens have displayed an increase in their ability to survive within *A. castellanii* (T4). The highest increase is seen in *E. coli* ESBL negative (0.01 to 0.1%) survival followed by ESBL negative and positive *K. pneumoniae* (0.6 to 1.7% and 1 to 1.7% respectively), ESBL positive *E. coli* (0.0009 to 0.001%) and *P. mirabilis* (16.05 to 16.68%). These results are significant at $p \leq 0.01$ level. CPC (5µg/ml) has significantly ($p \leq 0.01$) reduced the survival ability of all uropathogens except for ESBL positive strain of *K. pneumoniae* (0.6 to 1.08%) whereas CPC (10µg/ml) has reduced the survival ability of all uropathogens.

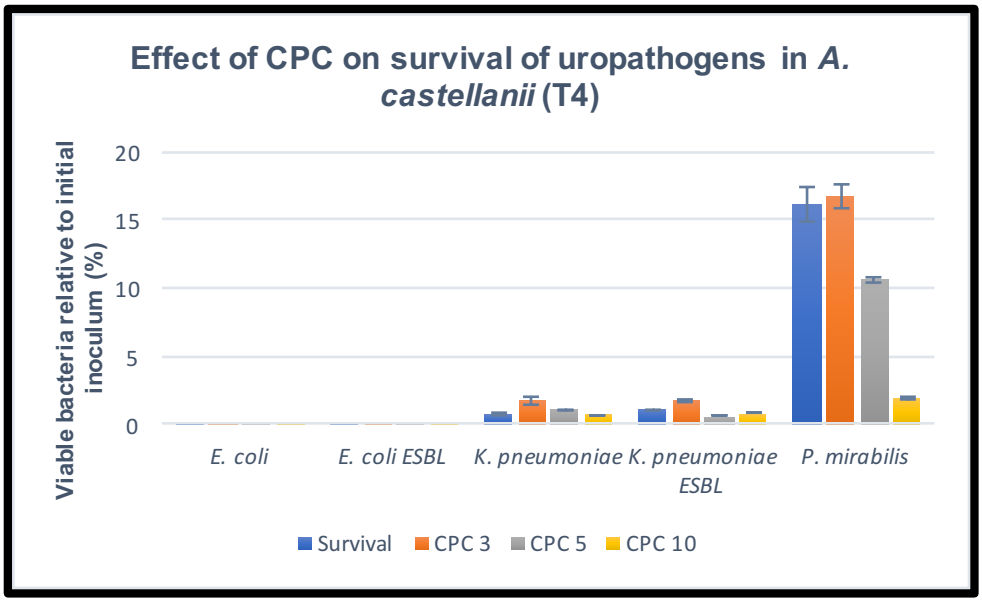

Figure 6.6: Effect of various concentrations of CPC on intracellular survival of uropathogens in *A. castellanii* (T4).

The percentages of bacteria relative to the initial inoculum are shown. Blue bars represent intracellular survival of uropathogens in the absence of CPC (control). CPC 3 - 3µg/ml; CPC 4 - 4µg/ml; CPC 5 - 5µg/ml; CPC 10 - 10µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. CPC 3 & 5 significant at $p \leq 0.01$ (paired two-tailed t test).

6.3.3.2 Uropathogens in TERT-NHUC line

TERT-NHUC survival assays were performed to determine the ability of CPC to kill intracellular bacteria. The bacteria which survived within urothelial cells were calculated as follows: number of viable bacteria recovered (CFU/ml) / number of bacteria in initial inoculum x 100 (Alsam *et al*., 2006; Yousuf *et al*., 2013). Figure 6.7 shows the comparisons between the various concentrations of CPC tested against the survival of uropathogens in TERT-NHUC line. It can be seen from the data that the survival ability of all uropathogens except for *E. coli* ESBL negative is reduced in the presence of CPC. In contrast with other uropathogens, *E. coli* ESBL negative seems to have acquired an increased ability to survive within TERT-NHUC line in the presence of 3 and 5μg/ml CPC. However, 10μg/ml CPC has significantly ($p \leq 0.01$) reduced its ability to survive intracellularly. For the other uropathogens, the decrease in survival is dose dependent with significant reductions in survival seen in the presence of all concentrations of CPC (3 and 5μg/ml significant at $p \leq 0.001$ level, paired two-tailed *t* test) (Figure 6.7).

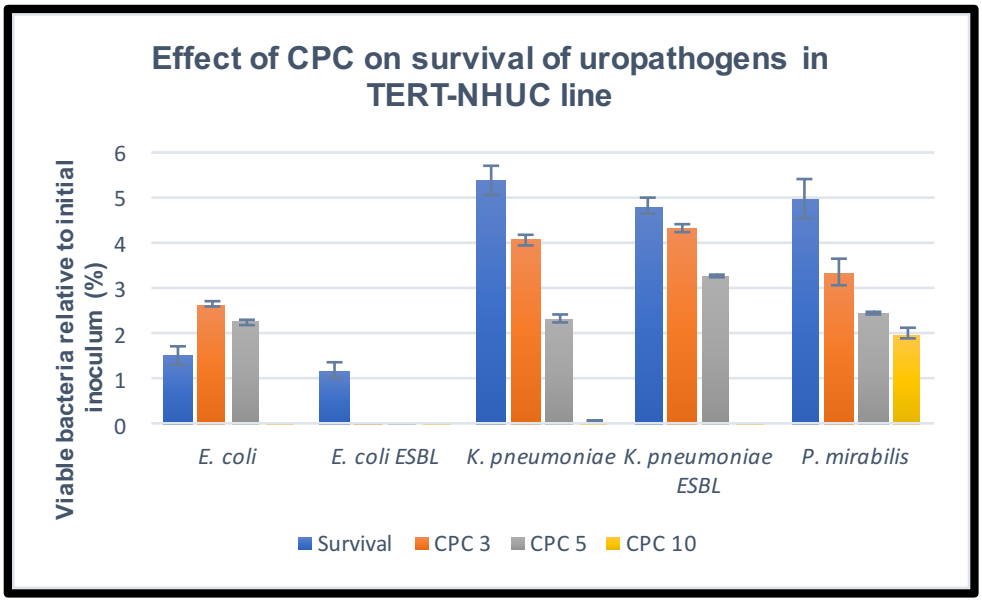

Figure 6.7: Effect of various concentrations of CPC on intracellular survival of uropathogens in TERT-NHUC line.

The percentages of bacteria relative to the initial inoculum are shown. Blue bars represent intracellular survival of uropathogens in the absence of CPC (control). CPC 3 - 3µg/ml; CPC 4 - 4µg/ml; CPC 5 - 5µg/ml; CPC 10 - 10µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. CPC 3 & 5 significant at $p \leq 0.001$ and CPC 10 significant at $p \leq 0.01$ (paired two-tailed *t* test).

6.3.4 Catheter impregnation assays

Although the minimal bactericidal concentration of CPC is 3µg/ml (Figure 6.1), the catheter coating solutions were prepared with various concentrations of CPC in the mg/ml range to promote maximum drug adsorption.

6.3.4.1 PTFE coated latex catheters

To assess the anti-biofilm effect of CPC coated PTFE latex catheters, uropathogens were allowed to form biofilms on catheter pieces for 24h. Uncoated catheters and coated catheters without CPC were used as control. Simple statistical analysis (two-tailed, paired *t* test) was used to compare the control and experimental groups.

As shown in Figure 6.8, all uropathogens were able to form biofilms on catheters coated with CPC (1 & 2mg/ml) albeit at varying levels. For CPC 1mg/ml coated catheters, the lowest percentage of biofilm formation was observed for *E. coli* ESBL negative (0.93%) followed by ESBL negative strain of *K. pneumoniae* (12.4%), ESBL positive strains of *K. pneumoniae* (13.2%) and *E. coli* (16.8%), and *P. mirabilis* (41%). The difference between the control groups and CPC 1mg/ml catheters was significant at $p \leq 0.05$ level. Biofilm formation on CPC 2mg/ml coated catheters is significantly ($p \leq 0.05$) reduced when compared with CPC 1mg/ml catheters for all uropathogens except *K. pneumoniae* ESBL positive. The latter strain has displayed less than 1% difference in their ability to form biofilms on CPC (1 & 2mg/ml) coated catheters. In comparison with the control group, these results are also significant at $p \leq 0.05$ level. Although the percentage of reduction in biofilms on catheters

coated with 3mg/ml CPC (p=0.05) is approximately similar (~>99%) for all uropathogens, the highest reduction is seen in the biofilm formed by ESBL negative strain of *E. coli* whereas the lowest in ESBL positive strain of *K. pneumoniae*. Catheters coated with CPC 4mg/ml (p≤0.05) has completely prevented the formation of biofilms (Figure 6.8).

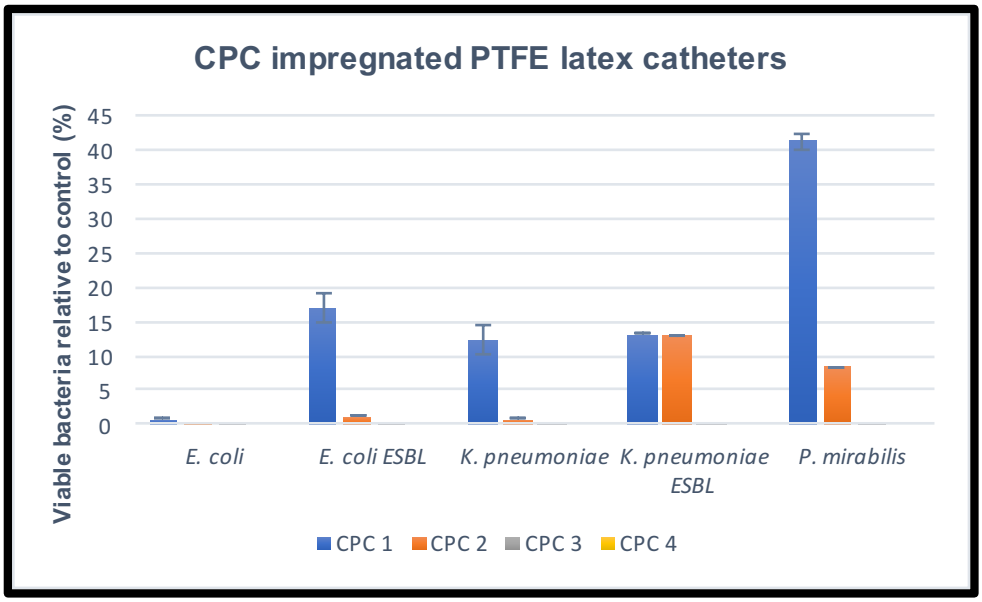

Figure 6.8: Biofilm formation (24h) on coated (with and without CPC) PTFE latex catheters.

Percentages of viable bacteria relative to control (coated catheter without CPC) are shown. CPC 1 – 1mg/ml; CPC 2 – 2mg/ml; CPC 3 – 3mg/ml; CPC 4 – 4mg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at $p \leq 0.05$ (paired two-tailed *t* test).

6.3.4.2 Silicone catheters

To assess the anti-biofilm effect of CPC coated silicone catheters, uropathogens were allowed to form biofilms on catheter pieces for 24h. Uncoated catheters and coated catheters without CPC were used as control. Simple statistical analysis (two-tailed, paired *t* test) was used to compare the control and experimental groups.

As shown in Figure 6.9, all uropathogens were able to form biofilms on catheters coated with 1mg/ml CPC albeit at varying levels. The lowest percentage of biofilm formation is observed for *K. pneumoniae* ESBL positive (0.04%) followed by ESBL positive and negative strain of *E. coli* (0.72% & 1.61% respectively), *P. mirabilis* (2.89%) and ESBL negative strain of *K. pneumoniae* (3.74%). The difference between the control groups and CPC 1mg/ml catheters is significant at p=0.05 level. Biofilm formation on CPC 2mg/ml coated catheters is significantly ($p \leq 0.05$) reduced when compared with CPC 1mg/ml catheters for all uropathogens. The highest reduction is seen in ESBL negative strain of *K. pneumoniae* and *P. mirabilis*. On the other hand, no biofilm was formed for ESBL positive strain of *K. pneumoniae*. In comparison with the control group, these results are also significant at p=0.05 level. CPC 3mg/ml catheters have significantly ($p \leq 0.05$) reduced the ability of all uropathogens to form biofilms. ESBL positive strains of *E. coli* and *K. pneumoniae* were not able to form any biofilm on CPC 3mg/ml coated catheters whereas their ESBL negative counterparts and *P. mirabilis* were able to form biofilms albeit at very low levels (<0.05%). Catheters coated with CPC 4mg/ml ($p \leq 0.05$) has completely prevented the formation of biofilms.

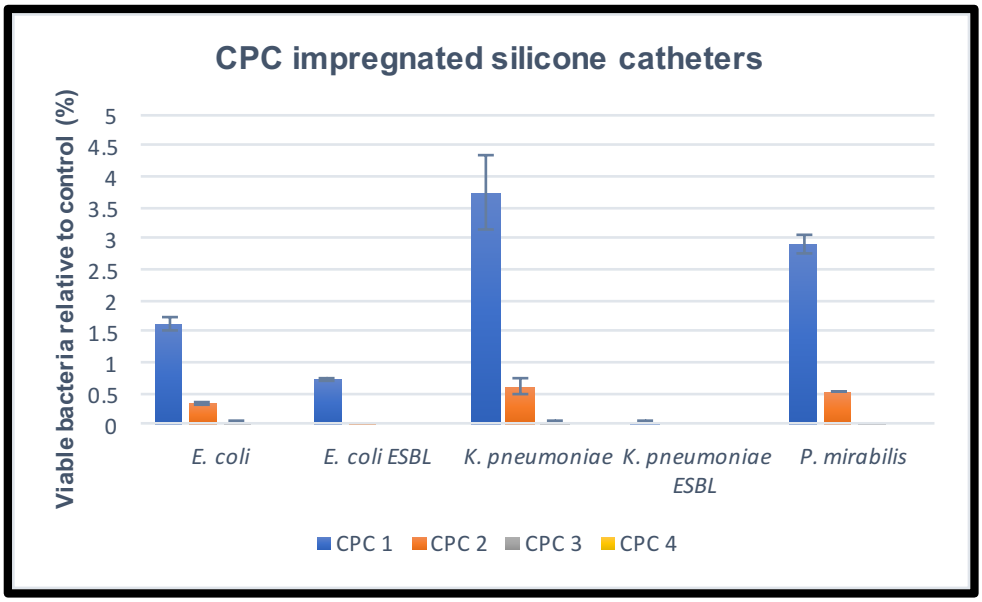

Figure 6.9: Biofilm formation (24h) on coated (with and without CPC) silicone catheters.

Percentages of viable bacteria relative to control (coated catheter without CPC) are shown. CPC 1 – 1mg/ml; CPC 2 – 2mg/ml; CPC 3 – 3mg/ml; CPC 4 – 4mg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at $p \leq 0.05$ (paired two-tailed *t* test).

6.3.4.3 Comparison of CPC impregnated PTFE latex and silicone catheters

Figure 6.10 shows the comparison of biofilm formation on 3mg/ml CPC coated PTFE latex and silicone catheters. As evident in the figure, CPC coated silicone catheters are more efficient in reducing biofilm formation than PTFE latex catheters for ESBL positive strains of *E. coli* and *K. pneumoniae*, and *P. mirabilis*. ESBL positive strains of *E. coli* and *K. pneumoniae* have displayed an inability to form biofilms on 3mg/ml CPC coated silicone catheters. Figure 6.11 shows the percentage change in biofilm formation between the two types of catheters. In comparison with PTFE latex catheters, the highest percentage reduction in biofilm formation on silicone catheters is seen for ESBL positive *K. pneumoniae* (-19.2%) followed by *P. mirabilis* (-11.8%) and ESBL positive *E. coli* (-0.29%). On the other hand, an increase in biofilm formation is seen for ESBL negative *K. pneumoniae* (+4.89%) and *E. coli* (+3.4%). The comparison suggests that the biofilm forming ability of ESBL positive strains is reduced on 3mg/ml CPC coated silicone catheters when compared with PTFE latex catheters. Although these results are very interesting, they are not statistically significant. Overall, when coated with CPC (3mg/ml), it is evident that both types of catheters are efficient in reducing biofilm formation (<99% in comparison with coated catheters without CPC). CPC (3mg/ml) coated PTFE latex catheters are used for all other analysis in this chapter since latex catheters are cheaper compared with silicone catheters and since 3mg/ml is the minimum catheter coating concentration required to prevent biofilm formation.

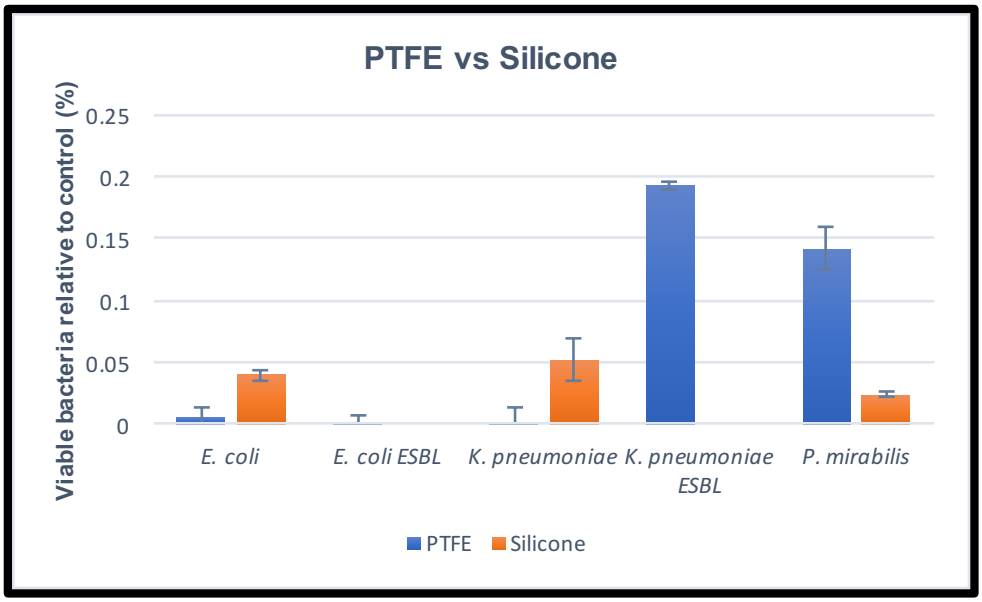

Figure 6.10: Comparison of uropathogenic biofilm formation on 3mg/ml CPC coated PTFE latex and silicone catheters.

Percentages of viable bacteria relative to control (coated PTFE latex and silicone catheters without CPC) are shown. Results are obtained from at least three independent experiments. Error bars represent standard error.

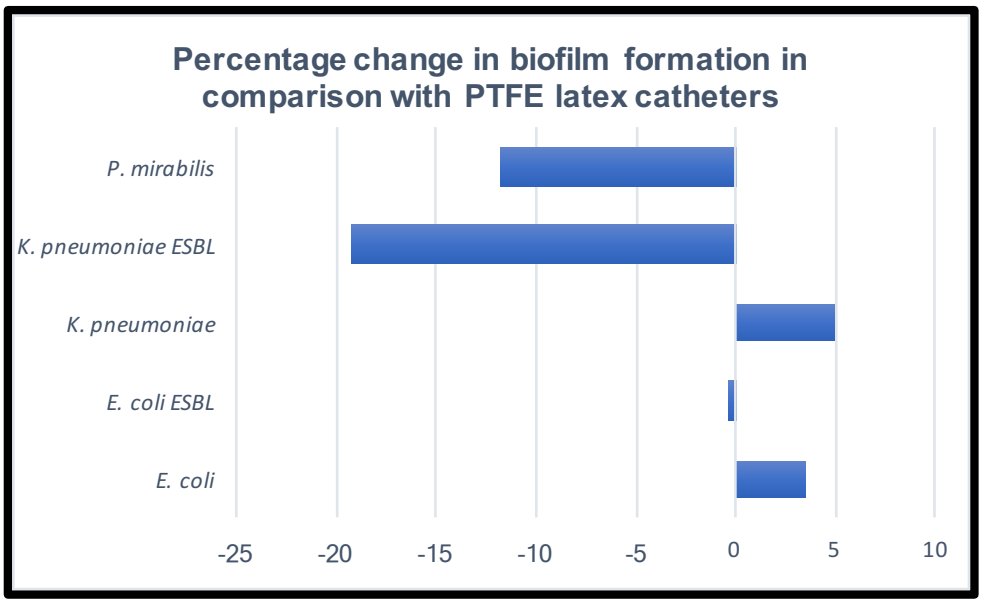

Figure 6.11: Percentage change (increase or decrease) in uropathogenic biofilm formation on 3mg/ml CPC coated silicone catheters.

The percentage difference was calculated in comparison with 3mg/ml CPC coated PTFE latex catheters.

6.3.5 Bacterial challenge test

To assess the anti-biofilm efficacy of CPC (3mg/ml) impregnated catheters on repeated bacterial challenge, CPC catheters were inoculated with bacteria for 24h and were then subjected to a fresh bacterial inoculum for a further 24h. The bacterial challenge test is continued until the drug coated catheters allow formation of biofilms. However, as evident in Figure 6.12, CPC (3mg/ml) coated catheters were positive for biofilm formation on day 2. This suggests that most of the drug is either used or released into the surrounding environment in the first 24h. None of the uropathogens were able to form biofilms on day 1, however, when subjected to a fresh bacterial inoculum on day 2, ESBL negative strain of *K. pneumoniae* (33.5 x 10^3 CFU/ml) has displayed the highest formation of biofilms followed by *P. mirabilis* (~6 x 10^3 CFU/ml), ESBL positive and negative strains on *E. coli* (2.5 and 2.4 x 10^3 CFU/ml respectively) and ESBL positive strain of *K. pneumoniae* (~1 x 10^3 CFU/ml).

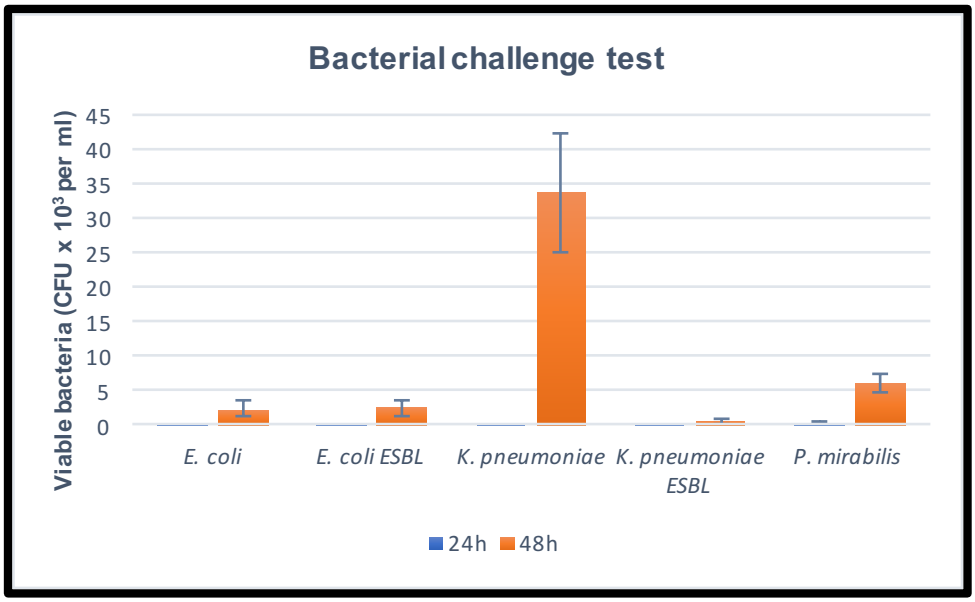

Figure 6.12: Evaluation of the anti-biofilm activity of CPC (3mg/ml) coated catheters via bacterial challenge tests.

CPC catheters were inoculated with bacteria for 24h and were then subjected to a fresh bacterial inoculum for a further 24h. The bacterial challenge test is continued until the drug coated catheters allow formation of biofilms. Results are obtained from at least three independent experiments. Error bars represent standard error.

6.3.6 Drug release from CPC impregnated catheters

From Figure 6.13, it is evident that catheters coated with 3mg/ml CPC were efficient in preventing biofilm formation on day 1, but allowed biofilm formation when subjected to microbial challenge on day 2. This could possibly mean that the CPC coated catheters released most of their drug in the first 24h of incubation in AUM. Planktonic assays were employed to determine if the CPC coated catheters indeed released any drug into their surrounding environment. CPC (1, 2, 3 & 4mg/ml) coated catheters were placed in AUM with uropathogens for 24h and viability of bacteria in the AUM suspension was enumerated. As evident in Figure 6.13, there is a significant ($p \leq 0.01$, paired two-tailed *t* test) decrease in the percentage of viable planktonic bacteria of the experimental groups (CPC coated catheters) compared with the control groups (coated catheters without CPC) suggesting that the CPC coated catheters have released some amount of the drug into their surrounding environment, thereby killing the planktonic bacteria. Although the percentage of reduction in planktonic bacteria for catheters coated with 1mg/ml CPC is approximately similar (~>99%) for all uropathogens, the highest reduction is seen in *P. mirabilis* planktonic bacteria whereas the lowest is seen in the planktonic bacteria of ESBL positive *K. pneumoniae*. Very low numbers (<0.0002%) of planktonic bacteria have survived in the presence of catheters coated with 2mg/ml CPC whereas no planktonic bacteria were able to survive in the presence of 3 & 4mg/ml CPC coated catheters. There is no significant difference between the survival abilities of ESBL positive and negative strains of *E. coli* and *K. pneumoniae* in the presence of CPC coated catheters (1 & 2mg/ml).

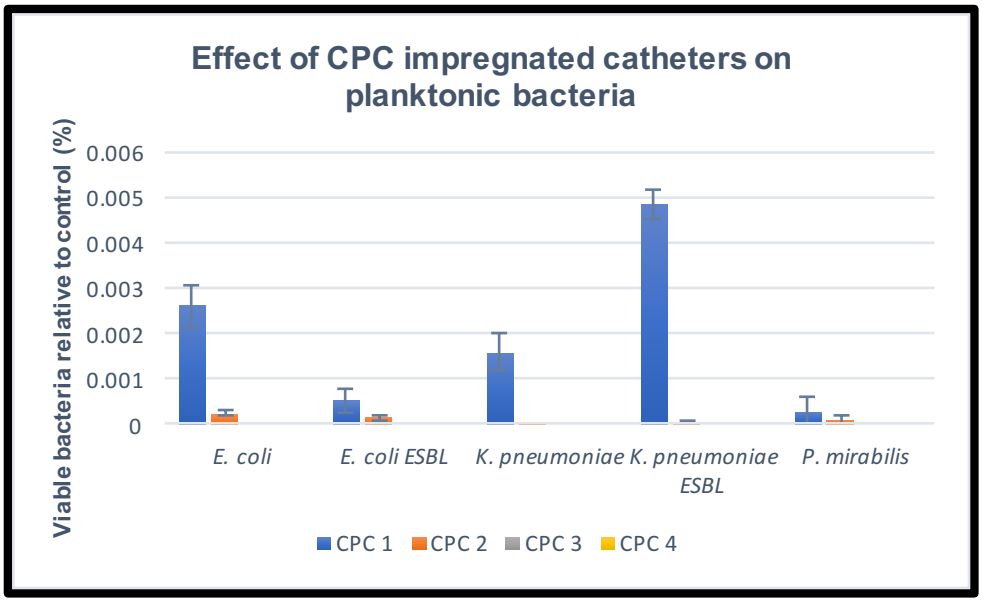

Figure 6.13: Effect of CPC impregnated catheters on planktonic bacteria.

CPC coated catheters were placed in AUM with uropathogens for 24h and viable bacteria from the planktonic suspension were enumerated. Percentage of viable bacteria relative to control (coated catheter without CPC) are shown. CPC 1 - 1µg/ml; CPC 2 - 2µg/ml; CPC 3 - 3µg/ml; CPC 4 - 4µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. CPC 1, CPC 2, CPC 3 & CPC 4 significant at $p \leq 0.001$ (paired two-tailed *t* test).

6.3.7 HPLC analysis

In order to determine the amount of drug released from the CPC (3mg/ml) coated catheters, HPLC analysis was performed. CPC coated catheters were placed in ds water and incubated at 37°C for 24h. After incubation, the ds water was collected and analysed via HPLC. The long gradient method was used and a positive control (1ml ds water spiked with 1mg CPC) was run to check for detection of CPC. As evident in Figure 6.14A, CPC (1mg/ml) has a peak absorbance of about 300 milli absorbance units (mAU) and a retention time of about 61min. From Figure 6.14B, C & D, it is also evident that all the samples (control and experimental groups) yielded a similar profile. A couple of tiny peaks can be seen in the CPC region with a peak height of about 6 mAU, whereas for 1mg/mL (positive control) it is about 300 mAU, so this makes a rough estimate of 2%, or in other words, 20 micrograms/ml. However, since the peaks also appear in the control groups, this detection is not significant and can therefore be considered as almost noise and hence unquantifiable. This could either mean that the catheters did not release any CPC or the amount of CPC released was very low for detection.

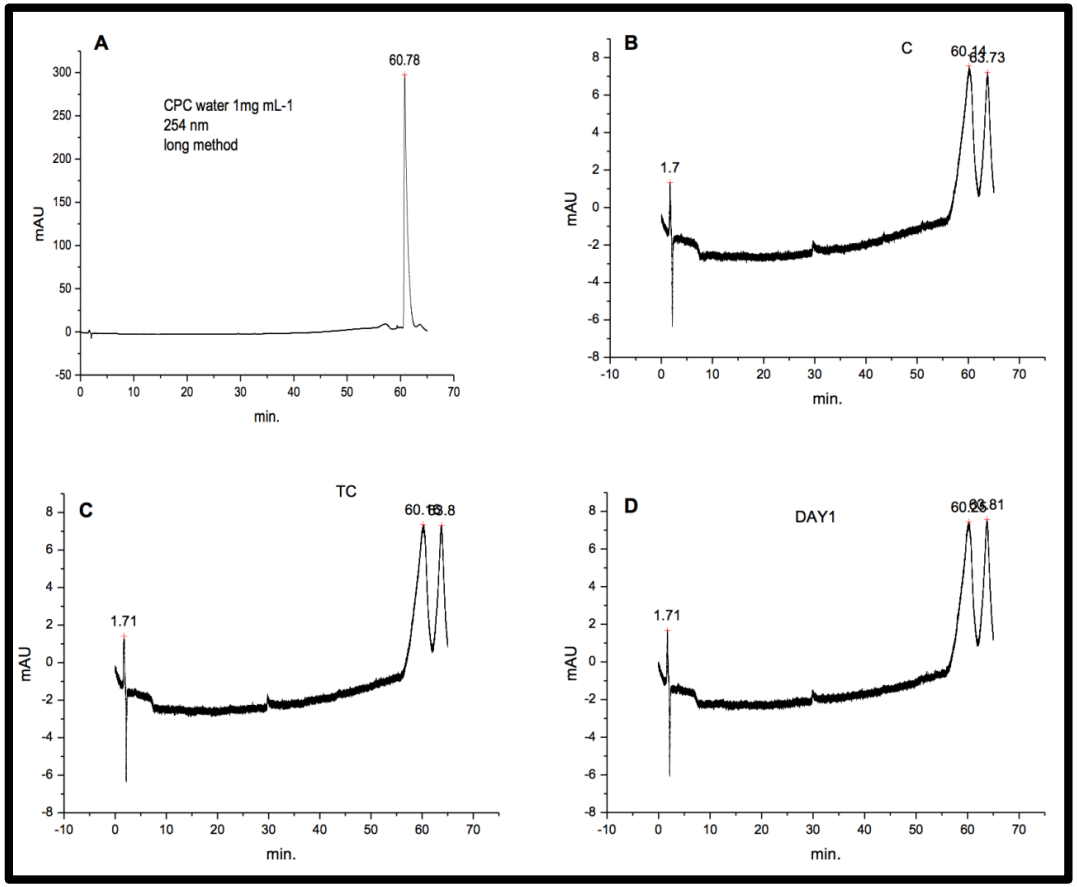

Figure 6.14: HPLC analysis of CPC (3mg/ml) coated catheters. Uncoated and coated catheters without CPC were used as control.

The long gradient method was used and a positive control (1ml ds water spiked with 1mg CPC) was run to check for detection of CPC. A – Positive control (CPC 1mg/ml); B – Uncoated catheter; C – Coated catheter without CPC; D – CPC (3mg/ml) coated catheters; mAU – milli absorbance units. HPLC chromatograms are representative of at least three independent experiments.

6.3.8 Further drug release analysis

In order to explore the experimental evidence from HPLC analysis, further drug release analysis was performed.

From Table 6.1, it is evident that CPC (3mg/ml) coated catheters released some amount of drug, sufficient to kill (100%) planktonic bacteria. However, HPLC analysis (Figure 6.14) did not detect any CPC released from coated catheters. Therefore, to further confirm that the drug coated catheters indeed released some amount of drug (undetectable on HPLC), CPC (3mg/ml) coated catheters were incubated in ds water for 24h at 37°C. After the incubation, uropathogens were inoculated in the ds water for 24h at 37°C. Uropathogens in fresh ds water were used as control. As evident in Table 6.1, no bacterial growth was observed in the experimental group (ds water incubated with CPC coated catheters). This further confirms that CPC (3mg/ml) coated catheters do release CPC into their surrounding environment. These results are significant at $p \leq 0.01$ (paired two-tailed *t* test). It is reasonable to assume that the amount of drug released is at least 3µg/ml since that is the minimum concentration required to kill more than 99% of bacteria (Figure 6.1).

A second analysis was performed to determine if the drug eluted catheter from the above analysis still had the potential to prevent biofilm formation. After the 24h incubation of CPC (3mg/ml) coated catheters in ds water, the catheter pieces were placed in AUM and inoculated with uropathogens for 24h at 37°C. Non-eluted CPC (3mg/ml) coated catheters were used as control. From Figure 6.15, it is evident that drug eluted catheters have lost the ability to prevent

biofilm formation when compared with the control groups. This further confirms that the CPC coated catheters do release their drug into the surrounding environment. These results are significant at $p \leq 0.05$ (paired two-tailed *t* test).

Finally, data from Figure 6.16 reveals that, although the drug eluted catheter failed to prevent biofilm formation, the percentage of biofilm formation is still significantly ($p \leq 0.05$, paired two-tailed *t* test) lower that the control (without drug elution) catheters. This suggests that in the first 24h, most of the drug is released from the catheters into the surrounding environment. However, there is still some amount of drug adsorbed in the catheter which is still reducing but not preventing biofilm formation.

Table 6.1: Effect of eluted drug on 24h bacterial growth.

CPC was eluted from coated catheters (3mg/ml) in ds water and the ds water was used as the growth medium for bacteria. Percentage of viable bacteria relative to control (bacterial growth in fresh ds water) is shown. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at p≤0.01 (paired two-tailed t test).

Uropathogens	Percentage of bacteria relative to control (%)
E. coli (ESBL negative)	0
E. coli (ESBL positive)	0
K. pneumoniae (ESBL negative)	0
K. pneumoniae (ESBL positive)	0
P. mirabilis	0

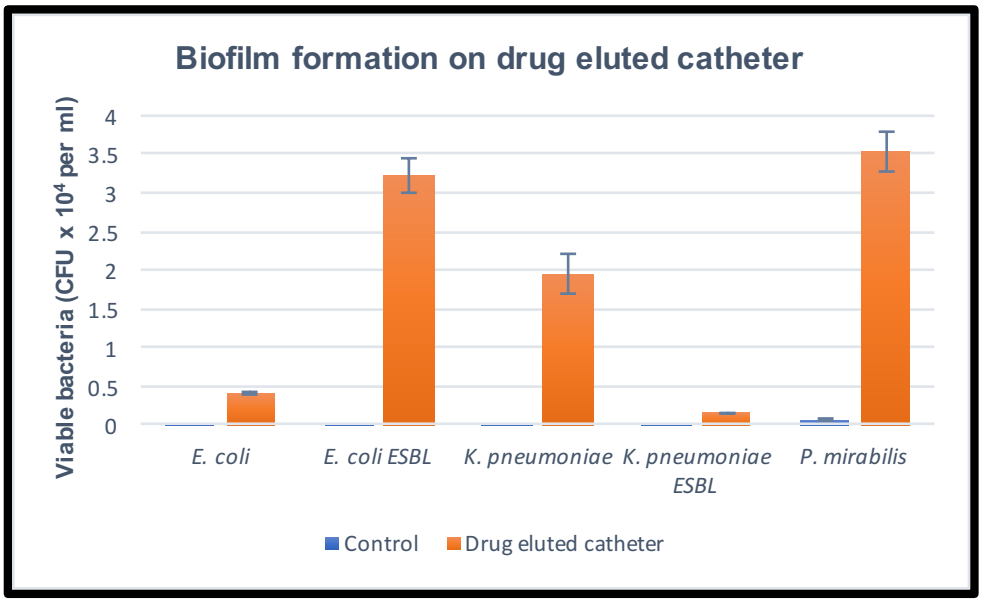

Figure 6.15: Biofilm formation on drug eluted catheter.

CPC was eluted from coated catheters (3mg/ml) in ds water and the drug eluted catheter was used to test uropathogenic biofilm formation over 24h. CPC (3mg/ml) coated catheters without drug elution were used as control. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at p≤0.05 (paired two-tailed *t* test).

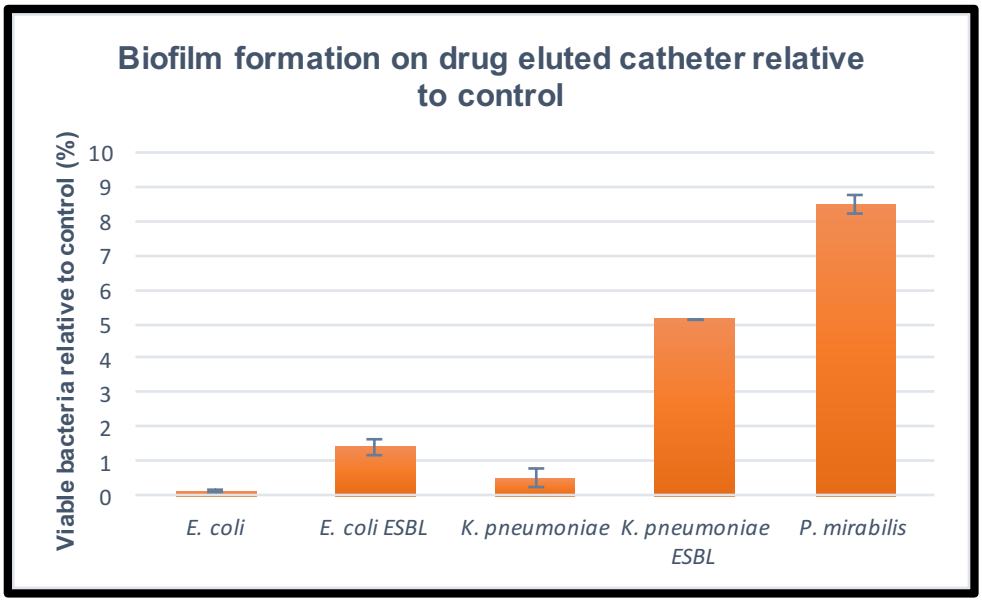

Figure 6.16: Biofilm formation on drug eluted catheter vs non-eluted catheter.

Percentages of viable bacteria relative to control (non-eluted 3mg/ml CPC coated catheter) are shown. CPC was eluted from coated catheters (3mg/ml) in ds water and the drug eluted catheter was used to test uropathogenic biofilm formation over 24h. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at $p \leq 0.05$ (paired two-tailed *t* test).

6.4 Discussion

This study set out with the aim of assessing the antimicrobial and anti-biofilm ability of CPC and its potential use in treating UTIs.

The first part of the chapter looked at the antimicrobial potential of CPC against uropathogenic planktonic and biofilm bacteria. CPC is a well-known antibacterial agent. Masadeh *et al*. (2013) have shown that CPC (0.05%) has potent antimicrobial activity against *E. coli* and *P. mirabilis*. Using the agar dilution and broth dilution method, Wu *et al*. (2015) found that zoonotic isolates of *E. coli* and *K. pneumoniae* were susceptible to CPC (0.1 to 1mg/ml). Although several other studies (Cutter *et al*., 2000; Ransom *et al*., 2003; Bosilevac *et al*., 2004; Lim and Mustapha, 2004; Baird *et al*., 2006) have investigated the antimicrobial effect of CPC on *E. coli*, all of them are related to the poultry and beef industry. In reviewing the literature, no data were found on the effect of CPC against uropathogens associated with UTIs. This study investigated the effect of CPC on uropathogenic bacteria and the results (Figure 6.1 and Figure 6.2) indicate that, at a concentration of 3µg/ml, CPC is bactericidal to all tested uropathogens over a period of 24h. The bactericidal effect of CPC is sufficiently understood. Electrostatic interactions between the positively charged hydrophilic quaternary ammonium group and negatively charged bacterial plasma membrane leads to cell lysis and bacterial death (Sandt *et al*., 2007).

Several studies have also investigated the anti-biofilm activity of CPC. Rao *et al*. (2011) have demonstrated the disruptive effect of 0.075% CPC-containing

mouthwash solution on multi-species oral biofilms. Nance *et al*. (2013) have demonstrated the dose dependent (0.5% to 0.001%) anti-biofilm activity of CPC against oral multi-species biofilms grown in human saliva. Latimer *et al*. (2015) have also shown that 0.075% CPC mouth rinses have significantly reduced the viability of oral biofilms. Apart from demonstrating the strong anti-biofilm activity of CPC against *S. mutans* biofilms, Pandit *et al.* (2015) have also shown that the anti-biofilm activity is dependent on the biofilm stage, CPC concentration and exposure time. All of the above studies, however, are related to oral biofilms. The current study found that at a concentration of 4µg/ml, CPC is a potent anti-biofilm agent (24h biofilms). It has previously been shown that CPC binds strongly with *S. mutans* biofilms (Marcotte *et al*., 2004). In a study conducted on the role of ammonium groups in the diffusion of CPC into *S. mutans* biofilms, Sandt *et al*. (2007) have concluded that the association of CPC with biofilms is irreversible and the strong CPC-biofilm binding ability is not only due to the interactions between the quaternary ammonium groups and EPS matrices but more importantly due to possible hydrophobic interactions determined by the length of the alkyl chains.

This study also confirms that planktonic bacteria are more susceptible to antimicrobial agents than biofilm bacteria (Figure 6.1 and Figure 6.3). This finding is in agreement with previous studies (Nickel *et al*., 1985; Van der Mei *et al*., 2006; Masadeh *et al*., 2013). A variety of factors can affect the anti-biofilm ability of antimicrobial agents but all of them relate to the protective role of biofilm EPS. EPS limits the penetration of drugs due to various factors such as size exclusion, interactions with components in the biofilm, differential

adsorption abilities, hydrophobic and electrostatic interactions, and enzyme neutralisation (He, 2014). Biofilm resistance can also be caused due to phenotype changes in biofilm bacteria (Tyerman *et al*., 2013; McCarthy *et al*., 2015).

The next part of the current study looked at the cytotoxic effect of CPC. Several studies have investigated the activity of biocides against free living amoeba. However, none have investigated the effect of CPC. A study conducted by Lukáč *et al*. (2013) is the closest study related to CPC and *Acanthamoeba*. Alkylphosphocholine and alkylphosphohomocholine derivatives of QACs such as cetyltrimethylammonium bromide, cetylpyridinium bromide, benzalkonium bromide (C16) and benzethonium chloride were tested for their anti-amoebic properties. They found the tested QACs to have high activity against *A. lugdunensis* and *A. quina*. Although the study did not specifically investigate the effect of CPC on *A. castellanii*, their findings are in contrast with the findings of the current study. CPC at concentrations of 3, 5 and 10µg/ml did not show any significant cytotoxicity against *A. castellanii* (T4) (Figure 6.4). Concentrations higher than 10µg/ml induced encystation and decreased surface adherence.

For human use, the US Food and Drug Administration Plaque Subcommittee has concluded that short term use of oral antiseptics containing up to 0.1% of CPC is safe (Feres *et al*., 2010). In humans, ingestion of CPC between 1 to 3g is considered as a fatal dose. Although there are no reported cases of CPC toxicity on the genitourinary system, it has been reported that the ingestion of

a large volume of benzethonium-containing wound disinfectant has caused delayed presentation renal failure (NLM, 2003). Benzethonium is a quaternary ammonium salt with a similar mechanism of action as CPC. In a study conducted by Fromm-Dornieden *et al*. (2015), CPC (> $3x10^{-3}$%) showed a high cytotoxic impact on human keratinocyte (HaCaT) and murine fibroblast (L929) cell lines. In another study conducted by Müller *et al*. (2017), oral mouth rinses containing CPC was found to have a high *in vitro* cytotoxic effect on oral fibroblasts, human oral epithelial carcinoma cells (HSC-2) and murine aneuploid fibrosarcoma cells (L929). The findings from the present study also indicate that CPC has significant cytotoxic effects on urothelial cell lines in a dose dependent manner (Figure 6.5). The cytotoxic investigation in the present study is mainly based on monolayer cultures. However, as described in chapter 3 (3.1), the human urothelium is made of 3 layers including the basal, intermediate and umbrella cells. Furthermore, the primary urothelial mediated response to infections and toxicity is the exfoliation of superficial cells and urothelial regeneration (Nagamatsu *et al*., 2015). Therefore, it is difficult to extrapolate the *in vitro* results from the present study to the urinary tract. Further studies which take these variables into account will need to be undertaken.

Several studies have investigated the effect of genomic manipulation on IBCs (Wright *et al*., 2007; Sorg *et al*., 2016; Conover *et al*., 2016). This part of the study, however, investigates the effect of CPC on the intracellular survival of IBCs.

Previous studies have demonstrated the bactericidal activity of various antimicrobial products on intracellular bacteria inside *A. castellanii* (T4), including disinfectants like actichlor, virkon, biocleanse & ethanol (Cardas, 2014), iron (III) – selective hexadentate ligands of 3-hydroxypyridine-4-one chelators (Al-Rugaie, 2016), eugenols type 3 (Al-Ghamdi, 2016) and a combination of drugs (magainin 2, silver nitrate & vancomycin) (Tashmukhambetov, 2016). The results (Figure 6.6) from this study show that CPC (3µg/ml) has significantly increased the ability of uropathogens to survive within *A. castellanii* (T4) compared with the control groups. A possible explanation for this might be that the uropathogens were able to strengthen their virulence factors whilst inside the amoebae. Several studies have also reported the increase in virulence of bacteria grown inside *Acanthamoeba* (Barker *et al*., 1992; Cirillo *et al*., 1994; Barker *et al*., 1994; Scaife *et al*., 1996; Cirillo *et al*., 1999; Sandström *et al*., 2011; Van der Henst *et al*., 2016).

On the question of intracellular survival in urothelial cells, this study (Figure 6.7) found that except for *E. coli* ESBL negative strain, all uropathogens displayed a reduced ability to survive within TERT-NHUC line in the presence of CPC in a dose dependent manner. Berry *et al*. (2009) have demonstrated that UPEC has the ability to differentially regulate its virulence factors in the intracellular environment of TERT-NHUC line. Other studies have also confirmed the increase in virulence of *E. coli* found inside urothelial cells (Berry *et al*., 2009; Robino *et al*., 2014; Dikshit *et al*., 2015; Schreiber *et al*., 2017). Previous studies have also investigated the bactericidal effect of various antimicrobial

products on bacteria inside human epithelial lung carcinoma (a549) cell lines (Tashmukhambetov, 2016) and KB epithelial cell lines (Al-Rugaie, 2016).

The final part of the present study deals with the evaluation of a CPC coated anti-biofilm urinary catheter. Catheter related biofilm formation is a very complex process. Almost all medical implants are susceptible to colonisation by biofilm forming bacteria and this is the main contributing factor to CAUTIs. Developing novel urinary catheters that target biofilm formation is necessary to tackle this problem. To this end, several studies have evaluated the efficacy of impregnating catheters with different antimicrobial products. Products like triclosan (Jones *et al*., 2006), gendine (Hachem *et al*., 2009), benzalkonium-chloride (Tebbs and Elliott, 1993), chlorhexidine-silver sulfadiazine (Maaskant *et al*., 2009) have shown promising results in eradicating biofilm formation. The main approaches to antibacterial surfaces include released-based coatings, contact-killing coatings and anti-adhesion coatings (Cloutier *et al*., 2015). The current study employs the approach of release-based coatings.

Darouiche and Raad (1997) devised a novel method of impregnating medical implants with an antimicrobial agent. Using their method, albeit slightly modified, the antimicrobial effect of CPC coated catheters was evaluated. Results from this study (Figure 6.8 and Figure 6.9) show that urinary catheters impregnated with CPC (3mg/ml) completely prevented biofilm formation over a 24h period for all five uropathogenic strains when compared with CPC impregnated catheters at a concentration of 1 & 2mg/ml. These findings suggest that in a 3mg/ml CPC catheter coating solution, urinary catheters have

adsorbed sufficient amounts of CPC to kill bacteria and thereby prevent biofilm formation. Our study (Figure 6.10) did not find any significant difference between the anti-biofilm activity of CPC coated PTFE latex and silicone catheters. This finding accords with earlier observations (Pratt *et al*., 2001).

Kinetics of drug elution from the antimicrobial catheters depends on the physical-chemical properties of the antimicrobial agent, catheter materials and the spatial loading profiles of the agents in the catheter (Viola *et al*., 2016). An optimised drug delivery system consists of two phases: an initial burst release of drugs from the catheter followed by a secondary sustained drug release profile (Daneshpour *et al*., 2013). If the drug molecules are located on the surface of the biomaterial, an initial burst of drug release is highly likely (Chao *et al*., 2015). In vitro drug release experiments (Figure 6.13, Table 6.1, Figure 6.15 and Figure 6.16) from the present study have confirmed such a release pattern.

It is vital to ensure that the chosen drug has a low minimum bactericidal concentration to prevent cytotoxicity and to reduce systemic drug load. An encouraging aspect of the present findings is that low concentrations of CPC was sufficient to kill >99% of uropathogens in their planktonic (3µg/ml) and biofilm states (4µg/ml). Even though the optimal catheter coating solution (>99% reduction in biofilm formation) is 3mg/ml (Figure 6.10), the amount of drug adsorbed into the catheter is very low as confirmed by HPLC analysis (undetected) (Figure 6.14) and further drug release studies (Table 6.1).

Further work needs to be undertaken to strengthen the findings from the current study related to the use of CPC impregnated catheters to prevent biofilm formation. Firstly, during the impregnation process, the time taken for maximum drug adsorption into the catheter needs to be investigated. Post impregnation, the total drug content in the catheter (percentage recovery in chloroform) and drug release profiles should be studied. These studies can be undertaken using Gas chromatography–mass spectrometry (GC-MS), which is more sensitive than HPLC and can detect drugs at the nanogram level. Secondly, the distribution of the drug in the catheter can be studied using secondary ion mass spectroscopy. Finally, the surface anatomy of CPC impregnated catheters can be studied using atomic force microscopy to determine if there are any changes in the catheter surface (intra and extra luminal) post impregnation. The anti-biofilm efficacy of CPC impregnated catheters can be clearly understood through these investigations. This is an important area for future research in this field.

As encountered in the present study, one of the main limitations of drug delivery systems is their inability to release drugs over a longer period of time. Not only did the CPC (3mg/ml) coated catheter fail to prevent biofilm formation on repeated bacterial challenge (Figure 6.12) but it also did not prevent biofilm formation post 24h drug elution (Figure 6.15). However, another encouraging aspect of these findings is that the drug eluted catheter significantly reduced biofilm formation in comparison with the control (catheter not subjected to drug elution) indicating the presence of some drug in the catheter. Sustained release delivery systems can be employed to overcome this limitation. The drug

delivery mechanism is based on two aspects; drug molecule transfer and release of entrapped drug by the detachment of polymeric chains (Shenderovich *et al*., 2015). Several studies have employed the use of sustained release mechanisms to improve the efficacy of antimicrobial coated urinary catheters including surface particulate localisation method for pH-dependent drug release (Irwin *et al*., 2013), use of polyurethane nanocomposites (Fong *et al*., 2012), sustained release varnishes (Segev *et al*., 2013; Shenderovich *et al*., 2015), photo-chemical deposition of silver coatings (Cooper *et al*., 2016), pre-irradiation methods (Islas *et al*., 2015; Magaña *et al*., 2016; Zuñiga-Zamorano *et al*., 2017), use of biodegradable poly(ether-ester) urethane acrylates (Feng *et al*., 2017) and the use of sustained release microspheres (Wu *et al*., 2003). To this end, research questions that could be asked include the suitability of the polymer in enabling strong cross linkage of CPC and adapting contact-killing rather than release-based approaches. Further work is required to establish this.

According to our knowledge, this is the first study to investigate the use of CPC in treating UTIs, particularly CAUTIs. This study has successfully employed the use of CPC to prevent catheter biofilm formation albeit for a shorter time period. Apart from the sustained release of drugs, another issue emerging from this study relates specifically to cytotoxicity. This issue has serious clinical implications since urinary catheters maintain direct contact with the urinary tract and therefore a haematogenous dissemination of CPC is possible. Previous *in vitro* studies have demonstrated the denaturing effect of CPC on human haemoglobin (Mitjans *et al*., 2008). However, the clinically approved use of

CPC in oral hygiene products is a promising factor in its potential use for UTIs. A further study with more focus on drug release kinetics to ensure slow release of minimal bactericidal drug concentration is therefore suggested. Nevertheless, the combination of findings from the current study provides some support for the conceptual premise that CPC coated catheters can be used to prevent uropathogenic biofilm formation and therefore CAUTIs.

Chapter 7

General discussion

7 General discussion

The present study was primarily designed to explore various underlying issues associated with UTIs. The main issues investigated in this study are the formation of biofilms (linked with CAUTIs), formation of IBCs (linked with rUTIs) and the possible role of *Acanthamoeba* in the pathogenesis of UTIs. Furthermore, use of CPC to tackle the issue of uropathogenic ABR was also evaluated.

7.1 Underlying issues

7.1.1 ABR

Penicillin was the first antibiotic to be mass produced and distributed in 1945. Using inhibition zones to test the antimicrobial efficacy of antibiotics was Flemming's idea and since then it is one of the most widely used mass screening methods. He was also one of the first person to advise caution against the overuse of penicillin to avoid potential microbial resistance (Aminov, 2010). Since the mass production of penicillin, a variety of antibiotics have been produced and successfully used. However, the last class of new antibiotics were produced in the 1980s and since then there has been a discovery void (WHO, 2014). This coupled with the development of ABR genes due to increased selection pressure from the overuse of antibiotics (Su *et al.,* 2017) has increased the need to develop novel antibacterial drugs (WHO, 2014). One of the primary causes of ABR is bacterial production of ESBL enzymes. ESBL producing Gram-negative bacteria are resistant to most β–lactam antibiotics and are often resistant to non-β-lactam groups such as aminoglycosides and

quinolones (Wragg *et al.,* 2017). There are more than 200 characterised ESBLs (Rawat and Nair, 2010) out of which TEM and SHV are most commonly found in Gram-negative bacilli (Turner, 2005). In the present study, three (TEM, SHV and CTXM) primers were used for ESBL screening of uropathogens. One strain of *E. coli* and *K. pneumoniae* were positive for TEM and SHV genes respectively. EUCAST antimicrobial susceptibility testing revealed that *E. coli* ESBL positive strain was resistant to all tested antibiotics (n=7) except imipenem whereas *K. pneumoniae* ESBL positive was sensitive only to imipenem, gentamicin and cefoxitin. These findings not only confirmed the presence of ESBL genes in uropathogens but also highlighted the potency of TEM-type ESBLs. The WHO has recognised ESBL producing *E. coli* and *K. pneumoniae* as bacteria of international concern due to their widespread ABR (WHO, 2014).

7.1.2 CAUTIs

CAUTIs are implicated in a majority of nosocomial infections (Fasugba *et al.*, 2017). Urinary catheters are used for diagnostic and therapeutic purposes (Ramakrishnan and Mold, 2005). Bacteria can use the intraluminal route (via urine reflux from contaminated drainage bags) to colonise the urinary catheter. After catheter insertion, the risk of bacteriuria increases by 3% to 10% every day (Tenke *et al.,* 2017). The pathogenesis of CAUTIs is related to the formation of biofilms on catheter surfaces (Hatt and Rather, 2008). Biofilms are communities of microorganisms which are enclosed in a protective, complex extracellular matrix known as EPS (Fish *et al*., 2017). EPS are primarily composed of proteins and polysaccharides which are responsible for adhesion,

protection, cell-to-cell communication and retention of a variety of compounds like water and nutrients (Lemus and Rodríguez, 2017). Bacteria express differential phenotypes in their biofilm and planktonic states. Biofilm bacteria are usually characterised by reduced growth rates, resistance to antimicrobial drugs and host immune response, and altered gene expressions (Azevedo *et al.,* 2017).

Biofilms are formed in several stages. Surface attachment of bacteria is one of the first steps in biofilm formation (Díaz *et al.,* 2011). However, biofilm formation in indwelling medical devices such as the urinary catheter has an additional step which is the formation of a conditioning layer (Garrett *et al.,* 2008). It has been suggested that a conditioning layer begins to form within minutes of substrate exposure to the surrounding medium (Moreira *et al.,* 2017). In the urinary tract, adsorption of urine components onto the catheter surface promotes microbial adhesion (Tieszer, 1998; Trautner and Darouiche, 2004). The findings from the present study confirms this. All uropathogens exhibited increased levels of adhesion on PTFE latex catheters coated with AUM for 3h. THP is one of the most abundant proteins in urine and previous studies have reported that THP binds to catheter surfaces and promotes adherence of uropathogens (Raffi *et al.,* 2012). Adherence is initially reversible and depends on repulsive and attractive forces. However, it has been suggested that the formation of a conditioning layer accelerates irreversible adhesion (Garrett *et al.,* 2008).

Irreversible adhesion is followed by microcolony formation and biofilm maturation (Crouzet *et al.,* 2014). This stage is characterised by an initial lag phase and then an exponential growth phase of biofilm bacteria (Garrett *et al.,* 2008). The present study has shown that the initial lag phase for all uropathogens except *K. pneumoniae* ESBL negative and *P. mirabilis* was between 0h and 3h. The latter two strains exhibited exponential growth phase at 2h and 1h respectively. These results indicate that the lag phase for all uropathogens is very short, suggesting strong uropathogenic ability to form biofilms in a relatively short period of time and therefore, increased susceptibility of substrates such as catheters to biofilms. This can have significant implications particularly in STC.

Maturation of biofilms is influenced by several factors including nutrient availability, nutrient perfusion into biofilms, removal of waste, internal pH, carbon source, osmolarity and oxygen perfusion (Dunne, 2002). A mature biofilm is characterised by a multilayered 3D structure made up of EPS (Karim *et al.,* 2013; Kostakioti *et al.,* 2013). Biofilm biomass assays have revealed high amounts of biomass in 48h *E. coli* biofilms, 24h *K. pneumoniae* biofilms and 24h & 48h *P. mirabilis* biofilms. Biofilm biomass consists of both bacteria and EPS. These findings reveal that under static conditions, uropathogens form mature biofilms 24h to 48h post colonisation. These mature biofilms contain a variety of polymers.

Although proteins are considered as one of the major constituents of EPS (Jiao *et al.,* 2011), carbohydrates also play an important role in maintaining the

overall structural integrity of biofilms (Andersson *et al.,* 2011). EPS analysis from this study has confirmed the presence of both macromolecules in all uropathogenic biofilms. The findings also indicate that proteins are a major biofilm component.

In a study conducted by Welin *et al*. (2004), out of the 33 adhesion related proteins in *S. mutans* biofilm cells, the rate of synthesis of 25 proteins was significantly enhanced in comparison with planktonic cells. Another study found an overexpression of 57 biofilm-related proteins in *S. mutans* (Svensäter *et al.,* 2001). Apart from proteins needed for surface adhesion, biofilm bacteria are also known to enhance the production of a variety of surface proteins which aid in nutrient and waste transport (Garrett *et al.,* 2008). Comparing intracellular proteins of planktonic and biofilm bacteria, the present study has revealed that the latter has expressed higher amounts of proteins. Taken together, these findings suggest an important role for proteins in promoting biofilm maturation.

The final stage of biofilm formation is cellular detachment and dispersal (Rendueles and Ghigo, 2012). Following maturation, once the biofilm reaches critical mass, bacteria in the outermost layer are dispersed in their planktonic state (Dunne, 2002). It is well known that biofilm dispersal depends on nutrient availability (Chao *et al.,* 2015). Some biofilms disperse bacteria in response to nutrient depletion whereas others induce dispersal on availability of nutrients (Chao *et al.,* 2014; Uppuluri and Lopez-Ribot, 2016). Our findings have confirmed that biofilms from all uropathogens employed in the present study dispersed bacteria in response to nutrient availability.

Apart from planktonic and biofilm states, bacteria also express different forms of motility including swimming and swarming (Kearns, 2010). It has been suggested that bacterial motility can promote biofilm formation (Maya-Hoyos *et al.,* 2015). However, the results of this study does not indicate any positive correlation between motility and ability to form biofilms. In fact, even though *P. mirabilis* was able to swim and swarm, it formed lesser biofilms compared with other uropathogens.

The use of indwelling catheters not only cause CAUTIs but can also initiate bladder UTIs by allowing bacteria to migrate along the catheter surface into the urinary bladder (Sabbuba *et al.,* 2002; Jones *et al.,* 2004). Latex (PTFE and hydrogel) and silicone catheters, which are commonly used in the NHS (Pickard *et al.,* 2012), were tested for their susceptibility to biofilms and bacterial migration. The evidence from this study shows that silicone catheters were more prone to bacterial biofilms whereas hydrogel catheters promoted bacterial migration.

Overall, these findings enhance our understanding of uropathogenic biofilm formation and the factors that influence it. The research will serve as a base for future studies in preventing CAUTIs particularly by targeting specific stages of biofilm formation.

7.1.3 rUTIs

IBC formation is mainly implicated in the pathogenesis of rUTIs. Formation of IBCs particularly in the deeper layers of the bladder urothelium can serve as

stable reservoirs for rUTIs (Ejrnæs, 2011; Hannan *et al.,* 2012). An IBC cycle typically involves association, invasion and survival of uropathogens within urothelial cells (Scott *et al.*, 2015). IBCs are protected from host immune response and antimicrobial drugs (Conover *et al.,* 2016). Moreover, bacteria can flux from IBCs and the turnover and shedding of epithelial cells can also release bacteria from quiescent intracellular reservoirs causing rUTIs (Glover *et al.,* 2014). The results from the present investigations show that all uropathogens were able to interact with TERT-NHUC line albeit at varying degrees. In terms of survival and formation of IBCs within urothelial cells, *P. mirabilis* exhibited the highest ability followed by both strains of *K. pneumoniae and E. coli*. This is an interesting finding since most studies have reported the potent ability of UPEC in forming IBCs compared with other pathogens (Justice *et al.*, 2003; Rosen *et al.*, 2007; Garofalo *et al.*, 2007; Berry *et al.*, 2009; Robino *et al.*, 2013). Another interesting finding to emerge from this study is that all uropathogens except *E. coli* (ESBL positive) were able to induce significant dispersal from 24h IBCs. The ability of all uropathogenic IBCs to disperse bacteria confirms their relevance to rUTIs.

A variety of toxins produced by *E. coli* (HlyA and CNF-1), *K. pneumoniae* (capsule and outer membrane vesicles) and *P. mirabilis* (HlyA and HpmA) are responsible for inducing cytotoxicity on urothelial cells (Podschun and Ullmann, 1998; Gupta *et al.*, 2003; Bien *et al.*, 2012; Lee *et al.,* 2012; Baldo and Rocha, 2014; Flores-Mireles *et al.*, 2015). Cytotoxicity assays from the present study show that all uropathogens induced significant cytotoxicity on TERT-NHUC line. The highest cytotoxicity was induced by *P. mirabilis*, possibly due to its

ability to cleave urea and form struvite crystals (Jacobsen *et al.,* 2008). Interestingly, flow cytometry assays have revealed that *P. mirabilis* induced the highest amount of primary necrosis on urothelial cells. It is well-known that primary necrosis is a result of direct toxic effect on cells rather than a transition from apoptosis (Poon *et al.,* 2010), implicating struvite formation by *P. mirabilis*. Clinically, this can have profound implications since necrosis is most often characterised by adjacent cell inflammation (Robins and Cotran, 2015). Comparison of IBC formation and cytotoxicity has revealed that the percentage of late apoptosis and or secondary necrosis induced on urothelial cells is directly related to the amount of IBCs formed. This finding suggests that uropathogens not only establish protective reservoirs in the bladder epithelium but in the process, also induces significant cytotoxicity.

IBCs are the primary targets in the prevention of rUTIs. ABR conferred by IBCs through phenotypic changes in their quiescent intracellular localisation and through the prolonged use of low dose antibiotic therapy (Dason *et al.*, 2011; Barber *et al.*, 2013; Lüthje and Brauner, 2016) has compounded the need for alternative treatment strategies. Current rUTI prevention strategies including the use of lactobacillus preparations (Foxman and Buxton, 2013), D-mannose (Beerepoot and Geerlings, 2016) and cranberries (Kucheria *et al.*, 2005) have shown limited but promising results.

7.1.4 Role of *Acanthamoeba* in UTIs

Acanthamoeba is a free-living opportunistic protist (Ortega-Rivasa *et al.,* 2016). It can exist in two forms: trophozoites and cysts and in their trophozoite stage

they can feed on a variety of bacteria through phagocytosis (Alsam *et al.,* 2005a). In recent years, *Acanthamoeba* has garnered a lot of interest primarily due to its endosymbiotic relationship with bacteria. A wide variety of pathogens are known to survive and multiply within *Acanthamoeba*: *F. tularensis* (Abd *et al.*, 2003), *V. cholera* (Abd *et al.*, 2004), *E. coli* (Alsam *et al.*, 2006; Yousuf *et al.*, 2014) and *S. aureus* (Cardas *et al.,* 2012).

Three rare but life-threatening human infections are associated with *Acanthamoeba*: GAE, AK and DI. However, the protozoa has been previously isolated from healthy people or people suffering from non-*Acanthamoeba* infections such as pneumonia. *Acanthamoeba* antibodies have also been found in healthy people. This suggests that *Acanthamoeba* can live in humans without causing any of the well-known amoebic infections. Do they have any other role in the human body? Can they influence the recurrence of infections such as UTIs?

Several lines of evidence suggest a possible role of *Acanthamoeba* in UTIs. Firstly, it is a FLA found in a variety of environments including natural water bodies (Lorenzo-Morales *et al.*, 2005; Liu *et al.*, 2006; Nuprasert *et al.*, 2010, public water systems (Kilvington *et al.*, 2004; Jeong and Yu, 2005; Sente *et al.*, 2016), swimming pools (Toczołowski *et al.*, 2000; Caumo *et al.*, 2009; Al-Herrawy *et al.*, 2014), bottled water (Visvesvara *et al.*, 2007; Siddiqui and Khan, 2012; Maschio *et al.*, 2015) and the general hospital environment (Costa *et al.*, 2010; Fukumoto *et al.*, 2015), which greatly increases the chances of the amoeba entering the human body. Secondly, *Acanthamoeba* is known to feed

on bacteria (Alsam *et al.,* 2006), which in the human body could include normal microflora such as those found in the intestinal tract and bladder (Lewis *et al.,* 201). However, as mentioned above, bacteria can survive within the amoeba and in fact can become more virulent (Lovieno *et al.*, 2010; Bennett *et al.*, 2014) and therefore difficult to treat. Thirdly, *Acanthamoeba spp.* has been previously isolated from urine samples collected from indwelling catheters of critically ill patients (Santos *et al.,* 2009). All of this suggests that *Acanthamoeba* may play an important role in the pathogenesis of UTIs particularly causing recurrent infections.

In order to explore the nature of the relationship between uropathogens and *Acanthamoeba*, interaction, amoeba conditioned medium and co-cultivation assays were performed. Interaction assays have revealed that all uropathogens were able to interact with *A. castellanii* (T4). Of particular significance is the ability of uropathogens to resist *Acanthamoeba* phagocytosis and survive within the amoebae. This supports the idea that uropathogens can use the amoeba as a protective reservoir.

An important finding from amoeba conditioned medium assays is that all uropathogens displayed higher planktonic growth in the presence of *A. castellanii* (T4) extracellular products. Extrapolating these findings to the urinary bladder suggests the possibility of increased bacteriuria and therefore infection rates when the amoebae are present in urine.

Besides being grazed by the amoebae and surviving within them, can uropathogens live alongside *Acanthamoeba*? To answer this question co-cultivation assays were performed. The results have revealed that all uropathogens can grow in their planktonic states and form biofilms in the presence of *A. castellanii* (T4). *P. mirabilis* has displayed a higher ability to form biofilms in the presence of amoebae compared with other uropathogens. Furthermore, CV stained light microscopy images of co-cultivated biofilms not only reveal that bacteria outcompete the amoebae in surface adherence but also possibly prefer amoebic grazing. This can be particularly seen around biofilm adhered amoebae. Cumulatively, these findings suggest that uropathogens can also live outside *Acanthamoeba* and in some cases with increased pathogenic ability.

One of the more significant findings to emerge from this study is the presence of *Acanthamoeba* in urine. Two hundred urine samples from patients suspected of UTIs were collected from CHUFT. Using NNA culture method and PCR analysis, *Acanthamoeba* was isolated from 21 urine samples. Sequencing of the mitochondrial small subunit rRNA gene (16s rRNA) showed that 19 samples positive for *Acanthamoeba sp*. (unclassified *Acanthamoeba*) and the other two samples were positive for *A. castellanii.* These findings not only confirm the findings from a sole previous study (Santos *et al.,* 2009) but also contributes additional evidence in support of our hypothesis. To the best of our knowledge, this is the first study in the UK to isolate *Acanthamoeba* from urine samples.

Taken together, these findings support the relevance of *Acanthamoeba* in UTIs and enhances our understanding of the relationship between *Acanthamoeba* and uropathogens.

7.2 Tackling the underlying issues

Bacteria can develop ABR through a variety of ways: genetic and mechanistic ways, forming IBCs and surviving within *Acanthamoeba*. As discussed in the literature review, these pathways also confer greater virulence.

One way of tackling the issue of ABR is by using non-antibiotic products to treat bacterial infections. To this end, many studies have employed the use of QACs (Jiao *et al.*, 2017). CPC is a QAC with potent wide spectrum antibacterial activity (Hurt *et al.,* 2016). However, most studies investigating the antibacterial ability of CPC have only focused on oral care (Hwang *et al.*, 2013) and the poultry industry (Li *et al.*, 1997; Arritt *et al.*, 2002; Beers *et al.*, 2006). To the best of our knowledge, this is the first study to investigate the use of CPC against uropathogens associated with UTIs. Antimicrobial assays from this study has revealed that very low concentrations (3µg/ml) of CPC are sufficient to kill greater than 99% of uropathogens over a 24h period.

Biofilms confer increased resistance to uropathogens (Niveditha *et al.*, 2012). Firstly, it can act as a molecular filter by reducing penetration of antibiotics into the biofilm matrix. Secondly, biofilm bacteria with dormant phenotypes may act indifferently to antibiotic activity. Finally, the altered microenvironment of the biofilm itself can affect the potency of antimicrobial drugs (Stewart and

Costerton, 2001; Dunne, 2002). However, at a low concentration of 4µg/ml, CPC has displayed potent anti-biofilm activity against all uropathogenic biofilms.

Although this study has found that CPC is cytotoxic to urothelial cells, very low bactericidal and anti-biofilm concentrations, and the clinically approved use of CPC in oral hygiene products are promising factors in its potential use in treating UTIs.

A recognised way of preventing rUTIs is bladder instillation (Gugliotta *et al.,* 2015). Several studies have demonstrated the successful use of hyaluronic acid (Constantinides *et al.,* 2004; Lipovac *et al.,* 2007; Damiano and Cicione, 2011; Son *et al.,* 2011; Ząbkowski *et al.,* 2015) and heparin (Ablove *et al.,* 2013) bladder instillations in reducing rUTIs. However, the main limitation of this strategy is drug washout and limited attachment and penetration into the bladder wall. To circumvent this, GuhaSarkar *et al*. (2017) have devised a novel liposome-in-gel system (LP-Gel) wherein drug loaded liposomes were incorporated into gellan hydrogel and instilled in the urinary bladder. On contact with urine, LP-Gel undergoes ion-triggered gelation which enhances attachment to the mucin layer of the urothelium. The mucoadhesive properties of gellan also promotes mucin adhesion. A similar mechanism can be used with CPC as the drug to target IBCs formed in the bladder urothelium.

Although the current study also revealed that higher concentrations of CPC are required to reduce IBCs in TERT-NHUC line and *A. castellanii* (T4), there are

ways in which QACs can be modified to improve their antimicrobial activity. This can be done by modifying their alkyl chain lengths through the substitution of aromatic ring hydrogen with chlorine, methyl, and ethyl groups (Melin *et al.*, 2014; Morkaew *et al.,* 2017). It is widely recognised that QACs with long chain lengths are more efficient in their antimicrobial activity (Ioannou *et al.*, 2007). Therefore, development of novel QACs by modifying their alkyl chain length can prove as a useful strategy to target IBCs and therefore rUTIs. Another significant finding from the bacteria-amoebae intracellular survival assays is the increased patterns of uropathogenic growth inside *Acanthamoeba* in the presence of CPC (3µg/ml) indicating a possible increase in virulence.

With regards to targeting catheter biofilms, this study found that CPC (3mg/ml) coated catheters were efficient in the prevention of biofilms over a 24h period. A burst release of CPC from the catheter reduced its efficacy on day 2. Using contact-killing method to develop CPC coated catheters as opposed to the release-based approach used in this study is one way of overcoming this limitation (Cloutier *et al.,* 2015). There are several ways in which antimicrobial coated urinary catheters can be modified to promote sustained release of drugs (1.3.2). Of interest is gamma radiation method due to their strong ability to embed QACs into polymers (Fan *et al.,* 2015).

Despite the limitation regarding drug release, a few encouraging aspects have emerged from these findings. Firstly, the amount of drug adsorbed into the catheter is very low as confirmed by HPLC analysis (undetected) and further drug release studies which is encouraging in terms of the low concentrations

required to prevent biofilm formation. Secondly, even though the drug eluted catheter allowed biofilm formation, in comparison with the control (catheter not subjected to drug elution) there was a significant reduction in biofilm indicating the presence of some drug in the catheter. Overall these findings substantiate the potential use of CPC coated catheters in CAUTIs.

7.3 Conclusion

In conclusion, this study has shown that:

1. Uropathogens can form biofilms in a relatively short period of time indicating their relevance to STC. Proteins are an important constituent of mature biofilms indicating their relevance as a potential target in anti-biofilm treatment. Biofilm dispersal is nutrient dependent and therefore limiting nutrition through strategies like iron chelation can prove useful in preventing colonisation of new surfaces.

2. Uropathogenic cytotoxicity on urothelial cells is dependent on the number of IBCs formed and uropathogens complete their IBC pathogenic cycle in a relatively short window of 24h.

3. *Acanthamoeba* plays an important role in the pathogenesis of UTIs particularly rUTIs. Furthermore, uropathogens have exhibited an endosymbiotic relationship with *Acanthamoeba.*

4. CPC can be potentially used in treating UTIs particularly CAUTIs.

7.4 Recommendations

The present study confirms previous findings related to biofilm and IBC formation and adds to the growing body of literature. The study also contributes additional evidence that suggests a possible role of *Acanthamoeba* in the pathogenesis of UTIs. Moreover, this research will serve as a base for future studies on the use of QACs in treating UTIs. It is recommended that further research be undertaken in the following areas:

1. The current investigation on uropathogen induced cytotoxicity was limited in terms of time course experiments. It would be useful to explore the cytotoxic effects of uropathogens at various incubation periods so that a correlation between different stages of IBC formation can be made. It would also be interesting to assess *in vivo* IBC formation as it is more challenging to extrapolate *in vitro* studies to the urinary tract.

2. The current research was not specifically designed to evaluate factors related to biofilm formation under flow conditions. It is suggested that the effect of flow dynamics such as shearing forces on biofilm survival is investigated in future studies. Another aspect that could be incorporated is multispecies biofilm formation.

3. Further investigations on the ability of uropathogens to interact with *Acanthamoeba spp.* isolated from urine samples will shed more light on the exact nature of their relationship in the urinary tract. Future amoeba isolation studies should also evaluate the complete urine profile and clinical history of

patients to fully explore health implications related to the presence of the amoeba in urine. Checking for the presence of *Acanthamoeba* antibodies in such patients will also provide useful insights into host immune response.

4. Finally, further investigation and experimentation into the use of CPC or other potent antimicrobial QACs in preventing catheter biofilm formation particularly through contact-killing methods is strongly recommended.

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Appendices

Appendices

Appendix A

1. Microorganisms and cell line

E. coli (ESBL positive and negative), *K. pneumoniae* (ESBL positive and negative) and *P. mirabilis* isolated from patients diagnosed with UTIs were kindly provided by Dr. Tony Elston, CHUFT
TERT-NHUC line was kindly provided by Prof. Knowles, Cancer Research UK Clinical Centre, St. James's University Hospital, Leeds.
A. castellanii T4 (ATCC 30234) isolated from a patient diagnosed with *Acanthamoeba* keratitis used in this study was kindly provided by Dr. S Maciver, University of Edinburgh.

2. Urinary catheters

Catheters were kindly provided by Dr. Tony Elston, CHUFT.

Hydrogel coated latex Ch 12	Teleflex H310112
PTFE coated latex Ch 12	Teleflex P310112
Silicone Ch 12	Teleflex S310112

3. Protocol for storing, thawing and re-culturing of bacteria

By using a disposal sterile loop, about 20-30 isolated colonies of bacteria were picked up from the agar plate and inoculated in a 10ml of bacterial freezing medium [70% Luria-Bertani broth and 30% glycerol v/v]. Then, the mixture of freezing medium with bacteria were divided into cryotubes final volume of each tube was 0.5ml and stored in a -20°C freezer.
To re-culture bacteria, a disposable sterile loop was inserted into the frozen cryotube and then streaked on fresh agar plate and incubated at 37°C air incubator overnight. After the incubation, the plate was carefully examined if the colonies have similar morphologies and stored at 4°C for up to a month.

4. Protocol for storage and thawing of cells and *Acanthamoeba*

Cells and *Acanthamoeba* were stored as axenic cultures at -80°C and liquid nitrogen. Briefly, growing cells or amoebae were re-suspended at a density of 1-5 x 10^6 cells/mL in the freezing medium [90 % FBS, and 10 % DMSO v/v for human cells; 90 % PYG, and 10 % DMSO v/v for *Acanthamoeba*] and placed in a cryotubes. The protocol was carried out gradually on ice, then -20°C for 60min followed by their storage at -80°C freezer. Some of the cryotubes were transferred after overnight incubation in -80°C to store in liquid nitrogen.
Thawing and recovery of stored cells and *Acanthamoeba* from both liquid nitrogen and -80°C freezer was done quickly. The cryotubes were placed immediately in the warmer incubator maintained at 37°C for 3min. Cells or *Acanthamoeba* were transferred into a 15ml tube with warmed medium KGM-2 for TERT-NHUC line and PYG for *Acanthamoeba* and centrifuged at 2000 x *g* for 5 min. Next, supernatants were discarded, pellets were resuspended in growth medium KGM-2 for TERT-NHUC line and PYG for *Acanthamoeba* and transferred into a T25 tissue culture flask. Flasks were incubated at 37°C in 5% CO_2 and 95% humidity for TERT-NHUC line or 30°C air incubator for *Acanthamoeba* until the cells were confluent.

5. Final supplement concentrations of KGM-2 media

Bovine Pituitary Extract	0.004 ml/ml
$CaCl_2$	0.06 mM
Epidermal Growth Factor recombinant human	0.125 ng/ml
Epinephrine	0.39 µg/ml
Hydrocortisone	0.33 µg/ml
Insulin recombinant human	5 µg/ml
Transferrin, holo human	10 µg/ml

6. Disinfectants used in the lab

1% virkon w/v: 1mg in 100ml PBS
70% ethanol v/v: 70ml in 100ml H_2O

Appendix B

1. Reagents for AUM

Ammonium chloride	Sigma A9434
Calcium chloride dihydrate	Sigma C3306
Citric acid	Sigma 251275
Creatinine	Sigma C4255
Di-potassium hydrogen phosphate	Sigma
Iron II sulphate heptahydrate	Sigma F8633
Lactic acid	
Magnesium sulphate heptahydrate	Sigma M2773
Peptone L37	Oxoid LP0034
Potassium dihydrogen phosphate	Sigma P5655
Sodium bicarbonate	Sigma S5761
Sodium chloride	Sigma S7653
Sodium sulphate heptahydrate	Sigma 71969
Urea	Sigma U5378
Uric acid	Sigma U2625
Yeast extract	Sigma Y1625

2. Reagents for PCR and gel electrophoresis

Agarose	Fisher Scientific 10776644
dNTP Mix	Thermo Scientific TM R0241
GeneRuler 1 kb DNA Ladder	Thermo Scientific TM SM0311
SafeView Nucleic Acid Stain	NBS-Biologicals NBS-SV1
Taq DNA Polymerase	Thermo Scientific TM EP0401

3. Reagents for flow cytometry

Annexin V Binding Buffer	BioLegend 422201
Annexin V-FITC	BioLegend 640906
Cell staining buffer	BioLegend 420201
Zombie NIR	BioLegend 423106

4. Reagents for TERT-NHUC line

Accutase	Sigma A6964
Cell lytic M	Sigma C2978
Keratinocyte growth medium 2 ready-to-use	PromoCell C-20011

5. Antibiotics and antimicrobial agents

Antibiotic discs	Oxoid
Cetylypyridinium chloride	Sigma C0732
Gentamicin sulfate	Sigma G1914
Meropenem trihydrate	Sigma M2574
Penicillin-Streptomycin	Sigma P4333

6. Kits

Bradford assay kit	Bio-Rad 5000001
Cytotoxicity detection kit^{plus}	Roche 11644793001
QIAamp DNA micro kit	Qiagen 56304
QIAquick PCR purification kit	Qiagen 28104

7. Agars

Mueller Hinton agar	Sigma 70191
Bacteriological agar	Sigma A5306
CLED	Sigma 55420

8. Other reagents used

Acetic acid	Fisher Scientific 10375020
Acetonitrile	Sigma 271004
Ammonium persulphate	Sigma A3678
Bis/acrylamide solution for electrophoresis	Sigma A3574
Bovine serum albumin	Sigma A2153
CHAPS	Sigma C9426
Chelex	Sigma C7901
Chloroform	VWR international ltd C_CHLORO
Coomassie brilliant blue	Sigma B0149

Crystal violet	Sigma C0775
Ethanol	Fisher Scientific BP2818
Glacial acetic acid	Fisher Scientific 10139689
Methanol	VWR international ltd C_METHANOL_AR
PBS	Sigma P4417
Peptone water	Sigma 70179
Phenol	Sigma P1037
Potassium chloride	Sigma P9541
RIPA buffer	Sigma R0278
Sodium azide	Sigma S2002
Sodium dodecyl sulfate SDS	Sigma L3771
Sulfuric acid	Fisher Scientific 15481817
TEMED	Sigma T9281
Trifluoroacetic acid	Sigma 74564
Triton X100	Sigma X-100
Trypan blue	Sigma T6146
Virkon	Fisher Scientific 12358667
Yeast extract	Sigma 92144

Appendix C

1. Lysis buffer for protein extraction

7M urea, 2M thiourea, 4% 3-[3-cholamidopropyl dimethylammonio]-1-propanesulfonate (CHAPS), 15 mM pH 8.5 TRIS

2. Chelex solution for DNA extraction

10% (w/v) chelex in 0.1% Triton X-100 and 10mM Tris buffer [pH 8.0]

3. Growth medium (w/v)

PYG: Proteose peptone 0.75 %, yeast extract 0.75 %, and glucose. Add 1.5 % distilled water prior to autoclaving 121°C for 15min.
LB broth: 10g tryptone, 5g yeast extract and 10g NaCl in water to a final volume of 1000ml, adjust the pH to 7.5 before autoclaving at 121°C for 15min.

5. Agar (w/v)

CLED: 36.15g of CLED agar dissolved in 1000ml distilled water prior to autoclaving at 121°C for 15min.
Non-nutrient: 30g bacteriological agar dissolved in to 1000ml Ringers solution prior to autoclaving at 121°C for 15min.

7. Ringers solution (1/4 strength tablets)

One tablet is sufficient for 500ml distilled water. One tablet contains calcium chloride (0.12 g/l), potassium chloride (0.105 g/l), sodium bicarbonate (0.05 g/l) and sodium chloride (2.25 g/l)

8. Phosphate buffer saline (PBS tablets)

One tablet dissolved in 200ml deionized water giving 0.01M phosphate buffer containing potassium chloride (0.0027M) and sodium chloride (0.137M). Autoclave at 121°C for 15min. Adjust pH 7.4 at 25°C

Appendix D

1. SDS-PAGE buffers

1.5M Triethylamine hydrochloride (Tris) pH 7.4	18.1g dissolved in 100ml distilled water, adjust pH to 7.4 using HCl.
0.5 M Tris pH 6.8	6.057g in 100ml distilled water, adjusted pH to 6.8 using HCl.
10% (w/v) SDS	10 g SDS in 100ml distilled water.
Running buffer for SDS-PAGE stock solution 5X	Tris 15g, glycine 72g, SDS 5g, make up to 1000ml with distilled water. Adjust pH to 8.3 with HCl. Dilute to 1X with distilled water prior to use.
Sample buffer 2X	0.06 M Tris, 2.5% glycerol (Sigma G5516), 0.5% SDS, 1.25% 2- mercaptoethanol (Sigma M6250) and bromophenol blue 0.001% (Sigma B0126) (pH=6.8).
Coomassie brilliant blue stain	0.5% w/v Coomassie brilliant blue, 40% methanol v/v, 10% acetic acid v/v, 50% distilled water v/v.
De-stain	40% methanol v/v, 10% acetic acid v/v, 50% distilled water v/v.
Ammonium persulphate	4% w/v dissolved in distilled water

2. Running gel (10% SDS)

Distilled water	4.2ml
Bis/acrylamide solution	3.3ml
SDS 10%	100µl
Tris 1.5M pH 7.4	2ml
10% Ammonium persulphate	400µl
TEMED solution	6µl

3. Stacking gel (5%)

Distilled water	4.3ml
Bis/acrylamide solution	0.8ml
Tris 0.5M, pH 6.8	1.88ml
SDS 10%	60µl
10% Ammonium persulphate	750µl
TEMED solution	3µl

4. SDS-PAGE protocol

The Biorads mini-protein electrophoresis unit 67S12533, Biorad, Hemel Hempstead, UK is assembled according to the manufacturers' instructions. The running gel reagents were mixed and loaded into the sealed gel caster overlaid with 70% ethanol to exclude oxygen, this is a vertical SDS-PAGE system that cast gels of 0.75mm thickness. After polymerisation, the ethanol was decanted and the stacking gel was mixed and layerd on top of the running gel. Plastic lO-well combs holds up to 20 ..ll/well were inserted into the stacking gel and polymerisation allowed to occur min. The combs were then removed and gels were assembled into the electrophoresis tank Biorad 165-2949 and the tank filled with 1 X running buffer as mentioned above. Samples mixed with sample buffer 1:1 were added to the wells and electrophoresed for 2h, 115V. Gels were then stained with 50ml coomassie blue stain, with gentle shaking and then destained for 30 min with gentle shaking to visualise protein bands on the same day.

Appendix E

1. TAE buffer (50X)

Tris base w/v	242g
Glacial Acetic Acid v/v	57.1ml
0.5M EDTA pH8 v/v	100ml
Distilled water	Make up to 1000ml

2. Agarose gel (1%)

40g agarose dissolved in 400 mL 1X TAE buffer at 50°C then add 1ml of safeview before pouring in the electrophoresis tray.

3. Primer preparation

Spin the tubes at top speed for 10min to ensure that there are no lyophilised primers stuck to the cap. To make a typical 100mM 100X stock concentration of primers, dissolve the primers in a volume of sterile distilled water that is 10X the amount of nmoles in the tube, using µl of water. This value is printed on the side of the tube. Store all sets of primers at -20°C. Typically use 0.1-1.0µM final concentration in a PCR mix.

Appendix F

Ethics approval - HRA

NHS
Health Research Authority

Dr Aiden Matthew Jayanth
PhD Student
University of Essex
School of Biological Sciences
University of Essex
Wivenhoe Park, Colchester
CO4 3SQ

Email: hra.approval@nhs.net

14 September 2016

Dear Dr Jayanth

Letter of HRA Approval

Study title:	**Role of Acanthamoeba in Urinary Tract Infections**
IRAS project ID:	**210020**
Protocol number:	**N/A**
REC reference:	**16/YH/0266**
Sponsor	**University of Essex**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

IRAS project ID	210020

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The document *"After Ethical Review – guidance for sponsors and investigators"*, issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application

IRAS project ID	210020

procedure. If you wish to make your views known please email the HRA at hra.approval@nhs.net. Additionally, one of our staff would be happy to call and discuss your experience of HRA Approval.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **210020**. Please quote this on all correspondence.

Yours sincerely

Catherine Adams
Senior Assessor

Email: hra.approval@nhs.net

Copy to: *Ms Sarah Manning-Press, Sponsor Contact*
Mr Ayres Caldeira, Colchester University Hospital NHS Trust

CHUFT assurance letter

Colchester Hospital University NHS
NHS Foundation Trust

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19/09/2016

Dr Aiden Matthew Jayanth
PhD Student, University of Essex
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Southend on Sea
SS2 6JH

REC ref: 16/YH/0266
R&I ref: 2016/009
IRAS ref: 210020

Dear Dr Jayanth

Re: Role of Acanthamoeba in Urinary Tract Infections

Research & Innovation (R&I) has reviewed the documentation for this project and undertaken an assessment based on the complete document set provided by the sponsor/sponsor representative.

On behalf of the Research Steering Group, The Director of Research has no objection and is pleased to confirm that Colchester Hospital University NHS Foundation Trust, participating organisation, has the capacity and capability to deliver this study.

Sponsor: University of Essex

Funder: Self-funded

Study end date: 31/12/2016

Protocol: Project proposal v1.3 dated 04/07/2016

Conditions of Assurance Letter

- The project must follow the agreed protocol and be conducted in accordance with all Trust policies and procedures especially those relating to research and data management.
- You and your team must ensure that you understand and comply with the requirements of NHS Confidentiality Code of Practice, Data Protections Act 1998, Trust's Caldicott Principles and any further legislation released during the time of this study.
- Members of the research team must have appropriate substantive contract or a letter of access with the Trust prior to the study commencing. Any additional researchers who join the study at a later stage must also hold suitable HR documentation.
- Under ICH – GCP (International Conference of Harmonisation of Good Clinical Practice), a Central Investigator File, containing essential study documents should be set up for this study.

Colchester Hospital University NHS
NHS Foundation Trust

- Performance in Initiating Clinical Research: The trust will measure and publish data on the days elapsing between the time a valid research application is received and the time when the first patient is recruited to the trial (Benchmark: 70 days) and the reason(s) for not meeting the target.

- Performance in Delivering Clinical Research: The trust will monitor the study performance and publish information on the target number of patients it has agreed to recruit to the trial, the date by which it has agreed to recruit to the trial, trial status and if trial recruitment has finished, whether or not the agreed target number of patients was recruited within the agreed time.

Should you require any further information please do not hesitate to contact the R&I Department.

May I take this opportunity to wish you every success with this research.

Yours sincerely

Dr ~~Angela Tillett~~
Medical Director

Poster and oral presentations

Poster and oral presentations

Jayanth, A. M., Elston, T., Mitchell, G. and Alsam, S (2016) 'Role of *Acanthamoeba* in Urinary Tract Infections' in Health Research Authority, NHS. Available at http://www.hra.nhs.uk/news/research-summaries/role-of-acanthamoeba-in-urinary-tract-infections/

Jayanth, A. M., Mitchell, G. and Alsam, S (2015) 'A Novel Antimicrobial Urinary Catheter' in Congress of the Institute of Biomedical Science, ICC Birmingham, 29 September.

Jayanth, A. M., Mitchell, G. and Alsam, S (2015) 'Role of Biofilms and *Acanthamoeba* in Urinary Tract Infections' in Molecular and Cellular Biology Group Semina, University of Essex, 23 September.

Jayanth, A. M. (2014) 'The Scientist in You' in National Science and Engineering Week, North Hertfordshire College, 11 March.

Alsam, S., **Jayanth, A. M.** and Mitchell, G. (2014) 'Uropathogens associated with Urinary Tract Infections: A Role for *Acanthamoeba*?' in Essex Biomedical Sciences Institute Conference, Colchester Hospital University Foundation Trust, 15 January.

Jayanth, A. M., Mitchell, G. and Alsam, S (2013) 'The Role of *Acanthamoeba* in Recurrent Urinary Tract Infections' in the 12th Graduate Forum, University of Essex, 11 September.

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