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The Nature of Uropathogenic Escherichia coli ST127

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The Nature of Uropathogenic *Escherichia coli* ST127

By

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Abstract

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The nature of uropathogenic *Escherichia coli* ST127

Uropathogenic *Escherichia coli* ST127 is a recently emerged clone that is reported to cause a small, but significant, proportion of urinary tract infections. Paradoxically, the low prevalence of ST127 in most published studies is also accompanied by a virulence potential significantly higher than many of the leading uropathogenic lineages (cf. ST131) using an established uropathogenic PCR virulence assay. This study explores the contradictory nature of these findings by evaluating some of the major aspects concerning the diagnosis, genomic repertoire and pathogenicity of strains of this sequence type in relation to its recorded prevalence. Analysis was performed to determine if UPEC ST127 had a propensity towards a certain group of individuals, was underrepresented due to diagnostic criterion or possessed a novel polymicrobial aetiology concealing the true prevalence of this clone in the general population. To enable evaluation, rapid and robust PCR tests were designed for identifying this sequence type within a collection of 475 urine isolates collected from clinical laboratories. Specimens underwent genomic examination and were subject to three virulence assays in an attempt to validate results and establish the true value of these assays. Further examination to determine the virulence for members of this sequence type was performed using a bladder cell culture infection model. Results suggest that the presence of a highly motile phenotype may dictate the prevalence of UPEC in the population and the ability to invade the uroepithelium. A widespread frameshift mutation present in the *flhB* gene in

the majority of ST127 isolates suggests that motility may be supressed in this lineage and the reason for the reported low prevalence of ST127. This report supports the long suspected, but never proved, notion that bacterial motility is one of the most important virulence traits relating to the pathogenicity of *Escherichia coli* within the urinary tract.

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List of abbreviations

ABU	Asymptomatic Bacteriuria
ACOG	American Congress of Obstetricians and Gynaecologists
ACT	Artemis Comparison Tool
Adk	Adenylate kinase
Ag	Antigen
APEC	Avian Pathogenic *Escherichia coli*
AT	Autotransporter
AUM	Asymmetric Unit Membrane
bla	β-lactamase
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BRIG	Blast ring Image Generator
BSAC	British Society for Antimicrobial Chemotherapy
BSI	Blood Stream Infection
BURST	Based Upon Related Sequence Types
CC	Clonal Complex
CFU	Colony Forming unit
CGE	Center for Genomic Epidemiology
CNF1	cytotoxic necrotizing factor 1
COG	Clusters of Orthologues
CPE	Cytopathic Effect
CPD	Critical point Drying
Csr	Carbon storage regulatory system
CU	Chaperone Usher
DAEC	Diffusely Adherent *Escherichia coli*
DF	Derriford Hospital
DFV	Discoid/Fusiform Vesicles
DHFR	Dihydrofolate reductase

DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
Ebp	Endocarditis and biofilm associated pilus
ECM	Extracellular Matrix
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extra-intestinal pathogenic *Escherichia coli*
FBS	Foetal Bovine Serum
FCA	Flow Cytometry Analysis
fimH	Type 1 Fimbrial Adhesin
fliC	Flagellin protein
fliD	Flagellin capping protein
fumC	Fumarate hydratase
fyuA	Ferric Yersiniabactin uptake receptor
GP	General Practitioner
GPI	glycosylphosphatidylinositol
gyrB	DNA gyrase
hly	Haemolysin
IBC	Intracellular Bacterial Communities
icd	isocitrate/isopropylmalate dehydrogenase
IQA	Internal Quality Control
IS	Insertion Element
kDa	Kilodalton
LB	Lysogeny broth
LPS	Lipopolysaccharide
MALDI-TOF MS	Matrix-assisted laser desorption Ionization-Time Of Flight Mass Spectrometry
mbp	Million Base Pairs

mdh	malate dehydrogenase
MEM	Minimum Essential Medium
MIC	Minimum Inhibitory Concentration
ML	Maximum Likelihood
MLEE	Multi-locus Enzyme Electrophoresis
MLST	Multi-locus Sequence Typing
MOI	Multiplicity Of Infection
MPS	Massive Parallel Sequencing
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NMEC	Meningitis-associated *Escherichia coli*
NPV	Negative Predictive Value
ORF	Open Reading Frames
OS	Open Source
P pilus	Pyelonephritis-associated pili
PAI	Pathogenicity Island
pap	Pyelonephritis associated pilus
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PHE	Public Health England
PMN	Polymorphonuclear leukocytes
PPV	Positive Predictive Value
prs	Pap related sequence
purA	adenylosuccinate dehydrogenase
recA	ATP/GTP binding motif
rUTI	Recurrent Urinary Tract infection
SA	Saudi Arabia
Sc	Scanty

SDEM	Standard Error of the Mean
SEM	Scanning Electron Microscopy
SfaS/H	S-fimbrial Tip Adhesin
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
ST	Sequence Type
STEC	Shiga-toxigenic *Escherichia coli*
TCS	Two Component System
TTC	Triphenyltetrazolium chloride
T3SS	Type 3 Secretion System
TAE	Tris acetate-EDTA
TEM	Transmission Electron Microscopy
TLRs	Toll-like receptors
TPS	Two-Partner Secretion
UP	Uroplankin
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary Tract Infection
VF	Virulence Factor
WGS	Whole Genome Sequencing

Chapter One

1. General Introduction

1.1 *Escherichia coli*

The bacterium *Escherichia coli* (*E. coli*) is by far the most studied and best understood member of the microbial kingdom. Ubiquitous in nature and present in the digestive tract of humans and other vertebrates, this prototypic commensal organism has become one of the most clinically important bacterial species in modern medicine.

E. coli was originally identified as *Bacterium coli* commune in the late 19th century by the Bavarian paediatrician Theodor Escherich during his Habilitation (a postdoctoral qualification required to lecture at a German university) studies in Munich (Shulman, Friedmann & Sims, 2007). His pioneering work on the intestinal flora of infants revealed that meconium was sterile and that bacterial colonisation of the gastrointestinal tract was dependant on the child's environment and occurred within a few hours post-partum. *Bacterium coli* commune was one of 19 bacteria isolated during the Escherich studies and was subsequently renamed in his honour (Kaper, 2005; Shulman, Friedmann & Sims, 2007).

E. coli is a Gram negative, facultative anaerobic bacillus. It is often motile and can grow in temperatures ranging from 7°C to 50°C. It can be quickly isolated using basic culture media and can tolerate acidity to pH 4.4 (Lin *et al.*, 1995). These and other attributes have made *E. coli* the model organism for studies in molecular microbiology with molecular research dating back to the 1960's and the discovery of the *lac* operon induction mechanism (Jacob & Monod, 1961; Jacob *et al.*, 1960)

1.1.1 Pathogenic *E. coli*

The majority of *E. coli* strains are generally non-pathogenic, coexisting in a relationship beneficial to both itself and its host (Blount, 2015). However, the possession of specific virulence determinants, which are often present on pathogenicity islands or mobile genetic elements, have led to highly adaptive clones with the ability to cause a wide range of diseases (Alkeskas *et al.*, 2015; Johnson & Stell, 2000; Kaper, Nataro & Mobley, 2004; Kuhnert, Boerlin & Frey, 2000). Classification of *E. coli* pathotypes is broadly divided into two major groups, those that mainly cause enteric/diarrhoeal disease and those that cause infection outside the gastrointestinal tract; the latter termed Extra-intestinal pathogenic *E. coli* (ExPEC) (Russo & Johnson, 2000). *E. coli* pathotypes that cause enteric/diarrhoeal disease are well-described and subdivided into six categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) ('Centres for Disease Control and Prevention: E.coli,' 2015; Kaper, Nataro & Mobley, 2004). The ExPEC group can also be subdivided into at least two main categories: uropathogenic *E. coli* (UPEC) and the strains responsible for meningitis and sepsis meningitis-associated *E. coli* (NMEC) (Kaper, Nataro & Mobley, 2004; Russo & Johnson, 2000; Wijetunge *et al.*, 2015). In general, each pathotype falls into a clonal group dictated by either their O (somatic) and H (flagellar) antigens (Kauffmann, 1946; Kauffmann, 1947; Kauffmann, 1964) or by genetic sequencing techniques such as multilocus sequence typing (MLST) (Wirth *et al.*, 2006) or, more recently, whole-genome phylogenic single

nucleotide polymorphism (SNP) analysis (Kaas *et al.*, 2014). This study will focus on the UPEC group of ExPEC.

1.2 Overview of Urinary tract infections (UTI)

Urinary tract infections (UTI) are one of the most common bacterial infections treated by general practitioners (GP) (Sarkar *et al.*, 2014) with the diagnosis and treatment costing billions per year (Foxman, 2010). A UTI is generally defined as any infection of the bladder (cystitis) and/or kidneys (pyelonephritis) (Isaacs, 2014). Although the bacteria that cause UTI can be transmitted sexually, it is thought that most originate endogenously (Foxman *et al.*, 1997; Nielsen *et al.*, 2014; Ulleryd *et al.*, 2015; Yamamoto *et al.*, 1997). The occurrence of UTI in the female gender is much higher due to the shorter fatter urethra and its close proximity to the anus. (Hickling, Sun & Wu, 2015; Minardi *et al.*, 2011).

The majority of UTIs are diagnosed at the GP surgery with assessment of dipstick results and accompanying characteristic symptoms such as increased frequency, dysuria, cloudy appearance and pelvic tenderness. In such situations, empiric antibiotic treatment is prescribed by the GP surgery. Laboratory analysis is only recommended under certain circumstances such as pregnancy, UTI in men, suspected pyelonephritis, failed antibiotic treatment, recurrent UTI or the presence of renal impairment (McNulty, 2017).

It is estimated that up to 50% of women and 5% of men will suffer from at least one UTI over the course of their lifetime (Ulett *et al.*, 2013). Among common infections, UTI are the most frequent laboratory-diagnosed urological disease

resulting in an immense financial burden on national healthcare systems (Foxman, 2002; Lau *et al.*, 2008b; Loveday *et al.*, 2014). In the year 2000, studies from the United States estimate the cost of evaluation and treatment of UTIs was $3.5 billion (ACOG, 2008). Several bacterial pathogens can be responsible for UTIs, but *E. coli* is by far the most common aetiological agent accounting for approximately 90% of all community acquired UTI and 50% of all nosocomial infections (Kuhnert, Boerlin & Frey, 2000; Srinivasan, Foxman & Marrs, 2003).

1.2.1 UTI caused by UPEC

UPEC isolates gain entry to the urinary tract (Figure 1) via migration from the periurethral area or through sexual activity (Foxman *et al.*, 1997). Colonisation of the bladder can first result in cystitis and continued ascension up the ureters to the kidneys can lead to pyelonephritis and sepsis (Flores-Mireles *et al.*, 2015; Floyd *et al.*, 2012). The invading bacteria may have the ability to invade the superficial epithelial cells and replicate, forming large intracellular inclusions, which may serve as a source for recurrent acute infections (Anderson *et al.*, 2003; Mulvey, Schilling & Hultgren, 2001; Schilling & Hultgren, 2002).

The ability of UPEC to bind to uroepithelia is a critical first step in the pathogenesis of UTI (Flores-Mireles *et al.*, 2015). The array of bacterial adhesins available to UPEC isolates is extensive, ranging from long hair-like polymeric protein fibres that can extend far into the surround environment, to small monomeric proteins that are directly anchored to the cell surface (Chahales & Thanassi, 2015). The various adhesins have higher specificity for

Figure 1 has been removed due to Copyright restrictions

Figure 1. Urinary tract infection disease progression. Figure adapted from Flores-Mireles *et al., 2015.*

certain cell types within the uroepithelia. The Type 1 pili are one of the best studied fimbriae expressed by most UPEC. These fimbriae often present with a FimH mannose binding adhesin that allows attachment to uroplankins coating the bladder epithelia, initialising the first stages of cystitis, whereas the P pilus (denoted originally due to the observed association with kidney disease,

‘Pyelonephritis-associated pili’) has three predominant alleles of the PapG adhesin. Each binds to a variation of the membrane-associated antigens with Gal(α1–4)Gal moieties within glycolipids, with the PapGII specifically binding to the globo-series variety found on the kidney epithelial cells (Chahales & Thanassi, 2015; Mulvey, 2002).

UPEC strains contain an arsenal of virulence factors specifically selected or adapted to cause disease within the urinary tract and elicit a potent immune response within the host. In cystitis, attachment to bladder epithelia triggers apoptosis and exfoliation in response to the potential invasion of UPEC into bladder umbrella cells (Kaper, Nataro & Mobley, 2004). The binding also induces potent induction of innate responses such as local inflammatory events that induce cytokine production and neutrophil induction (Ulett *et al.*, 2013). UPEC also secrete a number of toxins that can damage or kill host cells, such as α-haemolysin. The death and subsequent exfoliation of epithelial cells expose deeper layers of the urothelial for colonisation (Wiles, Kulesus & Mulvey, 2008).

An essential nutrient for UPEC metabolism is iron, a commodity that is deficient in the bladder environment. The lysis of host cells by toxins (such as α-haemolysin) release essential nutrients into the bladder lumen, including iron. A common characteristic of UPEC is the ability to efficiently scavenge for iron molecules from a variety of sources. This is reflected by the possession of several siderophores iron scavenging systems present within the UPEC genome (Flores-Mireles *et al.*, 2015).

Cystitis can be a very painful experience but it is usually treated successfully with empiric antibiotics. A much more serious form of UTI is pyelonephritis, a potentially life-threatening infection in which delayed treatment can lead to irreversible kidney damage and septicaemia. Patients suffering with acute pyelonephritis may present with the typical symptoms of cystitis but may also present with flank pain particularly in the lower back, fever, chills, nausea and vomiting (Orenstein & Wong, 1999). Historically, studies have shown that variants of UPEC possessing particular virulence factors (such as papG), are predominant with this type of infection (Lane & Mobley, 2007; Lund *et al.*, 1988; Roberts *et al.*, 1994); the serum from patients suffering from pyelonephritis containing P-fimbrial antibodies (de Ree & van den Bosch, 1987). However, no one UPEC virulence trait can be solely responsible for a particular aetiology, with reported incidences of non P-fimbrial isolates causing pyelonephritis and urosepsis (Gisela *et al.*, 1993; McNally *et al.*, 2013). Additionally, the expression of virulence factors in the urinary tract is multifactorial and can be modulated by the host response. UPEC often carry chromosomal gene clusters of virulence factors on pathogenicity islands (PAI) whose expression can be co-regulated by environmental stimuli (Hacker *et al.*, 1997; Wullt, 2003).

1.2.2 Uncomplicated and complicated UTIs

Urinary tract infections are often characterized as complicated or uncomplicated. This designation is used commonly in the nosocomial setting when predisposing factors are relevant when considering treatment options. Uncomplicated UTI include acute cystitis or acute pyelonephritis in otherwise healthy, non-pregnant, premenopausal females with no underlying conditions.

The vast majority of UTIs are uncomplicated and treated empirically at the GP surgery. Complicated UTIs are those that occur in combination with numerous predisposing conditions such as congenital abnormalities, prior urological surgery, increased age, foreign bodies in the urinary tract (catheter), pregnancy and diabetes mellitus (ACOG, 2008; Stamm & Norrby, 2001).

1.2.3 Asymptomatic Bacteriuria

The presence of bacteria in the urine is common. Urine in itself is a very good medium for bacterial cultivation (Asscher *et al.*, 1966). Therefore, it is not surprising that urines sent for clinical microbiological examination frequently grow one or numerous strains of bacteria. Often, the identification of more than one type of bacteria within a urine culture is considered as contamination; the organisms contaminating urine during micturition via the urethra lumen or the periurethral area.

Asymptomatic bacteriuria (ABU) is a condition in which high counts (often $>10^5$ cfu/ml) of bacteria are present in the urine but patients present with no urinary symptoms. *E. coli* are the most common organisms isolated with ABU and the high incidence increases with age. The incidence in premenopausal women ranges from 2% to 5% whereas the prevalence in long-term care facilities is extremely high, ranging from 25% to 50% for women and 15% to 40% for men (Nicolle, 2015). The resident bacteria can persist for days or years and only become problematic in circumstances such as pregnancy, genitourinary surgical procedures or immunocompetency (Nicolle, 2015). Strains of bacteria that cause ABU are thought to prevent the adherence of pathogenic bacteria,

increase the competition for nutrients and consequently prevent infection; a term known as bacterial interference. Often, one of the best defences against acquiring a UTI is the simple act of regular fluid intake and frequent urination; or a common prerequisite to UTI is dehydration. The continuous ascension of bacteria in the urinary apparatus is thwarted by the powerful, pressurised act of regular urination; physically flushing bacteria out of the bladder/urethra. If bacterial interference can lessen the frequency of attachment in the urethra and bladder epithelia, it is hypothesized the pathogenic bacteria will simply be washed away (Foxman & Frerichs, 1985; Reid, Howard & Gan, 2001). The use of avirulent bacteria to prevent UTI is an appealing notion, especially with rates of antimicrobial resistance ever increasing. ABU 83972 is well-documented strain of *E. coli* (Darouiche *et al.*, 2001; Hull *et al.*, 2000; Hull *et al.*, 1999; Trautner *et al.*, 2002) isolated from a young Swedish girl with ABU who had carried it for at least 3 years without symptoms (Hansson *et al.*, 1989). Studies have shown that this strain has the ability to prevent UTI by bacterial interference in a number of selected individuals for a specified period of time for both catheterised patients and patients who suffered from recurrent UTI (Darouiche *et al.*, 2011; Darouiche *et al.*, 2005; Sunden *et al.*, 2010; Trautner *et al.*, 2002).

1.3 Laboratory Diagnosis of Urinary Tract Infections

As previously mentioned, the presence of bacteria in urine is common. The distinction between asymptomatic bacteriuria, contamination and UTI is made on the basis of bacterial concentration coinciding with typical urinary symptoms. However, high bacterial counts in urine are common, especially in the elderly

and often not indicative of a UTI. Additionally, delays in specimen transit or incorrect specimen storage, can cause bacterial overgrowth and lead to false positive results. It was pioneering work by Kass in 1956 who first suggested that a significant bacteriuria was based on a quantitative urine culture of 100,000 colony forming units (CFU)/ml (Kass, 1956; 1957). Since then, further work has identified that a significant percentage of women possessing urinary symptoms can have bacterial counts as low as 100 CFU/ml, suggestive of 'early phase' UTI (Kunin, White & Hua, 1993).

The guidelines pertaining to the diagnosis of UTI in the UK involve urine cultures yielding a significant bacterial count of a single organism at $\geq 10^4$ CFU/ml (*Escherichia coli* or *Staphylococcus saprophyticus* at 10^3 CFU/ml). Positive urine cultures often yield concentrations $>10^5$ CFU/ml with an increased concentration coinciding with an higher positive predictive value (Public Health England, 2017). There are circumstances where a bacterial count of 10^2 CFU/ml may be regarded as significant (e.g. suprapubic aspirates) but each specimen must be considered individually taking into account the patients clinical information, urinalysis results and clinically relevant symptoms. Guidelines to aid diagnosis are issued by Public Health England (PHE) (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/619772/Urinary_tract_infection_UTI_guidance.pdf).

1.4 Uropathogenic *Escherichia coli*

Many of the major virulence factors important in UTI have been identified in UPEC (Table 1) and established PCR screens exist for their detection (Johnson

& Stell, 2000; 2002). Both host and bacterial factors contribute to the pathogenesis of UTI (Sussman & Gally, 1999) but higher proportions of these specific bacterial virulence traits have been detected in cystitis and pyelonephritis isolates (Er *et al.*, 2015).

The combination of virulence genes and antigenic diversity distinguish UPEC from commensal and diarrheagenic strains (Kaper, Nataro & Mobley, 2004) with a relatively small number of O serogroups consistently identified from UPEC isolates (O1, O2, O4, O6, O7, O8, O15, O16, O18, O21, O22, O25, O75 and O83) (Kaper, Nataro & Mobley, 2004; Li *et al.*, 2010).

The reservoir for UPEC is the mammalian gastrointestinal tract as the isolates responsible often match rectal isolates from the same patient (Russo *et al.*, 1995b). Here they endure without consequence and, as other members of the *Escherichia* genus, may have a beneficial function aiding digestion. But their capacity to disseminate, colonise the urinary tract and cause disease is what distinguishes them from commensal *E. coli*. Studies have shown that the transference of UPEC isolates can occur not only by sexual interaction (Foxman *et al.*, 2002; Ulleryd *et al.*, 2015) but also by ingestion of contaminated food (Manges *et al.*, 2001) or, potentially, even by environmental exposure (Leonard *et al.*, 2018).

It is true to say that UPEC possess more virulence factors than commensal *E. coli* with virulence genes often encoded within PAIs; regions with GC nucleotide content distinct from the rest of the genome (Blum *et al.*, 1994; Hacker & Kaper, 2000). However, the high level of genetic overlap between pathogenic and non-pathogenic strains together with the diversity of known and unknown UPEC-

associated virulence factors makes it very difficult to attribute one distinct set of virulence factors to this group of organisms (Wiles, Kulesus & Mulvey, 2008).

Avian pathogenic *Escherichia coli* (APEC) share many virulence factors with UPEC. So much so that UPEC CFT073 and UPEC U17 have shown to cause disease in an avian infection models (Moulin-Schouleur *et al.*, 2007; Zhao *et al.*, 2009)

Table 1.Predominant virulence factors associated with UPEC

	Target gene(s)	Virulence Factor	Comments	References
Adhesins	*papG(II,III), papA, papH, papC*	P fimbriae	papG II strongly associated with pyelonephritis. Binds to Gal(α1-4)Gal moieties expressed within the glycolipids present on renal epithelia, specifically the P blood-group antigen. Known to bind to Bowman's capsule. papG III has been associated with cystitis.	(Chahales & Thanassi, 2015; Lane & Mobley, 2007; Stromberg *et al.*, 1990; Subashchandrabose & Mobley, 2015)
	fimH	Type 1 fimbriae	Monomannose *fimH* variants in UPEC have a high tropism for uroplankin which cover almost the entire luminal surface of the bladder.	(Chahales & Thanassi, 2015; Chia-Suei *et al.*, 2002; Mulvey, 2002; Zhou *et al.*, 2001)
	sfaS	S fimbriae	Closely related adhesins. S fimbriae binding to sialic acid residues present in the proximal and distal tubials amongst other renal tissues and bladder uroplankin III. F1C fimbriae binds to galactosylceramide and globotriaosylceramide present in the bladder, ureters and kidneys.	(Backhed *et al.*, 2002; Khan *et al.*, 2000; Kreft *et al.*, 1995; Malagolini *et al.*, 2000; Mulvey, 2002; Riegman *et al.*, 1990; Wurpel *et al.*, 2013)
	Sfa/focDE	S and F1C fimbriae		
	focG	F1C fimbriae		
	afa/draBC	Dr family	Family includes the fimbrial and non-fimbrial adhesins. The fimbrial Dr adhesin can bind to type IV collagen as well as short consensus repeat sequences within the complement receptors on the uroepithelium. Known to bind to renal tubular epithelia and the Bowman's capsule.	(Mulvey, 2002; Nowicki *et al.*, 1988; Nowicki, Selvarangan & Nowicki, 2001)

Table 1. Continued

	Target gene(s)	Virulence Factor	Comments	References
Toxins	*cnf1*	Cytotoxic necrotizing factor	Binds to laminin receptor and can induce host cell cytoskeleton rearrangements. Interferes with phagocytosis and causes apoptosis of bladder epithelia.	(Chung *et al.*, 2003; Emody, Kerenyi & Nagy, 2003)
	Sat	Secreted autotransporter toxin	Associated with pyelonephritis. A vacuolating cytotoxin that elicits damage to kidney epithelium	(Emody, Kerenyi & Nagy, 2003; Guyer *et al.*, 2002)
	hlyA	Haemolysin	α (exported) and β (membrane bound) Haemolysins cause lysis of both erythrocytes and leukocytes. α –haemolysin can also target endothelial and renal epithelial cells.	*(Emody, Kerenyi & Nagy, 2003; Wandersman & Delepelaire, 1990)*
Siderop-hores	*fyuA*	Yersiniabactin	Aids growth under iron restriction. Exported from bacterial cell and attach to ferric iron from iron chelator molecules. Iron siderophore complex transported back into the bacteria by membrane bound receptors.	*(Emody, Kerenyi & Nagy, 2003; Subashchandrabose & Mobley, 2015)*
	iutA	Aerobactin		
Capsule	*kpsMT II* and *III* *kpsMT K1* *kpsMT K5*	K-Antigen	The capsule provides protection against phagocytic engulfment and complement mediated bactericidal effect in the host	(Emody, Kerenyi & Nagy, 2003; Sarkar *et al.*, 2014; Wang, Wang & Reeves, 2010)
Lipopoly-saccharide	*wzx/wzy*	O-Antigen	Protect from bactericidal activity of human serum and aids survival.	(Emody, Kerenyi & Nagy, 2003; Sarkar *et al.*, 2014)
Motility	Various	Flagellum	Aids ascension of the urinary tract and allows bacteria to adapt to its environment. Also some evidence to suggest motility may contribute to renal epithelia cell invasion.	(Allison *et al.*, 1994; Harmon *et al.*, 1989; Pichon *et al.*, 2009)

1.5 Typing techniques for *Escherichia coli*

There is a multitude of *E. coli* strains and serotypes (Ochman & Selander, 1984) including commensal strains and pathogenic groups capable of causing a wide variety of human infections, a fact reflected in the antigenic diversity of this pathogen. It was the pioneering work by Fritz Kauffman beginning in the 1940's that originally introduced the serotyping scheme for members of the *Enterobacteriacae* (Kauffmann, 1946; 1947; 1964). The classification scheme uses the presence and identification of O-antigens (Somatic or cell wall antigens), H-antigens (flagella protein) and K-antigens (Capsular polysaccharide antigen) to identify serological types of *E. coli* and other *Enterobacteriacae*. The variation within these serological targets is enormous. For example, the polyoligosaccharide length, composition and linkages between the sugars, give the O-antigen its structural diversity with nearly 200 different variations for just *E. coli* (Iguchi *et al.*, 2015; Samuel & Reeves, 2003). Typing *E. coli* serologically is a useful tool for the purpose of clinical and epidemiological investigations but requires numerous versions of expensive antisera and is not sufficient for defining phylogenetic relationships amongst *E. coli* strains (Diamant *et al.*, 2004).

The *E. coli* reference (ECOR) collection (Ochman & Selander, 1984) consists of 72 commensal and pathogenic strains selected to represent the genetic diversity of the species. Multi-locus enzyme electrophoresis (MLEE) (Selander *et al.*, 1986) of these isolates originally identified four distinct phylogenic groups (A, B1, B2 and D) with different pathotypes belonging to each. Further investigations have increased the phylogeny to eight groups (A, B1, B2, C, D, E, F and the cryptic clade I) (Beghain *et al.*, 2018; Clermont *et al.*, 2013).

Commensal strains usually belonging to groups A and B and ExPEC strains generally belonging to groups B2 and D (Carlos *et al.*, 2010).

The most popular and useful method for typing *E. coli* is currently Multi-locus sequence typing (MLST). MLST is a molecular technique that relies on the sequence variation from multiple 'housekeeping' genes and allows the assignment of an allele number to each gene locus. The collection of allele numbers for each bacterial isolate denotes the allelic profile or sequence type (ST). MLST was first introduced for *Neisseria meningitidis* (Maiden *et al.*, 1998), the pathogen responsible for meningococcal meningitis and septicaemia. MLST schemes are now available for most pathogenic bacteria (https://pubmlst.org/databases/) and the approach holds advantages over other typing techniques as the data produced is considered unambiguous, portable and can aid meaningful evolutionary analysis.

There are three main MLST databases for *E. coli*, the *Ec*MLST hosted at Michigan State University (East Lansing, USA), which originally focused on enteropathogenic *E.coli* (Reid *et al.*, 2000), the Pasteur scheme hosted at the Pasteur Institute (Paris France), and the Acthman scheme (Wirth *et al.*, 2006) hosted at the Warwick Medical School (Warwick, UK), the latter two schemes not focusing on any particular group of *E. coli*. The congruence between phylogenies derived from concatenated allele sequences from each MLST scheme and the phylogeny based on WGS has advocated the use of the Acthman scheme for *E. coli* (Clermont, Gordon & Denamur, 2015); now the most widely used MLST method. The Acthman scheme uses the internal fragments of seven house-keeping genes (*adk*: adenylate kinase; *fumC*: fumarate hydratase; *gyrB*: DNA gyrase; *icd*: isocitrate/isopropylmalate

dehydrogenase; *mdh*: malate dehydrogenase; *purA*: adenylosuccinate dehydrogenase; *recA*: ATP/GTP binding motif) to determine the allelic profile of each isolate.

MLST has become the most common and valuable molecular method for typing UPEC isolates. This method has allowed researchers to identify dominant community and nosocomial UPEC isolates. Reports from around the world have consistently identified specific STs or clonal complexes (CC) (isolates with at least 6 of the 7 identical alleles), which predominate study collections. The most prominent STs or CCs identified are ST131, ST73, ST69, ST95, ST10, ST14 and ST127 (Croxall *et al.*, 2011a; Gibreel *et al.*, 2012b; Nuesch-Inderbinen *et al.*, 2017; Salipante *et al.*, 2015; Yamaji *et al.*, 2018; Yun *et al.*, 2015) with the antibiotic resistant ST131 currently attracting the most attention (Cha *et al.*, 2016; Gibreel *et al.*, 2012a; Lau *et al.*, 2008a; Lau *et al.*, 2008b).

1.6 *Escherichia coli* Multi-locus sequence type 127

Recent analysis using MLST data and the Based Upon Related Sequence Types (BURST) clustering algorithm (eburst.mlst.net) suggests that UPEC ST127 is a founder of a distinct and newly emerged clonal complex (Gibreel *et al.*, 2012b). Using an established PCR screen for virulence factors known to contribute to UTI (Johnson & Stell, 2000; 2002), studies have shown that ST127 possess significantly more virulence factors than other UPEC ST (Alghoribi *et al.*, 2014; Beyrouthy *et al.*, 2013; Croxall *et al.*, 2011a; Gibreel *et al.*, 2012b; Salipante *et al.*, 2015). UPEC ST127 are responsible for a significant proportion of extra-intestinal infections primarily of the urinary tract (Gibreel *et al.*, 2012b)

but have also been implicated in blood stream infections (BSI) (Adams-Sapper *et al.*, 2013; Ciesielczuk *et al.*, 2016) and necrotizing enterocolitis in preterm infants (Ward *et al.*, 2016). Members of the ST127 clone possess the common uropathogenic O6 serotype (Johnson, 1991) and display an increased lethality in comparison to the more common UPEC ST lineages (ST73, ST131, ST95) with an *in vivo* insect model of infection (*Galleria mellonella*) (Alghoribi *et al.*, 2014).

Surprisingly, the incidence of isolates from ST127 in most epidemiological studies of UPEC seems to be relatively low when compared to other STs with considerably lower virulence potential. Additionally, ST127 has a notable lack of antibiotic resistance when compared to other UPEC (Alghoribi *et al.*, 2015; Banerjee *et al.*, 2013; Croxall *et al.*, 2011a; Gibreel *et al.*, 2012b; Hertz *et al.*, 2016).

However, reports of emerging resistance to antibiotics, including the cephalosporins (Beyrouthy *et al.*, 2013; Darling *et al.*, 2014; Ferjani *et al.*, 2017; Gomi *et al.*, 2017; Zurfluh *et al.*, 2016), suggests members of this UPEC ST127 lineage are increasingly becoming a cause for clinical concern and give this strain the potential to emerge as a significant threat to human health. The combination of high virulence potential with antibiotic resistance could lead to a substantial increase in cases of infection caused by ST127 strains.

1.7 Polymicrobial urine culture

It is believed that a true UTI involves a single infecting organism, with the current national clinical guidelines reflecting this opinion (McNulty, 2017). Urine

cultures with multiple organisms are often regarded as contamination with the culture reported as ‘mixed faecal flora’. This is understandable when considering the close proximity of the urethral opening to the anus, faeces containing billions of bacteria per gram (Franks *et al.*, 1998) and urine being an excellent culture medium (Kass, 1956). However, polymicrobial infections are possible. The isolation of more than one organism in what is considered a true UTI is a relatively common occurrence (Siegman-Igra *et al.*, 1994). The debate surrounding polymicrobial infections is one that is difficult to answer; is the presence of another bacterial species aiding the survival or disease progression with some kind of symbiotic interaction? Or does mixed growth represent contamination? Mixed growth from urine is especially common in the elderly. This may be due to a number of factors related to age, including the increased likelihood of faecal incontinence, an increased post voiding residual volume, changes in colonising organisms associated with increased vaginal alkalinity in postmenopausal women and changes in pelvic musculature (Cove-Smith & Almond, 2007; Norton *et al.*, 2010).

The current PHE guidelines for the diagnosis of UTI do not specifically refer to polymicrobial infections directly, but do refer to cultures indicative of a UTI with one predominant organism at a concentration of 10^5 CFU/ml. Dependant on the criteria employed, this may or may not be determined as a polymicrobial infection.

Although polymicrobial UTI have been identified in research articles, the UK national guidelines do not directly recognise or propose any therapeutic options relating to such an infection. This is understandable as conclusive evidence regarding polymicrobial UTI has yet to be presented. Furthermore, often it can

be impossible for a clinician to distinguish between asymptomatic urological colonisation, faecal contamination and a true mixed infection; each organism acting individually as a pathogen or possibly synergistically. There still remains limited information on the difference in clinical and bacteriological features between monomicrobial and polymicrobial urinary infections, but the progress of a polymicrobial UTI leading to septicaemia is well documented with evidence to suggest that polymicrobial septicaemic infections, which can often originate from a UTI, have higher rates or mortality (McNally *et al.*, 2013; Siegman-Igra *et al.*, 1994; Spencer & Nicol, 1986).

As bacteria in the urinary tract are thought to gain entry via an ascending manner, the motility of invading organisms is an important virulence factor that is often overlooked. However, *Staphylococcus saprophyticus* and *Enterococcus faecalis* are two non-motile, Gram positive organisms often isolated in urine specimens whose ascendance can be attributed to urethral massage (ACOG, 2008). *S. saprophyticus* is often isolated in young newly sexually active females (earning this type of infection the nickname ‘Honeymoon cystitis’) (Sanya Tahmina Jhora, 2011) and *Ent. faecalis* which can cause UTI (albeit at a low prevalence) occur mainly in hospitalised patients with underlying disease or with catheter *in situ* (Hall *et al.*, 1992). *Ent. faecalis* has also been identified as an organism frequently isolated with *E. coli* in urine specimens (Croxall *et al.*, 2011b), this being one of the many aspects studied in this thesis.

1.8 *Enterococcus faecalis*

The enterococci are Gram-positive, (generally) non-motile, facultatively anaerobic cocci that possess the ability to survive under harsh environmental conditions (Arias & Murray, 2012; Fisher & Phillips, 2009). They are a common commensal of the mammalian gastrointestinal tract and periurethral area (Hooton *et al.*, 2013), and are generally considered non-pathogenic in most immunocompetent hosts (Kommineni *et al.*, 2015). However, the enterococci are known to be a common cause in several clinical infections such as UTI, bacteraemia, neonatal infections and endocarditis (Arias & Murray, 2012; Madsen *et al.*, 2017; Whiteside *et al.*, 2018). *Enterococcus faecalis* is the predominant species of enterococci isolated from urine cultures with infections particularly associated with the elderly and in individuals with indwelling urinary catheters (Croxall *et al.*, 2011b; Huycke, Sahm & Gilmore, 1998; Kart *et al.*, 2017; Whiteside *et al.*, 2018). This association is partially due to the ability of enterococci to persist and form biofilms (Dale *et al.*, 2017). Enterococci are reported to cause 25% of all catheter-associated UTI (Ch'ng *et al.*, 2019) and several VFs (virulence factors) have been identified, with two of the most studied being the Ebp (endocarditis and biofilm-associated) pili and Esp (*Ent. faecalis* surface protein), both associated with attachment and biofilm formation (Flores-Mireles *et al.*, 2016; Garg, Mohan & Taneja, 2017; Shankar *et al.*, 2001; Tendolkar *et al.*, 2004). The gastrointestinal tract and periurethral area is known to be a natural reservoir for *Ent. faecalis*, therefore the presence of this organism in urine culture is often disregarded as contamination. However, a few authors have postulated a symbiosis with *E. coli* with regards to urinary infection

(Croxall *et al.*, 2011b; Lavigne *et al.*, 2008); an area of study further explored in this thesis.

1.9 General study aims and objectives

The purpose of this study is to generate a deeper understanding towards the clinical relevance of UPEC ST127, an emerging strain that remains largely susceptible to many antibiotics and which has enhanced virulence potential and could become a significant cause of morbidity and mortality in the future.

The first part of this study aims to determine the prevalence and microbial composition of polymicrobial UTIs, initially involving the examination of a large cohort of clinical urine specimens, which included recovery of *E. coli* in any capacity (UTI, polymicrobial infection, asymptomatic or contamination). Anecdotal evidence from clinical records (M Upton, personal communication) suggests that UPEC ST127 may be more commonly isolated in polymicrobial infections; a premise that will be further studied in this part of the study.

The further identification and quantification of all bacteria isolated from urine specimens will determine the number of mixed infections and whether any particular organisms are predominantly associated with *E. coli*.

To determine if the hypothesis that ST127 are frequently isolated in polymicrobial infections, a negative screen PCR will be designed and validated to quickly remove non-ST127 isolates from this large culture collection allowing MLST to be performed on the remaining specimens. From these studies it should be possible to determine the true prevalence of ST127 in mono- and poly-microbial UTIs.

The second part of this study will use whole genome sequencing technology and computational analysis to determine specific genetic features of both UPEC ST127 and a collection of *Ent. faecalis* isolated in the same urine as *E. coli*. The genomes of ST127 will be compared against other UPEC to detect and explore genomic regions specific to ST127. These regions will be examined to determine if they can be used to facilitate the design of a fully validated ST127 specific PCR. Further examination of ST127 genomes will allow the establishment of a full virulence profile and phylogeny characteristics for ST127 isolated from 3 different geographical locations. Additionally, the genomes for a set of *Ent. faecalis* will be examined for known virulence traits in an effort to distinguish pathogen from commensal. Using comparative genomics, each *Ent. faecalis* specimen will also be compared to other *Ent. faecalis* isolated from a range of diverse locations/pathologies in order to detect any phylogenetic similarities or common ST lineages between urine isolates and other pathogenic/non-pathogenic strains of *Ent. faecalis*.

The third part of this study examines the particular virulence characteristic of epithelial cell invasion and will attempt to assess if bacterial motility has any bearing on the ability of UPEC to bind to and invade bladder epithelia. Both the binding and invasive capability of ST127 will be correlated with the genomic sequence data in an attempt to determine if the presence, absence or variation of any virulence determinants has an effect on the pathogens ability to bind to and invade uroepithelium. All results from this assay will be confirmed using scanning (SEM) and Transmission (TEM) Electron Microscopy.

Chapter two

2. The prevalence of uropathogenic *Escherichia coli* multi-locus sequence type 127 in monomicrobial and polymicrobial urinary tract infections from hospital specimens in south west England.

2.1 Introduction

By definition, polymicrobial disease describes the pathological manifestation of disease caused by more than one type of microorganism. The microorganisms in question may often cause disease independently, but their ability to thrive or cause disease may be enhanced by the presence of additional organisms. Polymicrobial infections are well documented in the literature and are common in diseases such as periodontitis/gingivitis (Ingham & Sisson, 1984; Peters & Noverr, 2013; Sug Kim *et al.*, 2015; Wiksten *et al.*, 2015), abscesses (Bogdan *et al.*, 2015; Kommedal *et al.*, 2014; Sulaiman *et al.*, 2013), respiratory infections (Brealey *et al.*, 2015; Lijek & Weiser, 2012) and bacterial vaginosis (Pybus & Onderdonk, 1997; Pybus & Onderdonk, 1998; Rivers, Adaramola & Schwebke, 2011). The semantics surrounding this type of infection may infer an element of chance or a time period pertaining to the infection and, as such, may be labelled as a co-infection, complex infection, mixed infection, secondary infection, concurrent infection, synergistic infection or polymicrobial infection. The language describing these infections reflects the complex nature of the pathologies. Microbial synergy is well recognised but its role in pathogenesis is poorly understood and likely underestimated.

Some polymicrobial diseases may be somewhat easier to define and examine due to the site of infection. For example, if an aspirate is collected directly from

a brain abscess and two or more organisms are cultured from the specimen, a clinician can be confident that the organisms isolated from this (usually) sterile site will be the etiological agents responsible. However, when dealing with specimens from non-sterile sites, the interpretation becomes much more complex. Occasionally, when two known pathogens (such as *Streptococcus pneumoniae* and *Haemophilus influenza* from sputum) are isolated together in high numbers, it would be a fair assumption to deduce that both organisms would have a detrimental effect on the patient's well-being. However, when a commensal organism is isolated (which may be an opportunistic pathogen depending on circumstance) alongside a pathogen, from a site with significant numbers of commensal micro-organisms, the diagnostic assessment becomes much more complicated.

Dental plaques are polymicrobial biofilms and serve as a model example of how complicated it can be to identify the pathogen(s) causing a disease. It has been estimated that hundreds of species reside in the subgingival plaques in periodontal disease, and to further complicate the issue the organisms in question are indigenous, often have little pathogenic potential and can be isolated from healthy as well as diseased subjects (How, Song & Chan, 2016; Moore & Moore, 1994). Biofilms are structurally complex, dynamic systems which represent a significant and incompletely understood mode of growth for bacteria (Hall-Stoodley, Costerton & Stoodley, 2004). Additionally, polymicrobial infections may also include an interaction called microbial interference: the presence of one micro-organism establishes a niche in the host that supresses the colonisation and subsequent growth of another (Brogden, Guthmiller & Taylor, 2005).

Polymicrobial UTI can be a contentious subject because evidence must be sought which suggests that at least one of the micro-organisms directly benefits from the presence of another. As previously stated, the bacterial growth and subsequent clinical interpretation from a specimen originating from a site with significant levels of commensal micro-organisms can be difficult; urine is such a specimen. The organism(s) that cause a UTI will be thriving within the bladder/kidneys/ureters, however, for collection, a routine urine specimen must pass through the urethra and over the periurethral area, a region known to contain high concentrations of faecal bacteria (Hooton *et al.*, 2013). Consequently, it is not always possible to distinguish precisely where the bacteria originated from, the bladder, the urethra or the periurethra. Studies by Kass (Kass, 1956; Kass, 1957) introduced the concept of colony-count thresholds, correlating the bacterial concentration in urine with the symptoms of UTI, which in turn, has led to national guidelines for the interpretation of urine specimens (England, 2017). When following these guidelines, mixed growth is only reported if one organism is predominant. If two or more organisms are isolated in high numbers, it is likely the specimen will be considered contaminated. The only way to avoid urine contamination from areas external to the bladder is by temporary catheterisation or suprapubic aspiration. However both procedures are invasive, uncomfortable and not practical in today's healthcare system.

Mixed growth from a mid-stream urine specimen is very common in the elderly (Nicolle, 2001), as is asymptomatic bacteriuria (Nicolle, 2015) which further complicates the potential for diagnosing polymicrobial infection of the urinary tract. This project will attempt to generate a better understanding of

polymicrobial UTI by investigating a large cohort of urine specimens and identifying all the associated organisms isolated alongside *E. coli*, the most common etiological agent in UTI. To the best of the author's knowledge, this study will be the first of its kind and will include all age ranges and employ a strict criteria in regards to the exclusion of specimens contaminated with faecal flora. This study will also specifically look at urine isolates containing UPEC ST127, which will require the design and validation of a negative screen PCR assay that can quickly detect non-ST127 isolates. And finally, using MLST to identify ST127 isolates, the prevalence can be estimated, informing an answer to the question about whether this ST is seen more frequently in polymicrobial infections.

2.2 Methods

2.2.1 Clinical testing and isolation of suspect urinary pathogens

The majority of the clinical laboratory experimentation performed in this thesis was completed by the author. The thesis author is a registered HCPC Biomedical Scientist allowing access to limited clinical information and clinical laboratory equipment/protocols. The interpretation, bacterial identification (including MALDI-TOF analysis) and a limited amount of antimicrobial sensitivity testing were all performed by the author. Flow cytometry analysis, urine specimen inoculation and the majority of antimicrobial sensitivity testing was performed by the University Hospitals Plymouth (Derriford) NHS Trust clinical laboratory staff.

2.2.1.1 Sample collection

A total of 2897 urine specimens were included in this study. Patient urines were collected and processed routinely within the microbiology department by the clinical laboratory staff at Derriford hospital (University Hospitals Plymouth NHS Trust, Plymouth, Devon). Specimens for this study were collected over 3 time periods during April and May 2015. All specimens from each group consisted of consecutive laboratory numbers and were gathered from both hospital and community patients. All positive urine cultures that grew an *E. coli* along with up to two associated organisms were included, regardless of bacterial quantity, patient age, gender, symptoms, underlying conditions or location.

For the purpose of this study, urine cultures with >3 organisms were not included as it was postulated that urine specimens with increased numbers of

bacterial species would correlate with an increased likelihood of faecal contamination.

The first batch of urines examined consisted of 1888 consecutive specimens, the second 530 and the third 479. For the current study, all specimens were analysed after routine clinical laboratory investigation had been completed and data were collected anonymously, therefore, informed consent or ethical approval was not required.

2.2.1.2 Flow Cytometry Analysis.

The majority of specimens included in the study were negatively screened by the clinical laboratory staff using the Sysmex UF-100 urine analyser (Medical Electronics, Kobe, Japan). A small number of urine specimens were unable to be screened by Flow Cytometry Analysis (FCA) due to specific specimen protocols, insufficient volumes or urine consistency. Manual microscopy and culture were performed on such specimens. The Sysmex UF-100 determined urinary particles on the basis of their light scattering, fluorescence and impedance properties. Detection of the presence and number of leukocytes, bacteria, yeast-like cells, erythrocytes, epithelial cells, crystals, spermatozoa and pathological casts (Lun *et al.*, 1999) was established by FCA and only urine specimens that flagged positive were cultured.

2.2.1.3 Microbiological culture.

1µl of the FCA positive urines was streaked onto one quarter of BD™ CHROMagar™ Orientation Medium (BD, NJ, USA) using 1µl calibrated disposable loops and incubated aerobically at 35 to 37°C. BD™ CHROMagar™ Orientation Medium uses proprietary artificial substrates (chromogens) which release differently coloured compounds upon degradation by specific microbial enzymes, differentiating commonly isolated urinary pathogens (Becton-Dickinson, 2011). After 20 to 24 hours incubation, the Orientation medium was examined for the presence and quantity of bacterial growth. Analyses that formed part of this study were performed retrospectively (by the thesis author) and access to all urine cultures was only available once each laboratory specimen had been issued its final report.

2.2.1.4 Bacterial identification.

E. coli produced a characteristic dark rose to pink colony (Figure 2) on the Orientation medium and generally no further confirmatory tests were required. Strains of *Enterococcus*, *Streptococcus*, *Staphylococcus saprophyticus* and several members of the *Enterobacteriacae* all produced distinctive coloured colonies aiding rapid identification. However, when further confirmation was required for presumptive *E. coli*, the indole spot test (Becton-Dickinson, 2010) and/or Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI Biotyper, Bruker, Bremen, Germany) was employed to determine definitive identification.

Figure 2. Colony types on CHROMagar™ Orientation Medium. a, Characteristic dark rose to pink colonies of *E. coli*; b, Pink *E. coli* colonies mixed with another member of the *Enterobacteriaciae* (Green colony).

2.2.1.5 Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS).

Identification of all organisms associated with *E. coli* in polymicrobial urines and a small proportion of confirmatory testing for *E. coli* was performed using the MALDI Biotyper (Bruker) as per manufacturer instructions. Identifications were assigned by the MALDI Biotyper 2.0 software. On the occasions where 'no reliable identification' was obtained (either due to technical issues or an inadequate corresponding MS spectra), specimen analysis was repeated up to three times.

2.2.1.6 Bacterial quantification and diagnosis of UTI

The number of colonies of each organism isolated on the Orientation medium was recorded as shown in Table 2.

Table 2. Quantification of bacterial growth from urine culture

CFU count (1µl inoculum)	Interpretation	Equivalent concentration in urine (per ml)
1-10	Scanty	10^3
10-20	1+	10^4
21-99	2+	10^4
≥100	3+	10^5

Culture results and other clinical data were used in conjunction with current PHE guidelines (McNulty, 2017) for the diagnosis of a UTI (Figure 3). Patient details were not retained.

Criteria and evidence used to diagnose UTI in specimen collection

- Quantity of bacterial growth
- WBC count
- EPC count
- Clinical information
- Previous specimens and antimicrobial sensitivity results
- Catheter specimen considered significant if predominant organism isolated
- Removed if diagnosed as an Enterococcus UTI or *E.coli* was not the predominant organism.
- Most heavy mixed growth removed unless suspect E.coli recently isolated in blood culture
- PHE Guidelines
- Clinical laboratory Final report and comments

Figure 3. Overview of criteria used to determine if growth in urine was significant. WBC, White Blood Cell; EPC, Epithelial cell count; PHE, Public Health England.

2.2.1.7 Isolation and storage of bacterial isolates

Each organism was isolated by taking several individual colonies from the original Orientation medium culture plate and inoculating on to Columbia base blood agar (Oxoid, Basingstoke, UK). If the original urine culture was

considered a ‘pure’ *E. coli*, a sweep from the main inoculum on the Orientation medium was used for isolation on blood agar. Plates were incubated aerobically at 37°C for 20-24 hours and the entire growth removed and stored in 15% (v/v) glycerol (Sigma-Aldrich, Gillingham, UK) BHI broth (Oxoid) at -80°C until further testing was required.

2.2.1.8 Antimicrobial susceptibility of *E. coli*

All *E. coli* isolates suspected to cause UTI by clinical staff interpretation, along with all UPEC confirmed as ST127 isolates and UPEC isolated in the same urine specimen as ST127, were tested for antimicrobial susceptibility using the British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method for antimicrobial susceptibility testing

(http://bsac.org.uk/wp-content/uploads/2012/02/Version-12-Apr-2013_final.pdf).

Antibiotics tested (abbreviation and concentration (µg) in brackets) included Amoxicillin (AMO-10), Cefpodoxime (CPD-10), Cephradine (CEP-30), Co-amoxiclav (AMC-30 20 amoxicillin/10 clavulanate), Levofloxicin (LEV-1), Nitrofurantoin (NIT-200) and Trimethoprim (W-2.5). The choice of antibiotic susceptibility testing was in line with current clinical practice and protocol of University Hospitals Plymouth Teaching Hospitals, 2015.

2.2.1.9 Specimen information

The following anonymised information was collected from clinical computer databases for each specimen; age, specimen collection date, date specimen received, gender, specimen type, white blood cell count, epithelial cell count, antibiotic sensitivity results, requesting source (community/hospitalised patient) and information on previous urinary specimens received by the laboratory.

2.2.2 DNA extraction, molecular genotyping and screening for *E. coli* multi-locus sequence type 127

2.2.2.1 Primer design

In order to rapidly presumptively identify ST127 strains from the collection of *E. coli* isolated, a PCR screen was developed based on discriminatory SNPs in MLST loci for ST127. The nucleotide polymorphisms within each allele of UPEC ST127 were compared to all the corresponding alleles from all the known MLST strains by downloading each loci variant from the Warwick MLST *E. coli* database (https://enterobase.warwick.ac.uk/species/index/ecoli). Low frequency allele SNPs were identified using CLC genomics workbench 7 (CLC bio, Aarhus, Denmark) in five of the seven MLST alleles (*fumC*, *adk*, *gyrB*, *icd* and *purA*). Altogether, a total of 10 primer pairs were evaluated as possible ST127 allele specific primers. Amplification and optimisation studies were performed on all potential primer pairs. A specific guanine nucleotide in the *fumC*14 ST127 allele at position 368 was only observed in a small number of *fumC* alleles with the majority containing a cytosine base. A presumptive allele discriminatory forward

primer for *fumC*14 was designed based on the 368th nucleotide, with a primer to template mismatch of cytosine to cytosine placed at the 3' end of the forward primer to inhibit PCR amplification of none *fumC* ST127 alleles.

2.2.2.2 Multiplex PCR primers.

One primer pair, *fumC*368 (5'-CCCAGCGTTAACGGCGTC-3') and *fumC*R1 (5'-TCCCGGCAGATAAGCTGT-3') was identified as a potential allele specific discriminatory PCR. As UPEC ST127 have been shown to be predominantly associated with the O6 serogroup (Alghoribi *et al.*, 2015; Johnson *et al.*, 2008), O6 antigen specific primers (Li *et al.*, 2010) wl-14646 and wl-14647 (F 5'-GGATGACGATGTGATTTTGGCTAAC -3' and R 5'-TCTGGGTTTGCTGTGTA-TGAGGC -3', respectively) were used in conjunction with *fumC*368/*fumC*R1 to produce two distinct bands for any positive PCR result (547bp- fumC368 and 783bp-O6 Antigen).

2.2.2.3 Bacterial colony PCR extraction.

Stored *E. coli* specimens were grown on Muller-Hinton agar plates, streaked for single colonies and incubated aerobically at 37°C for 20-24 hours. Using bacterial mass taken from the lawn of growth in the main inoculum, or single colonies for confirmatory testing, a suspension equivalent to a 0.5 McFarland standard was prepared in molecular grade water. The 0.5 McFarland solution was then diluted 1/50 and the dilution used as the template for each PCR reaction. The initial stage of 98°C for 10 minutes, at the beginning of each PCR

reaction, lysed the bacteria in the PCR mixture releasing the DNA. The primer pair 27F and 338R for the 16S rRNA subunit (Hamady *et al.*, 2008) were used as extraction controls for each specimen and run alongside in a separate reaction on the same PCR plate. The same volume of the 1/50 dilution from a 0.5 McFarland standard solution of *E. coli* was used as the template in all PCR reactions. A PCR screening result was only considered valid with corresponding positive 16S rRNA PCR amplification.

2.2.2.4 *FumC*368/O6-Antigen (Ag) Multiplex PCR and 16S rRNA PCR.

The fumC368/O6-antigen negative screen PCR was performed using 0.8µl of each of the four primers at a concentration of 1pmol/µl, 10µl of Biomix™ Red, 1.6µl of dilute *E. coli* solution and molecular grade water in a final reaction mixture of 20µl. The 16S rRNA PCR control reaction was also performed using 0.8µl of the forward and reverse primers, at a concentration of 1pmol/µl, 10µl of Biomix™ Red, 1.6µl of dilute *E. coli* solution and molecular grade water in a final reaction mixture of 20µl. PCR parameters were as follows; an initial denaturation step of 10 minutes at 98°C, 30 cycles of 95°C for 30s, annealing at 55°C for 1 min, extension at 72°C for 1 minute with a final extension at 72°C for 5 minutes. All PCR reactions were performed on a T100 Thermal cycler (BIO-RAD, Hertfordshire, UK).

2.2.2.5 Visual detection of PCR products.

The presence of DNA bands were detected electrophoretically by the addition of 2µl of PCR mixture to a 1% (w/v) agarose (Promega Corporation, Madison, USA) Tris acetate-EDTA (TAE) buffer (Sigma, Dorset, UK) gel, incorporating 1µl of GelRed™ (Biotium, CA, USA) per 100ml agarose gel. The electrophoresis was performed at 80 V for 40 minutes, followed by visualisation under UV transillimination (UVP ChemiDoc 810 imager, CA, USA).

2.2.2.6 Validation of *FumC*368/O6Ag allele specific Multiplex PCR.

A total of 76 previously genotyped *E. coli* (Alghoribi *et al.*, 2015; Gibreel *et al.*, 2012b) were used to validate the fumC/O6Ag multiplex PCR. Nineteen ST127 were used along with 23 other specimens (8 different ST types) that either shared the same *fumC*14 allele as ST127, or shared the same SNP in the *fumC* allele at position 368. A total of 34 *E. coli* (20 different ST) that did not share the same allele or SNP were also used to assess the accuracy of the *fumC*/O6Ag multiplex PCR (Table 3).

Table 3. Sequence types of isolates tested during validation of multiplex PCR to screen out non-ST127 isolates.

E. coli ST (n) with *fumC* 14 allele	*E. coli* ST (n) with same SNP at pos. 368 as *fumC* 14	*E. coli* without *fumC* 14 allele or different SNP as *fumC* 14 at pos. 368 (n)	
ST14 (9)	ST809 (1)	ST10 (2)	ST405(1)
ST92 (1)	ST 372 (6)	ST58 (1)	ST783 (1)
ST127 (19)		ST69 (3)	ST784 (1)
ST404 (3)		ST73 (5)	ST787 (1)
ST550 (2)		ST80 (2)	ST804 (1)
ST807 (1)		ST88 (1)	ST805 (1)
		ST95 (3)	ST806 (1)
		ST104(1)	ST808 (1)
		ST131 (5)	ST999 (1)
		ST224 (1)	ST1000(1)

2.2.2.7 Allele and sequence typing of clinical *E. coli* specimens.

As there are isolates of ST127 that have been found with a large nucleotide deletion in the O6 antigen (Alghoribi *et al.*, 2014) and some reportedly belonging to other serogroups (Ciesielczuk *et al.*, 2016), all specimens that were *fumC368* positive had the potential to be ST127 regardless of their O antigen serogroup. As such, all *fumC368 positive* specimens along with specimens that were both *fumC368* and O6Ag positive, underwent sequence typing to determine if any were ST127. The sequences in both the forward and reverse direction were determined for each locus, and the results submitted to the Warwick MLST *E. coli* database. Alleles were assigned a specific allele number from the Warwick MLST *E. coli* database website and members of the UPEC ST127 lineage were confirmed using the allelic profile obtained from the database

2.2.3 MLST.

PCR amplification for the MLST loci was performed using primer pairs previously described by the Warwick MLST database (https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html).
The forward (F) and reverse (R) primers were used for the MLST with some additional primers (F1 and R1) used for optimisation of the negative screen PCR, specifically *fumC*-R1.

Each reaction was carried out in a final volume of 40µl including 20ul of 2x Biomix™ Red (Bioline ISA inc.), 1µl of each primer at a concentration of 2pmol/µl, 3.2µl of dilute *E. coli* solution and 14.8µl of molecular grade water.

The following PCR conditions were used consisting of 30 cycles at 1 min 95°C, 1 min at the specific annealing temperature (Table 4), extension for 1 min at 72°C, and a final extension for 5 minutes at 72°C.

Table 4. Primer sequences for MLST housekeeping genes obtained from the Warwick MLST database website.

Loci	**Primer (5'- 3')**	**Annealing temp. °C**	**Allele length (bp)**
adk	*F-* ATTCTGCTTGGCGCTCCGGG *R-* CCGTCAACTTTCGCGTATTT *F1-* TCATCATCTGCACTTTCCGC *R1*-CCAGATCAGCGCGAACTTCA	54	536
fumC	*F*-TCACAGGTCGCCAGCGCTTC *R*-GTACGCAGCGAAAAAGATTC *R1* –TCCCGGCAGATAAGCTGTGG	54	469
gyrB	*F*-TCGGCGACACGGATGACGGC *R*-ATCAGGCCTTCACGCGCATC *R1* –GTCCATGTAGGCGTTCAGGG	60	460
icd	*F*-ATGGAAAGTAAAGTAGTTGTTCCGGCACA *R*-GGACGCAGCAGGATCTGTT	54	518
mdh	*F*-ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG *R*-TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT *F1* 5'-AGCGCGTTCTGTTCAAATGC *R1* 5'-CAGGTTCAGAACTCTCTCTGT	60	452
purA	*F*-CGCGCTGATGAAAGAGATGA *R*-CATACGGTAAGCCACGCAGA *F1* –TCGGTAACGGTGTTGTGCTG	54	478
recA	*F*-CGCATTCGCTTTACCCTGACC *R*-TCGTCGAAATCTACGGACCGGA *F1* -ACCTTTGTAGCTGTACCACG *R1* –AGCGTGAAGGTAAAACCTGTG	58	510

2.2.3.1 Sequencing of amplified MLST alleles.

The clean-up and sequencing of PCR products was performed by LGC Genomics (Berlin, Germany). ExoSAP-IT (Affymetrix,CA, USA) was used to clean the amplified PCR products and an ABI 3730XL (Applied Biosystems, CA, USA) used to sequence the PCR products. The resultant chromatograms were obtained electronically from LGC before being examined and processed using Bionumerics 4.6 (Applied Maths, Belgium).

2.2.4 Statistical analysis

Statistical analysis was performed using Microsoft Excel and IBM SPSS Statistics 22 package. Calculation of the sensitivity and specificity was performed as previously described (Altman & Bland, 1994). The positive and negative predictive values were calculated assuming the prevalence was proportionate to the number of positive isolates within the validation strain collection.

2.3 Results

2.3.1 Raw data analysis.

A total of 4431 urine specimens passed through the clinical laboratory during the three week collection period. Overall, 2897 of these urine specimens were examined in this study and subsequently, a total of 503 anonymous specimens containing an isolate of *E. coli* were collected. Upon the removal of Internal Quality Control (IQA) specimens and void specimens, a total of 475 urine specimens containing *E. coli* isolates were acquired during the study period (See appendix I). Half of all the specimens collected (49.7%) had at least one other organism isolated alongside the *E. coli*. *Ent. faecalis* was the most prominent associated organism in all specimen age groups and was particularly high in the >65 age group. An overview of all results relating to the collected isolates is presented in Table 5.

Table 5. Analysis of monomicrobial and polymicrobial growth for the 475 samples with suspected *E. coli* associated UTI that were collected for this study.

Mono and polymicrobial growth of all urine specimens	Number of specimens **(%)**
All collected specimens	
Specimens from patient's ≥65 years of age.	261 **(54.9)**
Specimens from patients aged between 13-64 years of age.	183 **(38.5)**
Specimens from children ≤12 years of age.	31 **(6.5)**
Specimens from female patients	391 **(82.3)**
Specimens from male patients	84 **(17.7)**
≥65 years of age (261 specimens)	
Isolated in pure culture (>2 *E.coli* in same specimen recorded as pure)	117 **(44.8)**
Isolated in mixed culture with one associated organism	126 **(48.2)**
Isolated in mixed culture with two associated organisms	18 **(6.9)**
Specimens from female patients	201 **(77)**
Specimens from male patients	60 **(23)**
Prevalence of *Ent. faecalis* in all 261 specimens.	72 **(27.4)**
21 isolates in this group were unidentifiable	
Between 13-64 years of age (183 specimens)	
Isolated in pure culture	107 **(58.5)**
Isolated in mixed culture with one associated organism	66 **(36.1)**
Isolated in mixed culture with two associated organisms	10 **(5.5)**
Specimens from female patients	160 **(87.4)**
Specimens from male patients	23 **(12.6)**
Prevalence of *Ent. faecalis in 183 specimens*	32 **(17.4)**
Nine isolates in this group were unidentifiable	
≤12 years of age (31 specimens)	
Isolated in pure culture	15 **(48.4)**
Isolated in mixed culture with one associated organism	15 **(48.4)**
Isolated in mixed culture with two associated organisms	1 **(3.2)**
Specimens from female patients	30 **(96.8)**
Specimens from male patients	1 **(3.2)**
Prevalence of *Ent. faecalis* in 31 specimens.	5 **(16.1)**
One organism in this group was unidentifiable	

2.3.1.2 Significant UTI.

With the aid of the PHE guidelines (McNulty, 2017), laboratory results and available clinical information, specimen results were categorized in to one of 7 groups: Pure *E. coli* growth-Positive UTI (n=225); Pure *E. coli* growth-possible UTI (n=12); Pure *E. coli* growth-Negative UTI (n=2); Mixed growth likely positive UTI (n=123); Mixed growth possible UTI (n=68); Mixed growth likely negative UTI (n=35) and *E. coli* not the predominant organism-non *E. coli* UTI (n=10). In total, 348 of the 475 specimens were considered to be UTIs, 80 were considered as possible-UTIs and 47 were not significant or not an *E. coli*

infection. Table 6 displays the number of isolates considered as UTIs in relation to patient age groups.

Table 6. Monomicrobial and polymicrobial specimens considered to be clinically relevant UTIs in relation to patient age. Percentage in brackets (%) represented by coloured groups

	Patient age (%)		
	≤12 years	13-64 years	≥65 years
Diagnosed UTI Positive (n=348)	13 (3.7)	144 (41.4)	191 (54.9)
Pure culture of *E. coli* (n= 225)	12 (92.3)	101 (70.1)	112(58.6)
E. coli mixed with 1 organism (n=114)	1 (7.7)	42 (29.2)	71 (37.2)
E. coli mixed with 2 organisms (n=9)	0	1 (0.7)	8 (4.2)

The highest proportion of all UTIs and polymicrobial UTIs were seen in the ≥65 group (Table 6) with 41.4% of the diagnosed infections involving at least one other organism. The highest percentage (92%) of monomicrobial *E. coli* UTIs were observed in the ≤12 year age group with only one of the 13 specimens containing an additional organism.

Table 7. Number and type of associated bacteria isolated alongside UPEC in mixed positive UTI specimens

Associated organism	Number Isolated (n=132)	Percentage of total mixed organisms
Enterococcus faecalis	60	45.5
Streptococcus agalactiae	7	5.3
Enterococcus faecium	6	4.5
Staphylococcus haemolyticus	4	3.0
Staphylococcus epidermidis	3	2.3
Streptococcus gallolyticus	3	2.3
Enterobacter cloacae	2	1.5
Globicatella sulfidifaciens	2	1.5
Klebsiella pneumoniae	2	1.5
Bacillus lichenformis	1	0.8
Bacillus megaterium	1	0.8
Bacillus simplex	1	0.8
Candida tropicalis	1	0.8
Citrobacter freundii	1	0.8
Enterobacter asburiae	1	0.8
Enterococcus avium	1	0.8
Enterococcus raffinosus	1	0.8
Klebsiella oxytoca	1	0.8
Morganella morganii	1	0.8
Pseudomonas aeruginosa	1	0.8
Streptococcus anginosus	1	0.8
Staphylococcus cohnii	1	0.8
Streptococcus sciuri	1	0.8
Staphylococcus simulans	1	0.8
Streptococcus urinalis	1	0.8
Streptococcus vistibularis	1	0.8
Staphylococcus warneri	1	0.8
Green colony (organisms unable to isolate or without a reliable ID)	25	18.9

Table 7 shows the identification of all associated organisms (i.e. recovered in combination with a strain of *E. coli*) collected from mixed culture positive UTI specimens. Nearly half of the organisms isolated were of the genus *Enterococcus*, with *Ent. faecalis* accounting for 45.5%.

2.3.2 Validation of *fumC*368 allele specific PCR.

Figure 4 is an example of the results obtained from the validation of the *fumC*/O6Ag multiplex PCR. ST127 isolates displayed two distinct bands whereas other ST types produced only one band.

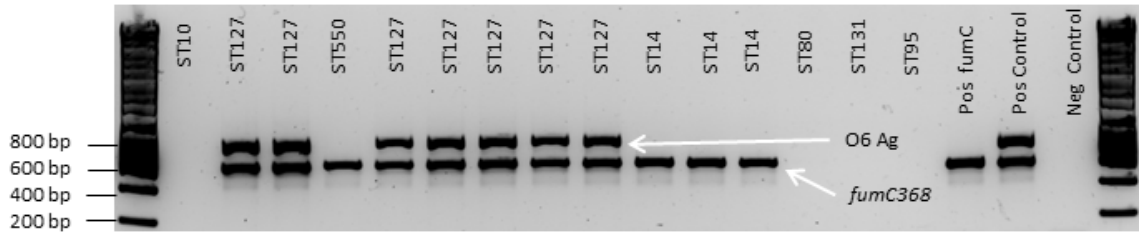

Figure 4. Results from the validation of the fumC/O6Ag allele specific PCR. Molecular marker-1kb (Bioline, London, UK); Positive fumC control, EC18 (ST127 O-antigen deletion); Positive control, EC41 (ST127); Negative control, EC958 (ST131).

The two positive controls were previously sequenced and identified as ST127 isolates (Alghoribi *et al.*, 2014). One isolate (EC18) contained a large deletion in the O antigen region thus functioning as a positive control for the *fumC* 368 SNP PCR and a negative control for the O6 antigen. The second positive control (EC41) functioned as a positive control for both PCR targets. Sensitivity and specificity results for the negative PCR screen were calculated using a positive result for only the presence of the *fumC* allele containing the 368 SNP (Table 9). An ideal score for a negative screen of 100% sensitivity was observed estimating a high probability that no false negative results would be observed using the PCR assay.

Table 8. Sensitivity and specificity results for the fumC368 specific allele.

	%	95% CI
Sensitivity	100	
Specificity	82.93	
Positive predictive value (PPV)	83.33	69.4 – 91.7
Negative predictive value (NPV)	100	89.9 - 100

2.3.3 Negative *fumC*368/O6Ag PCR Screen.

Multiplex PCR was performed on each of the *E. coli* isolates. A 16S rRNA extraction control was also performed alongside each screened specimen. Figure 5 shows one of the 25 electrophoresis gels displaying the type of results observed during the ST127 negative screen.

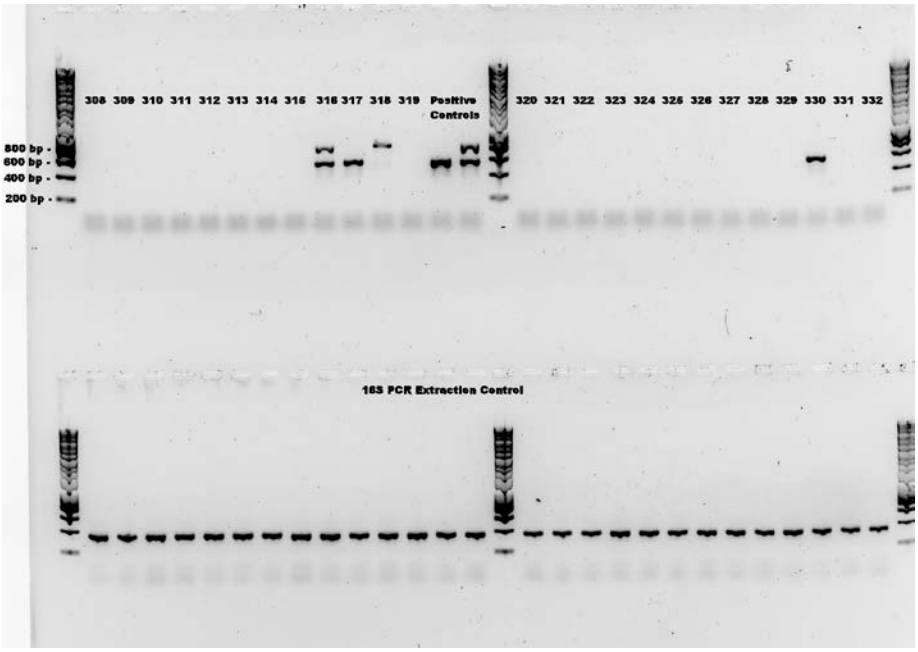

Figure 5. Results from one of the screening gels for the fumC/O6Ag allele specific PCR. Molecular marker-1kb (Bioline, London, UK); 1kb; Positive fumC control, EC18 (ST127 O-antigen deletion); Positive control, EC41 (ST127).

Isolate 316 displayed two DNA bands indicative of an ST127 isolate. Isolates 317 and 330 displayed a positive result for fumC368 SNP and isolate 318 displayed a positive result for O6 antigen only. The lower half of the gel image indicates the presence of positive PCR reactions for 16S rRNA primers for all samples.

2.3.3.1 Positive screening results.

A total of 10 specimens from 475 isolates were positive for the fumC368/O6 Ag multiplex PCR and another 99 specimens were positive for only for the fumC368 SNP. All positive isolates from original testing were repeated and confirmed positive.

2.3.4 Multi-locus sequence typing.

The 10 positive fumC368/O6Ag and 99 fumC368 positive O6Ag negative isolates were screened by sequencing 2 to 7 of the MLST alleles. The sequencing result for one allele determined the necessity for further allele identification i.e. if a fumC368 PCR positive strain generated sequence data indicating at least 2 MLST loci did not belong to ST127, no further sequencing was required. Following full MLST sequence analysis of all seven loci, It was found that 9 of the 10 fumC368/O6Ag positive specimens were ST127 (listed in Table 9), the one anomaly (specimen No. 260) was determined to be ST83.

Of the 99 specimens positive for fumC368 only, none were ST127. Fifty four of the positive fumC368 specimens, which had the *fumC* allele sequenced (n=55), produced allele numbers different from the ST127 *fumC*14 allele. Although each of these alleles possessed fumC368 SNP, validating selection of the priming site.

Two anomalies were recorded with the sequencing of the *fumC* allele for fumC368 PCR positive specimens. Isolate numbers 260 (fumC368/O6Ag positive) and 289 (fumC368 positive) possessed *fumC* alleles 38 and 22,

respectively, but neither allele contained the 368 SNP. Isolate 260 was a presumptive ST127 but upon repeat multiplex PCR testing, produced only an extremely weak fumC368 positive result. Multiplex PCR for isolate 298 was not repeated.

2.3.5 Variable results for *fumC*368/O6Ag PCR assay and the antimicrobial susceptibility of UPEC isolates.

Confirmatory testing with the fumC368/O6Ag PCR for specimens 124 and 263, gave variable results. Initial positive results from colony template DNA obtained from a sweep of the main inoculum, gave negative results when repeated with single colony DNA templates. Specimen 124 initially gave positive results for both PCR targets, but upon single colony testing; only the *fumC368* target was positive. Specimen 263 was positive for both *FumC368* and the O6 antigen upon initial testing, but negative for both targets when tested with single colony template. It was suspected that specimen 124 and specimen 263 contained multiple *E. coli* isolates. Further evidence for this hypothesis was provided by antimicrobial sensitivity results (see Figure 6).

Specimen 124 contained two ST127 isolates; one positive for the O6 antigen and one negative. It was observed that the O6 antigen negative 124 isolate produced a slightly larger colony (when grown on blood agar) than the positive O6 antigen strain and as such, each isolate was identified as ‘124S’ (Small - OAg positive) and ‘124L’ (Large - OAg negative). Furthermore, antimicrobial sensitivity testing of the two isolates showed that the 124L isolate was resistant to Trimethoprim whereas 124S was not (Figure 6a).

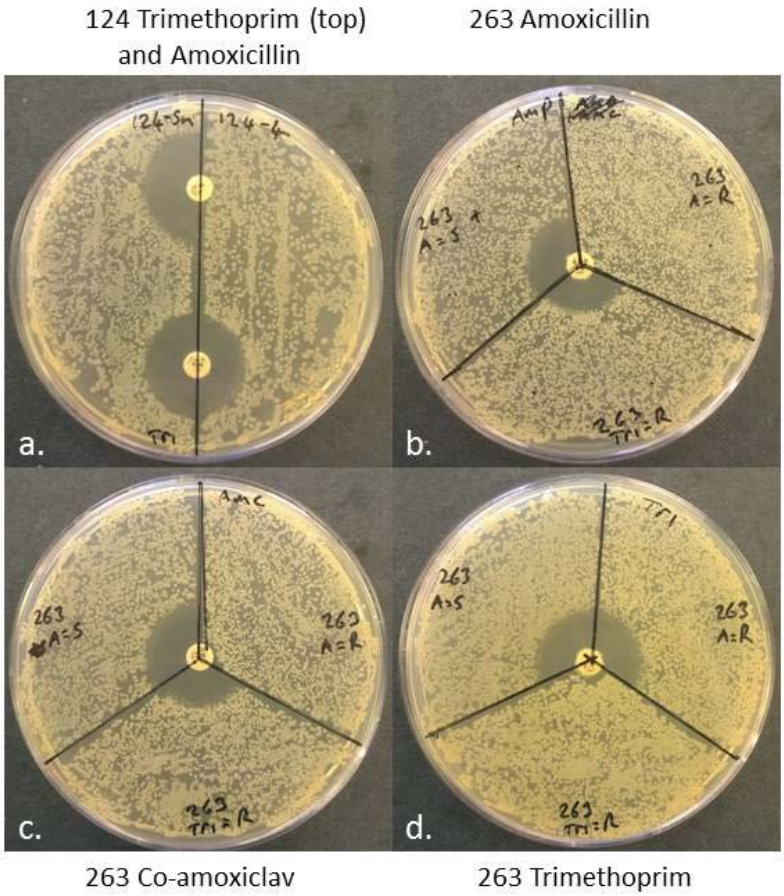

Figure 6. Antibiotic sensitivity results using the BSAC disc diffusion method for specimen numbers 124 and 263 tested against Amoxicillin, Co-amoxiclav and Trimethoprim. a, Specimen 124S (left) and 124L (right) inoculated on the same plate, tested against Trimethoprim disc (top) and amoxicillin disc (bottom); Images b, c and d, Three UPEC isolated from urine specimen 263 tested for susceptibility to amoxicillin (b); Co-amoxiclav (c); and Trimethoprim (d).

Specimen 263 contained three *E. coli* isolates, an ST127 that was fully sensitive to all tested antibiotics (Figure 6) and two ST73 isolates; one that was resistant to Amoxicillin and Co-amoxiclav but sensitive to Trimethoprim, and one that was sensitive to both Amoxicillin and Co-amoxiclav but was resistant to Trimethoprim (Specimens designated 263AS- ST127, 263AR- ST73 Amoxicillin resistant isolate and 263TR- ST73 Trimethoprim resistant isolate). A list of the urine specimens positive for ST127, along with specimen details are listed in Table 9.

Table 9. Initial clinical results of specimens containing ST127 isolated in monomicrobial and polymicrobial culture with patient information and associated clinical details and results.

UTI status	Isolate number	*E. coli* conc.	Associated organism	Age	Gender	WBC	EPC	Clinical details	Antibiotic profile						
									Am	Ac	Cf	Cp	Nit	Tri	Lev
Query UTI mixed	9	2++	Green colony Unable to identify (2++)	69	F	3+++	2++/3+++	None	**R**	**R**	S	S	S	S	S
Positive Mixed	39	3+++	*Ent. faecalis* (Scanty/1)	35	F	3+++	2++/3+++	UTI	**R**	S	S	S	S	S	S
Positive Pure	124 small	3+++		82	M	3+++	1+/-	UTI	S	S	S	S	S	S	S
	124 large	3+++							S	S	S	S	S	**R**	S
query UTI mixed	263 (ST127)	2++	*Ent. faecalis* (Scanty/9)	57	F	2++	2++/3+++	Dysuria	S	S	S	S	S	S	S
	263 (ST73)								**R**	**R**	S	S	S	S	S
	263 (ST73)								S	S	S	S	S	**R**	S
Positive Mixed	264	2++	*Ent. faecalis* (Scanty/2)	72	F	3+++	2++/3+++	recurrent UTI	**R**	**R**	S	S	S	S	S
Positive Pure	298	2++		44	F	3+++	1+/-	UTI	S	S	S	S	S	S	S
Negative Mixed	316	1+	*Ent. faecalis* (1+)	34	F	1+	2++/3+++	Leuks 3+++	S	S	S	S	S	S	S
Positive Mix	354	3+++	*Ent. faecalis* (Scanty/1)	86	M	3+++	1+/-	Confusion, Leukocytes +, nitrates present	S	S	S	S	S	S	S
Positive Pure	468	3+++		91	F	3+++	1+/-	None	S	S	S	S	S	S	S

WBC, White Blood Cell Count; EPI, Epithelial Cell Count; 3+++, Present in Large numbers; 2++, Present in moderate numbers; 1+/-, Not seen/Present in small numbers; Am, Amoxicillin; Ac, Amoxicillin/Clavulanic acid; Cf, Cefpodoxime; Cp, Cephradine; Nit, Nitrofurantoin; Tri, Trimethoprim; Lev, Levofloxacin; S, Sensitive; R, Resistant.

2.3.6 Prevalence of ST127 in monomicrobial and polymicrobial UTI.

Statistical analysis was performed using IBM SPSS Statistics 22 package. For the purpose of this analysis, specimen 124 was considered as a monomicrobial infection. Of the six UPEC ST127 that were considered to be a true UTI, three were seen in monomicrobial UTI and three in polymicrobial UTI. Altogether, ST127 was observed at a prevalence of 1.75%. Proportionally, ST127 was seen at a higher prevalence in polymicrobial infections (2.5%) than it was in monomicrobial infection (1.3%). Statistical analysis was performed using Fishers exact test, however, as isolate numbers were so low no statistical significance could be attributed (P value = 0.42) to these associations (see Table 11).

Table 10. Incidence of ST127 in monomicrobial and polymicrobial UTI.

	Non-ST127 *E. coli*	*E. coli* ST127	Total	P-Value (Fishers exact test)
Monomicrobial	223	3 (1.3%)	226	0.427
Polymicrobial	119	3 (2.5%)	122	
Total	342	6	348	

Additionally, all ST127 were isolated from mid-stream urine specimens. Each ST127 was isolated with only one associated organism species (Excluding other UPEC), *Enterococcus faecalis*.

2.4 Discussion

2.4.1 Clinical results.

This study focused on an area that is considered undervalued from a diagnostic and therapeutic viewpoint. The novel data collected in this study gives a greater understanding of the types of bacteria that are commonplace in and around the urinary tract. As the specimens were all obtained from patients suffering from a suspected UTI regardless of age, location, specimen type or any other prerequisite, these data give a true representation of the organisms associated with UTI, be it as a pathogen or commensal.

The raw data (Appendix I) from this study revealed that half of the *E. coli* cultures obtained from positive urinalysis were mixed with at least one other organism at a minimum concentration of 10^3 CFU/ml. Forty five different species were identified from 237 associated organisms with another 33 colonies observed in which it was not possible to isolate the organism or acquire a reliable identification. This illustrates the diversity of organisms isolated in high concentrations from urine. Some of the more obscure organisms included species such as *Globicatella sulfidifaciens*, *Corynebacterium minutissimum*, *Delftia acidovorans*, *Acinetobacter baumannii* and *Acinetobater towerii*. All these species have been associated with disease (Chotikanatis *et al.*, 2011; Fishbain & Peleg, 2010; Granok, Benjamin & Garrett, 2002; Lau *et al.*, 2006), and some, specifically with UTI although reports are uncommon and UTIs caused by these organisms will generally affect the aged or immunocompromised. (Ahmad & Ahmad, 2005; Calzada *et al.*, 2015; Chuang & Ratnayake, 2018; Craig *et al.*, 1994; Girija, Jayaseelan & Arumugam, 2018; Kam *et al.*, 2012; Matsunami *et al.*, 2012; Takahashi *et al.*, 2017). These

organisms may also be considered as periurethral, perineum or skin flora. The presence of these uncommon isolates in a relatively small cohort of specimens demonstrates the variability of species that may inhabit the urinary tract and surrounding tissues and the problem encountered by medical practitioners when making diagnoses based on mixed bacterial growth from urine culture. The importance of these types of organisms may be undervalued but, for the most part, it is beyond the remit of this study to examine them in more detail.

As this study has further confirmed, many organisms not usually implicated with urinary infection can be present in urine cultures with the colonising microbiological flora of the lower genital tract and perineal area the main source of contamination. The regulatory influences of such an area are complex and dynamic involving interactions between host biologic and behavioural factors, a subject that is individually variable and still not fully understood (Larsen & Monif, 2001). As the female urethra is short (2-4cm), the external 1-2cm is often colonised by commensal vaginal, perineum and enteric flora, justifying the rationale of a mid-stream urine specimen to lessen or avoid contamination.

Focusing on the 348 isolates that could be confidently associated with a diagnosed UTI, it was observed that a high percentage of mixed cultures were prevalent in both the over 65 group and in the 13-64 age group (41.4% and 29.9% respectively). In agreement with current opinion, a steady increase in UTI and mixed urine cultures (data not shown) was observed with age (ACOG, 2008; Cove-Smith & Almond, 2007; Najar, Saldanha & Banday, 2009). The study as a whole showed that 45% of all the associated organisms isolated in combination with *E. coli* were of the species *Ent. faecalis*. The same statistic in the over 65 age group was even higher (50%). This result is in accordance with the current

literature referring to mixed infections in the elderly (Croxall *et al.*, 2011a; 2011b). The increase in UTI and polymicrobial urine cultures in the elderly is thought to be due to several factors such as increased colonisation of the skin around the groin due to debilitation, a decrease in acidity of the vagina that leads to changes in colonising organisms and an increase in the postvoiding residual volume due to changes in pelvic musculature.

With reference to comparative studies in this area looking into the relationship between *E. coli* and *Ent. faecalis*, some have suggested that the presence of *Ent. faecalis* correlates with the isolation of UPEC possessing an increased pathogenic potential or suggested that *Ent. faecalis* may exacerbate the pathogenicity of *E. coli* during infection. These data were obtained from *in vitro* cell infection assays and the *in vivo C. elegans* nematode and rat models of infection (Croxall *et al.*, 2011b; Lavigne *et al.*, 2008; Montravers *et al.*, 1997).

Croxall *et al*. (2011b) found *E. coli* and *Ent. faecalis* frequently isolated in mixed cultures (36% of all specimens). The study focused on urine specimens from patients ≥70 years old and only on specimens that contained 3 or more organisms with no predominant count of one species. It is important to note that these types of culture results would nearly always be regarded in a clinical bacteriology laboratory as faecal contamination and results of this nature may not be pertinent to urinary infection. Evidence in support of a role for Enterococci exacerbating mixed infections in certain models is not overly persuasive or may be of limited relevance to UTIs. Croxall *et al.* (2011b) reported an increase in the invasive potential of *E. coli* isolated from polymicrobial infections and suggest that the genotype and/or phenotype of *E. coli* may be altered during co-infection. *Ent. faecalis* was the most commonly

associated organism isolated with *E. coli* in the Croxall study and collective evidence was presented from all mixed isolates to suggest an increase in the invasive potential of *E. coli* in these cultures. However, no detailed evidence was supplied with specific reference to an increased invasive capability of the *E. coli* isolated alongside *Ent. faecalis* in patient urine. Additionally, the methods used for cell invasion may be called into question. The expression of type 1 pili in UPEC isolates is known to mediate bacterial adhesion and invasion (Martinez *et al.*, 2000). Croxall and colleagues (Croxall *et al.*, 2011b) only incubated their UPEC isolates overnight although it had been shown that type 1 fimbriae expression may involve several rounds of subculture in static growth conditions (Kakkanat *et al.*, 2015; Martinez *et al.*, 2000) due to, for instance, the presence of a common transposon insertion in the *fimB* gene resulting in a slow off-to-on switching for type 1 fimbriae (Totsika *et al.*, 2011). This is a very important prerequisite to any invasion assay involving *E. coli* as type 1 fimbriae have been reported to be essential for uroepithelial binding and subsequent invasion leading to formation of intracellular bacterial communities (IBCs) (Kakkanat *et al.*, 2015). Furthermore, as this study's test isolates were heavily weighted in the favour of polymicrobial *E. coli* (129 isolates) to monomicrobial *E. coli* (21 isolates) a small increase in the number of invasive bacteria within the monomicrobial isolates would change the statistical analysis dramatically. It is also important to note that no specific synergistic mechanism pertaining to growth, fitness or pathogenicity was defined.

An increase in proinflammatory cytokines was observed in one model of peritonitis involving *E. coli* and *Ent. faecalis* in rats, as well as an increased recovery of *E. coli* from blood when the initial infection included *Ent. facalis*

(Montravers *et al.*, 1997). However, the increased *E. coli* recovery was only achieved from using a very high mixed concentration of *Ent. faecalis* (10^8 CFU/ml), *B. fragilis* (5 x 10^7) and E. coli (10^8); an initial infectious dose of individual bacteria that would rarely be observed in patients during the course of mixed intra-abdominal infections (Boehm & Sassoubre, 2014; Eckburg *et al.*, 2005). A similar study by Dupont and colleagues also found increased levels of bacteraemia from polymicrobial challenge, which included *Enterococcus*, compared to monomicrobial challenge. However, the increased frequency of bacteraemia was reported only for the *Enterococcus* isolates (Dupont *et al.*, 1998) and not *E. coli*. Similar studies in 1976 recorded no intra-abdominal abscess formation using only *E. coli* and *Enterococcus* in combination; abscess formation only occurring when anaerobes were included in the mixed infection (Onderdonk *et al.*, 1976), suggesting that any notable microbial synergy was only observed only between anaerobes and facultative bacteria.

In the *C. elegans* model (Lavigne *et al.*, 2008) a synergistic interaction was demonstrated with an increased death rate of this nematode. However, the lethality induced by the bacterial combination seemed to be influenced more by the virulence of *E. coli* strain used rather than the virulence or presence of the *Enterococcus*.

It is fair to say that the potential synergism between *E. coli* and *Ent. faecalis* in urinary tract infections remains uncertain. A typical *Ent. faecalis* UTI may not present in the same way as an *E. coli* infection, and in some cases will possibly present asymptomatically (Barros, Martinelli & Rocha, 2009). UTIs caused by *Ent. faecalis* are not frequent outside a hospital setting or in young patients but

exhibit a much higher prevalence in patients with an anatomical or functional obstructive uropathy (Hall *et al.*, 1992).

It is important to recognise not only the organisms present in the urine specimen, but also the concentration of said organisms. With reference to the 123 mixed positive UTI specimens, *Ent. faecalis* was found in 59 of these specimens, certainly a notable finding. However, 75% of these occurrences only recorded <10 colonies (10^3 per/ml) of *Ent. faecalis* per sample and another 20% recorded between 10-20 colonies per microlitre. These relatively low concentrations support the conclusion that the presence of *Ent. faecalis* in these specimens represent contaminants isolated from the urethra/periurethral area during micturition. Evidence in support of that finding can be found in a study by Hooton and colleagues that looked into the type of cultures isolated from premenopausal women with symptoms of cystitis (Hooton *et al.*, 2013). The study collected paired specimens from each patient consisting of an MSU and a catheterised specimen taken immediately afterwards. The paired specimen cultures were compared and of the 20 MSU specimens that contained Enterococci, only two were also found in the paired catheter specimen. Furthermore, the two catheter specimens that contained Enterococci, only yielded Enterococci.

It may be the case that *Ent. faecalis* (or possibly certain strains of *Ent. faecalis*) should soon be considered as the 'normal flora' of a mid-stream urine, causing urinary problems only when overgrowth of this commensal occurs due to circumstances that favour their proliferation, such as a catheter *in situ*. This is a scenario that is recognised with numerous commensals across the entirety of the human body; *Streptococcus pneumoniae* in the respiratory tract; *Clostridium*

difficile in the large intestines and *Gardnerella vaginalis* or other anaerobes in the vagina, but to name a few. It may be the case that growth of a UPEC isolate in the urinary tract may somehow encourage/facilitate the growth of *Ent. faecalis* to detectable levels, or possibly, *Ent. faecalis* may have a protective role for the elderly urinary tract, inducing microbial interference protecting the external urinary epithelia from UPEC colonisation.

Several further studies could be performed on this collection of urinary associated isolates. One such study, similar to the one performed by Croxall and colleagues (Croxall *et al.*, 2011b), could quantify the pathogenicity of *E. coli* isolated alongside *Ent. faecalis*. In this collection of 475 specimens, 15 specimens (data not shown) grew both *E. coli* and *Ent. faecalis* both at a concentration of 10^5 cfu/ml. Usually, this type of specimen result would be considered as faecal/periurethral contamination; however, studies could employ NGS on these *E. coli* and quickly determine their potential pathogenicity (e.g. carriage of virulence associated genes), confirming these isolates as commensals or potential pathogens. The pathogenic potential of the *Ent. faecalis* could also be determined by the same means, also confirming their ability to cause infection.

2.4.2 Allele specific screen for ST127 isolates.

The designated MLST allele sequence numbers for ST127 are *adk:* 13; *fumC:* 14; *gyrB:* 19; *icd*: 36; *mdh*: 23; *purA*: 11; and *recA*: 10. Although there were no SNPs that were unique to ST127, sequence analysis of all known *E. coli* allelic loci revealed several nucleotide positions in ST127, which were uncommon in

other UPEC alleles. As the presence of only one nucleotide difference at any primer site may not significantly reduce PCR efficiency, the complementary nucleotides of primer to template were used to identify mismatches that would dramatically reduce PCR efficiency in non ST127 alleles. It is well documented that mismatch extension efficiencies can vary significantly (Mendelman *et al.*, 1989) but it has been shown that it can be difficult to extend A·A, G·G, C·C, G·A, and A·G mismatches near or at the primer 3' end, under certain PCR conditions (Huang, Arnheim & Goodman, 1992). Of all the allele specific primers tested, only the *fumC*368 primer pair showed reliable and consistent amplification in alleles containing the specific SNP at position 368. The addition of the O6-antigen primers added a confirmatory element to the PCR as UPEC ST127 is thought to mainly carry the O6 antigen. However, as a urinary tract isolate of ST127 (EC18) has been identified to have a large deletion in the O-antigen gene cluster (Alghoribi *et al.*, 2014), the detection of *fumC*368 alone was used as the target for the ST127 negative screen PCR, with the sensitivity and specificity results relating to amplification of the *fumC*368 SNP only. It was found that all but one of the ST127 isolates obtained from this study were PCR positive for the O6 antigen, the one exception being the 124L ST127 isolate. Further study will be required to determine whether this organism possesses a different O antigen or has a similar deletion in the O6 antigen cluster as EC18 (124L O-antigen status reported in chapter 3). The multiplex *fumC*368/O6Ag negative screen produced a perfect sensitivity result of 100%, using a large collection of both ST127 and other isolates known to carry the *fumC368* SNP. As the results of this assay on the 475 urine cultures produced 109 positives, this assay can in no way be thought of as specific. However, the removal of 77%

of the *E. coli* isolates in the search for ST127 isolates proves the value of this PCR assay. The *fumC*368/O6Ag negative screen PCR assay proved to be a quick and reliable tool for identifying possible ST127 UPEC.

2.4.3 Significance of *E. coli* ST127 isolated in monomicrobial and polymicrobial culture.

ST127 was found at a prevalence of less than 2% across the entire strain collection. This is surprising and contradictory when considering the reported virulence potential of this lineage. Recent studies have shown ST127 to be highly virulent in comparison to other common UPEC isolates (Alghoribi *et al.*, 2014; Beyrouthy *et al.*, 2013; Croxall *et al.*, 2011a; Salipante *et al.*, 2015) and to have a higher prevalence in areas other than the south west of England (Alghoribi *et al.*, 2015; Gibreel *et al.*, 2012b; Yamaji *et al.*, 2018). The low prevalence may be just related to geography, but possibly, may be partly explained by specimen bias. UPEC ST127 has previously been shown to be very susceptible to common empirically prescribed antibiotics (Banerjee *et al.*, 2013; Croxall *et al.*, 2011a; Gibreel *et al.*, 2012b; Hertz *et al.*, 2016). The antimicrobial susceptibility results from the ten ST127 isolates in this study are in agreement with these findings, with resistance only to penicillin based antibiotics observed in three isolates and Trimethoprim resistance in one. The remaining ST127 UPEC isolates were fully susceptible to all tested antibiotics. It could be hypothesised that the susceptibility of ST127 to commonly used antibiotics for the empiric treatment of UTI may cause an under-representation of ST127 in most published prevalence surveys, given that such studies are

frequently based on UPEC isolates collected in clinical laboratories from individuals who have often failed antimicrobial therapy or have recurrent UTIs. Studies from Europe, Canada, Saudi Arabia and Japan (Adams-Sapper *et al.*, 2013; Alghoribi *et al.*, 2015; Croxall *et al.*, 2011a; Gibreel *et al.*, 2012b; Umene *et al.*, 2015) report ST127 at low, but often significant levels. A recent study by Yamaji and colleagues (Yamaji *et al.*, 2018) focused on Community acquired UTI (CA-UTI), with specimens taken from all patients at the point of care, within a Californian university community. This study found ST127 to be the second most prevalent strain increasing from 11% in 1999-2000 to 16% in 2016-2017. In light of this recent evidence, the high virulence potential and reports of emerging resistance to antibiotics, including the cephalosporins (Beyrouthy *et al.*, 2013; Darling *et al.*, 2014; Ferjani *et al.*, 2017; Gomi *et al.*, 2017), members of this lineage are increasingly becoming a cause for clinical concern and give this strain the potential to emerge as a significant threat to human health.

The current study revealed a higher prevalence of ST127 in polymicrobial culture than in monomicrobial culture, 2.5% and 1.3%, respectively. Thus, ST127 displayed almost double the incidence in polymicrobial infections. However, predictably, the low numbers of ST127 isolates obtained does not allow any statistical significance to be attributed to these data. Originally, only 200 urine specimens were to be collected for this study but a reappraisal of the current literature and the low incidence of ST127 led to this number being increased to 500. Unfortunately, even this cohort of specimens failed to achieve the required numbers of ST127 to reveal significant differences in these data. The data obtained presented another query with relevance to the incidence of *E. coli* ST127 in polymicrobial infection; what percentage increase of ST127

incidence in polymicrobial infection would be considered to be clinically relevant? This is a difficult question to answer without further study. A given ratio of *E. coli* ST127 found between mono- and polymicrobial culture is needed to determine the numbers required for such a study, allowing statistical relevance to be determined. As previously stated, a study with specimens collected at the point of care (as performed by Yamaji *et al*., 2018) may allow a much higher proportion of ST127 isolates to be collected and give some insight into the true prevalence of ST127 in the community, both in polymicrobial and monomicrobial culture.

Two interesting points of note were that five of the six specimens diagnosed as an ST127 positive UTI, were obtained from the community, a result that is consistent with the findings of a study carried out in the Northwest of England (Gibreel *et al.*, 2012b). Additionally, two of the six isolated ST127 were found in men (33%), which was above the average compared to the collection of all other isolates obtained in this study (20% male in pure isolates and 12% in mixed isolates). It is understood that ST127 strains generally carry more virulence factors than members of other UPEC lineages, therefore, it is reasonable to suggest that a higher prevalence in males may be associated with organisms with a higher virulence potential, or specifically, enhanced motility as the male urethra is much longer than the females. Further investigations in this area may also reveal particular virulence factors that may favour colonisation/infection in males. However, it must be again noted that due to the low numbers of ST127 isolates, no statistical relevance can be attributed to these results ($P = 0.27$).

2.4.4 Polymicrobial Infection involving multiple UPEC.

This study has provided evidence to suggest that a significant proportion of UTIs may be caused by multiple UPEC isolates. The idea of using multiple bacterial colonies for the original *fumC*368/O6Ag PCR inoculum was based on the hypothesis that multiple UPEC isolates may be present in a single urine specimen. Two specific examples of this phenomenon have been collected from just the 9 urine specimens containing ST127. Firstly, preliminary results for urine specimen 124 suggests that there were two variants of ST127; one possessing the O6 antigen serotype and fully sensitive to all tested antibiotics, the other with a negative result for the O6 antigen and resistance to Trimethoprim. Secondly, specimen 263 contained what is believed to be three different UPEC variants at a 10^4 concentration along with a scanty *Ent. faecalis*. This specimen contained one ST127 and two ST73 variants (both commonly associated UPEC lineages) distinguished only by their susceptibility to amoxicillin, Co-amoxiclav and Trimethoprim. The concentration of each UPEC variant in both urines could not be determined as the phenotypical characteristics of these organisms were indistinguishable upon initial culture. Additionally, with the entire collection of 475 urine specimens included in this study, there were 12 occasions (not including urines containing ST127 isolates) where it was suspected that more than one *E. coli* was present. These deductions were made based on visual observations, mainly determined by distinct variations in colony colour (some *E. coli* produce a green colony on the CHROMagar™ Orientation Medium, see Figure 2) and confirmation by MALDI-TOF analysis. However, this may be the proverbial ‘tip of the iceberg’ as cultured variants of *E. coli* colonies invariably look identical. There may be

phenotypic attributes that allow their differentiation such as α and β haemolysis, utilisation of proprietary chromogens, colony appearance, antibiotic sensitivity etc., but only molecular characterisation can truly determine the presence of UPEC variants. Unfortunately, to the best of the authors knowledge, no molecular test is available that can quickly distinguish multiple ST of UPEC in mixed culture. Previous studies have highlighted the limitations of culture-based methodology with the diagnosis of UTI (McNally *et al.*, 2013; Roberts, 2015). A study by McNally and colleagues (McNally *et al.*, 2013) identified two *E. coli* isolates (ST 127 and ST131) from a single urine specimen that was originally considered to contain only one isolate. Confirmation of the two isolates could only be verified retrospectively and using MLST typing from whole genome sequence (WGS) analysis. The scenario involving two or more UPEC ST causing a UTI with potentially different virulence traits, would almost certainly increase the pathogenic potential of the infection as a whole and increase the likelihood that synergistic processes between the organisms would take place *in vivo*. Published research regarding multiple variants of *E. coli* causing UTI is limited. Our study has provided a supporting key addition to the McNally study and suggests that UTI caused by multiple *E. coli* variants may be common; a scenario that has hitherto not been seriously considered. However, in relation to multiple *E. coli* variants in the same urine specimen, we must be wary with results based on phenotypic characteristics as colonial variation due to mutation, recombination or phage action can take place *in vivo* during infection (Nichols, 1975).

2.4.5 Concluding remarks

The diagnosis of UTI is often complicated by the presence of multiple organisms in urine culture. UPEC is the causative pathogen in the vast majority of cases and no overly persuasive evidence has been presented, in this or other studies, to suggest that any additional organisms isolated in the same culture have any effect on the pathogenesis of UPEC. However, as emphasised by other well documented polymicrobial diseases such as periodontal disease, polymicrobial infection is an area of study that is complex, dynamic, subtle and difficult to study *in vivo*. That being said, this novel study has highlighted both the high frequency at which *Ent. faecalis* is isolated from urine culture as well as the diversity of species present in the urinary tract and the surrounding tissues.

The national guidelines on the diagnosis of UTI do not seriously consider or comment on the possibility of polymicrobial UTI. As such, clinical decisions regarding therapeutic approaches may well be lacking, especially in the elderly, where mixed microbial cultures are common.

The question whether UPEC ST127 is isolated with increased prevalence in polymicrobial infection cannot be conclusively answered by this study due to the low prevalence of ST127 across the entire specimen cohort. However, this study did demonstrate a potential type of polymicrobial UTI that has not been significantly investigated in any published studies. This is understandable as routine diagnosis of UTI in clinical laboratory investigations focus mainly on the visual differentiation of bacteria and antimicrobial susceptibility. The presence of strains from more than one UPEC ST in the same infection has generally been overlooked with the guidance pertaining to UTI diagnosis focusing on uropathogens isolated in what is perceived to be pure culture. Mixed cultures of

E. coli has either been unidentifiable due to similarities of *E. coli* colonies or assumed to be due to colonial variation, the latter often overlooked by busy laboratory professionals and diagnosed as a negative mixed growth contamination. A large part of this study focused its attention on just 9 urine specimens containing UPEC ST127. The presence of what is highly likely to be multiple UPEC isolates from different STs within at least one of the nine specimens suggests that many more UTIs may be caused by multiple UPEC rather than a single organism; an aspect of polymicrobial UTI that merits further study, which may eventually have an impact on diagnostics and clinical therapy.

Chapter Three

3. Genomic analysis of Uropathogenic Sequence Type 127 *Escherichia coli* and *Enterococcus faecalis* Isolated from Monomicrobial and Polymicrobial Urinary Tract Infection.

3.1 Introduction

Over the last three decades, the speed, accuracy and availability of DNA sequencing technology has increased dramatically. Since the discovery of the Polymerase Chain Reaction (PCR) technique in 1985 (Saiki *et al.*, 1985), which allowed the exponential multiplication of small DNA regions, techniques in DNA sequencing have rapidly developed as the cost has steadily decreased. To give some scale to the level of development, the human genome project began in 1990 and took over ten years to complete at a cost of $2.7 Billion (National Human Genomic Research Institute, 2003). Today, current technology can sequence a human genome in a few hours at a price of less than £1000, soon to be, if not already, less than £100 (Durmaz *et al.*, 2015).

The Sanger sequencing technique was originally published in 1977 by Frederick Sanger and colleagues (Sanger, Nicklen & Coulson, 1977) and, with the introduction of automated DNA sequencers and simple to use dideoxynucleoside chain termination kits, Sanger sequencing became the most advanced and reliable method for DNA sequencing. Although the leading technology of the time, Sanger sequencing was labour intensive, expensive and limited in the amount of DNA that could be sequenced at any one time. Then, in the year 2000, a technology known as massively parallel sequencing (MPS) (Brenner *et al.*, 2000) was introduced that used multiple reactions to sequence

millions of templates simultaneously. This technology was the forerunner to the release of the Next Generation Sequencing (NGS) platforms in 2004 (2nd generation sequencing technologies) that are now almost commonplace in molecular laboratories.

The speed at which the sequencing technology has progressed is astounding whereas the software and computational knowledge required to analyse the huge amounts of data has struggled to keep pace, generating a bottleneck transit between the production of data and its analysis. Many programmes have been created to manage and aid the analysis of NGS data but many of the current open source (OS) programmes run on command line/Linux based operating systems and require specialist knowledge to compile, install, run and integrate into manageable interpretable outcomes; in the world of microbial NGS research the Bioinformatician is king.

Although NGS technology is creeping its way into clinical laboratories, and there are an ever increasing number of studies performed facilitating the proof-of-concept for the integration of NGS into diagnostics (Goldstein *et al.*, 2018; Moran-Gilad *et al.*, 2017; Pankhurst *et al.*, 2016; Rasko *et al.*, 2011; Underwood *et al.*, 2013), there still remains a lack of internationally recognised standards necessary for the validation of a NGS analysis bioinformatics workflow (Fricke & Rasko, 2014). However, recommendations for future standards are now coming to fruition (Endrullat *et al.*, 2016; Hardwick, Deveson & Mercer, 2017; Roy *et al.*, 2018).

Specialist knowledge in computer science is slowly becoming less essential in molecular microbiological research with the development of websites such as

the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/) that has incorporated complex algorithmic programmes into a freely available user-friendly format. This is a necessary step in the right direction as 3rd generation sequencing platforms (single-molecule sequencing) will soon be commonplace in molecular microbiology laboratories.

An on-going issue with 2nd generation NGS is the production of relatively short read lengths. There are tens of thousands of repetitive DNA sequence tracts (e.g. tandem repeats) in the *E. coli* genome (Gur-Arie *et al.*, 2000), which is problematic for computational analysis for short read data, creating ambiguities in alignment and assembly (Treangen & Salzberg, 2011). The cost and effort to produce 'closed' genomes from 'draft' genomes generated by NGS and subsequent computational assembly, is impractical and expensive as tandem repeats are over-represented in non-coding regions and under-represented in open reading frames (ORF) (Gur-Arie *et al.*, 2000). The majority of genomes published in GenBank are draft genomes and in 2015 Land and colleagues (Land *et al.*, 2015) determined that nearly 90% of all genomes in GenBank were not complete.

The emergence of PacBio® (Pacific Biosciences) and MinIon (Oxford Nanopore Technologies) sequencing platforms provides the ability to produce much longer reads with fewer contigs than 2nd generation sequencing. Assembly of 3rd generation sequence reads using paired de Bruijn graph assemblers (e.g. SPAdes) (Bankevich *et al.*, 2012), is much simpler and accurate as the long read raw sequence data can only be assembled into a single contig as the read length exceeds the size of the longest repeat sequence in the genome (Koren

et al., 2013). The speed at which genetic and computational technologies are evolving does not seem to be abating. There is a huge potential for microbiological genomic analysis in future diagnostics with the complexity of bacterial identification, pathotyping, quantification and microbial resistance profiling being available in one single rapid genomic test. Although we are still some years away, for the first time in our history we can see that these types of diagnostics are attainable.

E. coli represents one of the most frequently sequenced genomes in public databases (13, 728 total genomes in NCBI database, 66 complete – Nov 2018) with data from strains isolated from across the world, from different hosts and different diseases (Moriel *et al.*, 2016). The genome of an *E. coli* isolate consists of approximately five million base pairs with roughly five thousand genes (Rasko *et al.*, 2008). The core genome consists of approximately 2,300 genes, whereas the pan-genome has been estimated to be over 13,000 genes (Ford, 2014), demonstrating the flexibility of the *E. coli* genome and, in turn, its diversity as a pathogen.

Studies from the early to mid-1990's (Blum *et al.*, 1995; Blum *et al.*, 1994; Hacker, 1990) on the UPEC isolate 536 (now known to be MLST 127) were the first to report the identification of regions in the *E. coli* genome which carried virulence associated genes particular to the UPEC pathotype. These regions of the genome were termed Pathogenicity Islands (PAIs), which are mobile genetic elements acquired by horizontal transfer. They encode genes including fimbrial adhesins (P-related fimbrial adhesins, S-fimbriae), putative autotransporter genes (*sap*), toxins (α-haemolysin) and iron uptake systems (yersiniabactin) amongst others (Dobrindt *et al.*, 2002). Mobile genomic islands

are not just limited to enhance the pathogenicity of an organism, but can also to impart antimicrobial resistance or enhance an organism's fitness by conferring the ability to utilise new carbon sources. These genomic islands, as with the pathogenicity islands, are named and classified by the functions they impart. Genomic islands are identified by the following traits: a GC content that varies from that of the surrounding DNA; flanking repeat regions; the presence of an integrase (ability to recombine) and an association with tRNA genes (Tang, 2015).

In 2002, a urosepsis isolate known as CFT073 was sequenced and compared to EHEC O157:H7 EDL933. Distinct variation in gene complement was observed with 1800-CFT073 specific genes (Welch *et al.*, 2002). Further full genome sequences of UPEC isolates were made available in 2006 (UTI89 and 536) and comparisons with other members of pathogenic and non-pathogenic *E. coli* found distinct similarities in the clusters of orthologous groups (COG) under positive selection in UPEC isolates, including genes involved in iron acquisition and membrane surface structures (Chen *et al.*, 2006). These studies highlighted and confirmed the role of PAIs in differentiating UPEC from commensals and other pathogenic strains (Brzuszkiewicz *et al.*, 2006; Chen *et al.*, 2006; Lo *et al.*, 2017).

Just as the *E. coli* genome has a high level of plasticity, so do their PAIs. The same genes have been identified on various PAIs and are capable of horizontal transfer between lineages and even between species (Chen *et al.*, 2006; Dobrindt *et al.*, 2002; Johnson & Stell, 2000). The rate of mobilization and horizontal gene transfer of PAIs between bacteria coupled with the continuous

reorganisation of the PAI itself by recombination, promotes the continuous evolution of the ExPEC pathotype (Dobrindt *et al.*, 2002).

Although most uropathogenic isolates seem to be clonal, deriving chiefly from phylogenic group B2 and D (Johnson & Stell, 2000), there is no single phenotypic profile that correlates with the ability to cause UTI, with virulence factors found in varying quantities, on varying PAIs across sub-groups of ExPEC. What is certain is that the pathogenicity islands containing blocks of UPEC virulence genes are not present in the chromosome of faecal commensals (Kaper, Nataro & Mobley, 2004; Lo *et al.*, 2017).

3.1.1 Study aims and objectives

There are multiple objectives relating to this part of the research project. The use of WGS data from UPEC and UTI associated bacteria will allow a greater understanding of the genomics relating to UTI, urine associated *Ent. faecalis* and UPEC ST127. Various bioinformatics tools will be applied to compare and determine features associated with individual isolates of ST127 and *Ent. faecalis*, such as genomic regions specific to UPEC ST127, identification of VFs and comparison of lineages obtained from core genome analysis. Endeavours will be made to answer questions pertaining to the first part of the study, such as establishing whether the multiple UPEC isolates obtained from urine specimens 124 and 263 are the same or different UPEC variants.

The first objective in this part of the study was to identify regions specific to the ST127 clone for the purpose of designing an ST127 specific PCR. The MLST typing technique has proven to be very useful in identifying common lineages

associated with UPEC. However, MLST is costly and time consuming, therefore impractical for the rapid identification of members of the ST127 clone. A solution to these problems is the development of ST specific PCR assays, as have been designed for other STs (Clermont *et al.*, 2009; Doumith *et al.*, 2015; Li *et al.*, 2010). These rapid PCR assays have been shown to be very useful in surveillance and examination of large culture collections. With the use of comparative genomics, the ST127 UPEC strains isolated in the study, described in the previous chapter, will be compared to strains of other common UPEC lineages to discover genomic targets for ST127 specific amplification. To confirm the assay is fit for purpose, it will be validated using a large collection of non-ST127 *E. coli* isolates.

The second objective in this research project is to determine regions in UPEC ST127 and *Ent. faecalis* genomes, which possess VFs related to urovirulence. This will be done using various methods, some automated and some manual. A direct comparison will be made of the utility of various bioinformatic tools verses a well-established VF PCR assay (Johnson & Stell, 2000) for the UPEC ST127 isolates.

The third objective is to obtain additional information from genomic studies to identify any antimicrobial resistance mechanisms present in the ST127 and ST73 strains (isolated with ST127), compare phylogenies using core genome SNP analysis and endeavour to answer the questions relating to the first part of the study; is there any relationship between the UPEC and *Ent. faecalis* isolated from the same urine specimen? Are the multiple UPEC strains isolated from the same urine truly different strains of the same species or an adapted variant of the progenitor strain?

3.2 Methods

3.2.1 Clinical isolates selected for WGS.

In total, 14 UPEC ST127 isolates, two UPEC ST73 isolates and 18 *Ent. faecalis* isolates underwent whole genome sequencing by NGS. Included in the collection were the ten UPEC ST127 and two UPEC ST73 strains isolated from the University hospitals Plymouth NHS Trust (Chapter 2), two ST127 UPEC strains from Saudi Arabia (Alghoribi *et al.*, 2015) and two from Manchester (Gibreel *et al.*, 2012b). Eighteen *Ent. faecalis* isolates that were isolated alongside *E. coli* as part of the polymicrobial UTI study (Chapter 2) were also analysed. Details on all sequenced isolates for this chapter are listed in the results section in Table 17 (page 115).

3.2.1.1 DNA extraction and genomic sequencing.

All genomic DNA extraction and sequencing was completed by MicrobesNG (University of Birmingham and University of Sheffield) using 2x250bp paired-end reads with sequencing performed on an Illumina HiSeq 2500 platform.

3.2.1.2 MicrobesNG bioinformatics workflow.

All raw sequence data were processed through a standard analysis MicrobesNG sequencing pipeline. This involved using the Kraken programme (https://ccb.jhu.edu/-software/kraken/) (Wood & Salzberg, 2014) to identify the closest related genome, then the reads mapped to the reference genome to assess the quality of the data using BWA mem 0.7.15 (http://bio-

bwa.sourceforge.net/) (Li & Durbin, 2010). De novo assembly of the reads was completed using SPAdes 3.8.1 (http://bioinf.spbau.ru/spades) (Nurk *et al.*, 2013). Contigs were ordered using MUMmer 3.0 (http://mummer.sourceforge.net/) (Kurtz *et al.*, 2004) and annotated using Prokka 1.1 (http://www.vicbioinformatics.com/software.prokka-.shtml) (Seemann, 2014). The Illumina platform sequence coverage was obtained from MicrobesNG and the quality of sequence data produced was assessed using QUAST 4.0 (Gurevich *et al.*, 2013).

3.2.1.3 Additional assembly, alignment and annotation.

Specific output file formats derived from the MicrobesNG WGS analysis pipeline were incompatible with certain OS programmes used to further analyse the WGS data. As such, independent assembly, alignment and annotation were performed for the 14 UPEC ST127 and two ST73 isolates on an Ubuntu 14.04 BioLinux 8 operating software as follows: Fastq file raw sequence data obtained from MicrobesNG was assembled into contigs using SPAdes 3.9.0. The contigs were ordered and aligned using Mauve 2.4.0 (http://darlinglab.org/mauve/-mauve.html) (Rissman *et al.*, 2009) with UPEC ST127 536 (Accession ref |NC_008253) (Hochhut *et al.*, 2006) as the reference genome. Sequences were then concatenated using SeqHandler v0.5 (https://github.com/happykhan/-seqhandler) and annotated using prokka 1.11. Dependencies such as BLAST+ database and HMMER3 were required installs for processes necessary for the function of several of the programmes. Full lists of dependencies are shown in Table 11.

Table 11. List of installed dependencies required for bioinformatics analysis

Dependencies	Reference
GNU Parallel	(https://www.usenix.org/system/files/login/articles/105438-Tange.pdf)
BioPerl	(Stajich *et al.*, 2002)
Aragorn	(Laslett & Canback, 2004)
Barrnap	(http://www.vicbioinformatics.com/software.barrnap.shtml)
RNAmmer	(Lagesen *et al.*, 2007)
Prodigal	(Hyatt *et al.*, 2010)
SignalP	(Petersen *et al.*, 2011)
NCBI-BLAST+	(Camacho *et al.*, 2009)
HMMER3	(Finn, Clements & Eddy, 2011)
Infernal	(Kolbe & Eddy, 2011)
TBL2ASN	(https://www.ncbi.nlm.nih.gov/genbank/tbl2asn2/)
Java	(https://www.java.com/en/download/help/linux_x64_install.xml)
Python	(https://www.python.org)
BioPython	(http://biopython.org/DIST/docs/install/Installation.html)
Bcbio-gff	(https://github.com/chapmanb/bcbb/tree/master/gff)

3.2.1.4 Genomes used for comparative analysis.

In addition to the genomes of UPEC and UTI associated *Ent. faecalis* strains that were sequenced by MicrobesNG, a selection of publicly available genomes were downloaded from the NCBI (National Library of Medicine, Bethesda, Maryland, USA) database, including those for nine reference UPEC isolates, one asymptomatic *E. coli* bacteriuria strain (ABU83972; Table 12) and 32 *Ent. faecalis* genomes isolated from a variety of sources (Table 13).

Table 12. List of *E. coli* strains and their designated sequence types recovered from the publically available NCBI database and used for the comparative genomic analysis of UPEC.

UPEC Isolate	Sequence Type	NCBI Bioproject Accession No. / Reference sequence
ABU 83972	ST73	PRJNA38725
CFT073	ST73	PRJNA313
NA114	ST131	PRJNA66975
EC958	ST131	NZ_HG941718.1
UMN026	ST597	PRJNA33415
IAI39	ST62	PRJNA33411
EC536	ST127	NC_008253
UTI89	ST95	PRJNA16259
VR50	ST10	PRJEA61445
clone D i2	ST73	PRJNA52021

3.2.1.5 Confirmation of MLST results, serological results and antibiotic resistance mechanisms identified from WGS data

The MLST 2.0 programme (Bartual *et al.*, 2005) on the Centre for Genomic Epidemiology (CGE) website was used to confirm the MLST results for all *E. coli* (Acthman scheme) and *Ent. faecalis* (Ruiz-Garbajosa *et al.*, 2006) isolated in this study. The website was also used to determine and confirm the ST of all the publically available genomes downloaded from the NCBI database. Manual and automated genome searches were performed using known sets of antibiotic resistance genes using the CGE website, artemis (Carver *et al.*, 2005), blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and various other OS programmes and publicly available databases (listed in next section). Serological investigations using WGS data were performed using SerotypeFinder (Joensen *et al.*, 2015).

Table 13. Isolation site and BioProject number of *Ent. faecalis* genomes used for NGS analysis

Ent. faecalis Genome	Isolation Site	BioProject Number
ATCC 29212	Urine	PRJNA341407
T1	Urine	PRJNA32915
T2	Urine	PRJNA32917
T3	Urine	PRJNA32919
T11	Urine	PRJNA32949
	Medical	
V583	Blood	PRJNA70
OG1RF	Oral	PRJNA20843
DENG1	Trachea	PRJNA187445
CLB21560	Unknown (medical)	PRJNA368724
ATCC 4200	Rheumatic fever	PRJNA32923
CH19	Wound	PRJNA88881
MMH594	Blood	PRJNA89033
CH188	Liver	PRJNA32951
	Commensal	
62	Infant gut	PRJNA61185
DD14	Meconium	PRJNA380227
TX0630	Human Microbiome	PRJNA47171
TX0102	Human Microbiome	PRJNA47157
TX4244	Human Microbiome	PRJNA47141
	Animal/Agriculture	
D32	Porcine	PRJNA169860
KB1	Murine	PRJNA317592
L9	Porcine rectal	PRJNA352597
L12	Porcine rectal	PRJNA352597
Sorialis	Murine gut	PRJNA318633
AR01/DG	Canine mastitis	PRJNA395129
LD33	Dairy	PRJNA316564
NED10	Horse	PRJNA89041
ATCC27959	Bovine	PRJNA89081
	Miscellaneous	
Symbioflor 1	Oral medical suspension	PRJEB648
AMB05	Water purification plant	PRJNA324367
W11	Wakame (edible seaweed)	PRJDB5023
KI-6-1-110601-1	Aquatic	PRJNA179541
13-D-W-01	Environmental	PRJNA179531
E1Sol	Soloman Islands (Antibiotic naïve population)	PRJNA32941

3.2.1.6 Bioinformatic analysis of genomes.

Genomes were analysed using various programmes dependant on the objective. Comparative analysis was performed using Blast Ring Image Generator (BRIG 0.95-dev0004) (Alikhan *et al.*, 2011), the Artemis Comparison Tool (ACT)

(Carver *et al.*, 2005) along with nucleotide (blast+) (https://blast.ncbi.nlm.nih.gov) and protein blast (http://www.uniprot.org) searches to identify the prospective regions specific to the genome analysed. The presence and identification of virulence genes was performed using VirulenceFinder 1.5 on the CGE server (http://www.genomicepidemiology.org/) (Joensen *et al.*, 2014), Virulence Searcher on the old Health Protection Agency website (http://www.hpa-bioinfotools.org.uk/pise/virfactfind_small.html) and via a VF PCR assay (Johnson & Stell, 2000). All phylogenies were completed using CSI Phylogeny 1.4 and the maximum likelihood (ML) phylogeny inferred by FastTree and visualised using FigTree 1.4.3. (Kaas *et al.*, 2014). ISfinder (https://isfinder-.biotoul.fr/) was used to identify the presence of insertion sequences within the sequenced genomes (Zhang *et al.*, 2000).

3.2.2 Design and development of a specific multiplex PCR for the detection of UPEC ST127

Several prospective primer targets with a high specificity to UPEC ST127 were identified using BRIG, ACT and online databases. Final primer pairs were based on gene regions that showed little or no homology in non-ST127 genomes using the NCBI and UniProt databases. PCR primer pairs were designed using CLC Genomics Work bench 7.5.1 (www.qiagen-bioinformatics.com). Final PCR primer sequences, concentrations and amplicon size are listed in Table 14.

3.2.2.1 UPEC ST127 specific multiplex PCR Protocol.

Bacterial DNA template was obtained as described in the 'bacterial colony PCR extraction' section in Chapter 2 (2.2.2.3).

The well-established MLST locus *gyrB* (DNA gyrase subunit B) primers (Wirth *et al.*, 2006) were incorporated into the multiplex PCR to act as an extraction/PCR control (Table 14).

Each multiplex PCR reaction was performed using 10µl of 2X Biomix™ Red reaction mix (Bioline, London, UK) in a final PCR volume of 20 µl. A primer concentration of 1pmol/µl, 1.3pmol/µl and 0.85pmol/µl was used for each of the *upaG*, *fliC* and *gyrB* primers, respectively, and 0.8µl of this primer concentration was added to the PCR reaction. PCR was performed on a T100 Thermal cycler (BIO-RAD, Hertfordshire, UK) as follows: an initial denaturation at 98°C for 8 minutes followed by 36 cycles of 95°C for 30 seconds, 58°C for 1 minute and 72°C for 40 seconds, with a final extension of 72°C for 5 minutes. PCR amplification was confirmed by visualised electrophoresis of 5µl of the PCR product on a 1% (w/v) agarose (Promega Corporation, Madison, USA) Tris acetate-EDTA (TAE) buffer (Sigma, Dorset, UK) gel, incorporating 5µl (0.5µg/ml) ethidium bromide per 100ml of gel. Electrophoresis was performed at 90V for 40 minutes and the number and size of amplicon products visualised under UV transillimination (UVP ChemiDoc 810 imager, CA, USA).

3.2.2.2 Interpretation of ST127 specific multiplex PCR.

A positive ST127 isolate was identified by the presence of 3 distinct DNA bands (Figure 9) each of the expected amplicon sizes (Table 14). An ST127 negative reaction was determined by the presence of the *gyrB* band alone (to indicate successful PCR amplification) or with just one of the 2 specific targets amplified. Amplification of the *gyrB* gene was necessary to determine a true negative result.

Table 14. Oligonucleotide sequences and concentrations used for the multiplex UPEC ST127 specific PCR.

Gene	Primer direction	Primer Sequence (5'-3')	Final primer conc (µM)	Position on gene	Length (bp)	Ref.
upaG	Forward	GATAGGCAAGGACGCAAGA	0.04	489-1699	1219	This study
	Reverse	GGTCGCAATATCCGTAGT	0.04			
fliC	Forward	CATTAATACCAACAGCCTC	0.052	12-533	538	This study
	Reverse	TATTAGCCACAGCCCCTT	0.052			
gyrB	Forward	TCGGCGACACGGATGACGGC	0.034		911	(Wirth *et al.*, 2006)
	Reverse	ATCAGGCCTTCACGCGCATC	0.034			

3.2.2.3 Validation, sensitivity and specificity of ST127 specific PCR.

To determine the sensitivity and specificity of the assay, a total of 143 strains of *E. coli* (Table 15) were used to validate the multiplex PCR. Isolates were obtained from previous studies performed in Manchester, UK (Gibreel *et al.*, 2012b) and Riyadh, Saudi Arabia (Alghoribi *et al.*, 2015). The collection consisted of 29 ST127 strains and 114 UPEC isolates representing 98 different STs.

Table 15. List of all UPEC isolates and their designated ST used for validation of the UPEC ST127 specific PCR assay.

E. coli Isolate	MLST	*E. coli* Isolate	MLST	*E. coli* Isolate	MLST	*E. coli* Isolate	MLST	*E. coli* Isolate	MLST
SA014	10	NW45	104	NW224	127	SA067	624	SA060	1380
SA036	10	NW155	117	EC958	131	SA119	662	SA061	1431
SA097	10	D9	127	NW002	131	NW178	681	NW153	1529
SA098	10	D39	127	NW014	131	NW130	779	NW169	1532
SA135	10	D124A	127	NW59	136	NW140	780	NW174	1533
SA140	10	D124B	127	NW82	141	NW141	781	NW192	1534
NW006	10	D263A	127	SA053	153	NW57	782	NW193	1535
NW212	14	D264	127	SA005	155	NW016	783	NW203	1536
SA004	23	D298	127	SA013	162	NW007	784	NW220	1537
SA023	38	D316	127	NW39	224	NW63	785	NW221	1538
SA034	38	D354	127	SA082	315	NW74	786	NW225	1540
NW65	48	D468	127	SA035	347	NW34	787	NW226	1541
SA072	52	EC536	127	SA012	367	NW36	804	NW235	1542
SA217	57	NW18	127	SA010	371	NW40	805	NW236	1543
NW38	58	NW41	127	NW27	372	NW41	806	NW237	1544
NW79	59	SA009	127	NW80	393	NW42	807	NW244	1545
NW56	62	SA028	127	NW175	399	NW43	808	NW245	1546
NW019	69	SA033	127	NW53	405	NW47	809	NW245	1547
D263B	73	SA039	127	NW98	410	SA042	998	SA062	1611
D263C	73	SA126	127	NW165	420	NW011	999	SA137	2020
NW009	73	SA151	127	NW87	448	NW49	1000	SA109	2659
NW012	73	SA153	127	SA161	449	NW61	1001	SA198	3076
NW013	73	SA168	127	SA026	450	NW71	1002	SA030	3556
NW015	73	SA174	127	NW179	493	NW78	1003		
NW001	80	SA189	127	SA132	501	NW86	1004		
NW017	88	SA191	127	SA027	537	NW88	1005		
NW003	95	SA218	127	SA063	540	SA167	1196		
NW33	95	NW72	127	SA099	543	SA123	1266		
SA056	101	NW112	127	NW004	550	NW171	1303		
SA081	101	NW154	127	SA116	617	SA142	1312		

Abbreviations: SA, Saudi Arabia; NW, North West England; D, (Derriford) University Hospitals Plymouth NHS Trust

3.2.2.4 Virulence factor PCR assay and bacterial DNA extraction.

The ten UPEC ST127 isolated from Derriford hospital were additionally tested for the presence of VFs using an established PCR assay (Johnson & Stell, 2000). Template DNA was obtained using the DNeasy blood & tissue spin column kits (Qiagen) as per manufacturers' instructions and quantified using Nanodrop 2000™. Each PCR reaction consisted of 20µl of Biomix™ Red, 20ng of template DNA in a final reaction volume of 40ul.

Table 16. Primers for multiplex PCR virulence assay

Gene	Primer name	Primer sequence (5'-3')	Final Conc (μM)	Product length (bp)
Pool 1				
PAI	RPAi f	GGACATCCTGTTACAGCGCGCA	0.06	930
	RPAi r	TCGCCACCAATCACAGCCGAAC		
papAH	PapA f	ATGGCAGTGGTGTCTTTTGGTG	0.06	720
	PapA r	CGTCCCACCATACGTGCTCTTC		
fimH	FimH f	TGCAGAACGGATAAGCCGTGG	0.06	508
	FimH r	GCAGTCACCTGCCCTCCGGTA		
kpsMT III	KpsIII f	TCCTCTTGCTACTATTCCCCCT	0.06	392
	KpsIII r	AGGCGTATCCATCCCTCCTAAC		
papEF	PapEF f	GCAACAGCAACGCTGGTTGCATCAT	0.06	336
	PapEF r	AGAGAGAGCCACTCTTATACGGACA		
ibeA	Ibe10 f	AGGCAGGTGTGCGCCGCGTAC	0.06	170
	Ibe10 r	TGGTGCTCCGGCAAACCATGC		
Pool 2				
fyuA	FyuA f	F-TGATTAACCCCGCGACGGGAA	0.06	880
	FyuA r	R-CGCAGTAGGCACGATGTTGTA		
bmaE	bmaE f	ATGGCGCTAACTTGCCATGCTG	0.06	507
	bmaE r	AGGGGGACATATAGCCCCCTTC		
Sfa/focDE	sfa1	CTCCGGAGAACTGGGTGCATCTTAC	0.06	410
	sfa2	CGGAGGAGTAATTACAAACCTGGCA		
iutA	AerJ f	GGCTGGACATCATGGGAACTGG	0.06	300
	AerJ r	CGTCGGGAACGGGTAGAATCG		
papG allele III	Allele III-f	GGCCTGCAATGGATTTACCTGG	0.06	258
	Allele III-r	CCACCAAATGACCATGCCAGAC		
kpsMT K1	K1-f	TAGCAAACGTTCTATTGGTGC	0.06	153
	kpsII r	CATCCAGACGATAAGCATGAGCA		
Pool 3				
hlyA	hly f	AACAAGGATAAGCACTGTTCTGGCT	0.06	1177
	hly r	ACCATATAAGCGGTCATTCCCGTCA		
rfc	rfc-f	ATCCATCAGGAGGGGACTGGA	0.06	788
	rfc-r	AACCATACCAACCAATGCGAG		
nfaE	nfaE-f	GCTTACTGATTCTGGGATGGA	0.03	559
	nfaE-r	CGGTGGCCGAGTCATATGCCA		
papG allele I	Allele I-f	TCGTGCTCAGGTCCGGAATTT	0.03	461
	Allele I-r	TGGCATCCCCCAACATTATCG		
kpsMT II	kpsII f	GCGCATTTGCTGATACTGTTG	0.03	272
	kpsII r	CATCCAGACGATAAGCATGAGCA		
papC	PapC f	GTGGCAGTATGAGTAATGACCGTTA	0.03	200
	PapC r	ATATCCTTTCTGCAGGGATGCAATA		
Pool 4				
gafD	gafD-f	TGTTGGACCGTCTCAGGGCTC	0.06	952
	gafD-r	CTCCCGGAACTCGCTGTTACT		
cvaC	ColV-Cf	CACACACAAACGGGAGCTGTT	0.06	680
	ColV-Cr	CTTCCCGCAGCATAGTTCCAT		
cdtB	cdt-a1	AAATCACCAAGAATCATCCAGTTA	0.06	430
	cdt-a2	AAATCTCCTGCAATCATCCAGTTTA		
focG	FocG f	CAGCACAGGCAGTGGATACGA	0.06	360
	FocG r	GAATGTCGCCTGCCCATTGCT		
traT	TraT f	GGTGTGGTGCGATGAGCACAG	0.06	290
	traT r	CACGGTTCAGCCATCCCTGAG		
papG allele II	Allele II-f	GGGATGAGCGGGCCTTTGAT	0.06	190
	Allele II-r	CGGGCCCCCAAGTAACTCG		
Pool 5				
papG	pGf	CTGTAATTACGGAAGTGATTTCTG		
papG II, III	pGr	ACTATCCGGCTCCGGATAAACCAT	0.06	1070
papG I	pG1r	TCCAGAAATAGCTCATGTAACCCG		1190
afa/draBC	Afa f	GGCAGAGGGCCGGCAACAGGC	0.03	559
	Afa r	CCCGTAACGCGCCAGCATCTC		
cnfI	cnf 1	AAGATGGAGTTTCCTATGCAGGAG	0.03	498
	cnf 2	CATTCAGAGTCCTGCCCTCATTATT		
sfaS	SfaS f	GTGGATACGACGATTACTGTG	0.03	240
	SfaS r	CCGCCAGCATTCCCTGTATTC		
kpsMT K5	K5-f	CAGTATCAGCAATCGTTCTGTA	0.06	159
	kpsII r	CATCCAGACGATAAGCATGAGCA		

Primers were sorted into 5 pools with each primer used at a final PCR concentration of 0.06µM or 0.03µM (Table 16) (Johnson & Stell, 2000).

PCR was performed on the C1000 Touch™ thermal cycler with the following parameters; an initial denaturation step of 5 min at 95°C, 35 cycles of 95°C for 1 minute, annealing at 63°C for 1 min, extension at 72°C for 1 minute with a final extension at 72°C for 5 minutes. Reactions were visualised on a 2% (w/v) agarose (Promega Corporation, Madison, USA) Tris acetate-EDTA (TAE) buffer (Sigma, Dorset, UK) gel, incorporating 5µl (0.5µg/ml) ethidium bromide per 100ml of gel. Electrophoresis was performed at 90V for 60 minutes and bands visualised under UV transillimination (UVP ChemiDoc 810 imager, CA, USA).

3.3 Results

3.3.1 Quality assessment of genomic data.

The data relating to the quality of reads, depth of coverage and assemblies were obtained from the Illumina sequencing metrics and QUAST reports. A summary of these data are listed in Table 17. Data from the independent assemblies and annotation using Ubuntu 14.04 BioLinux 8 operating software were all at acceptable levels and in most cases were almost identical to the MicrobesNG assemblies (data not shown).

Table 17. List of quality raw data and assembly metrics for all sequenced isolates.

Organism (Strain No.)	Number of reads	Depth of coverage (x)	Total number of contigs	Number of contigs >=500bp	Size of assembled genome	N50
E. coli						
9	1074127	94.7	156	90	5030316	393870
39	558066	48.2	173	96	5213994	291752
124S	732202	62.2	204	89	5137408	279152
124L	875063	76.1	206	111	5134573	279152
263	635589	56.2	186	100	5098135	365405
264	1010107	88.4	196	93	5137206	288160
298	953639	83.3	162	86	5046317	371892
316	592517	51.5	182	81	5102852	272060
354	897233	78.7	193	104	5051726	476716
468	703655	59.1	188	108	5295847	288203
263AR	887635	73.8	242	130	5362399	361841
263TR	854671	75.0	151	82	5100112	209249
EC18	829475	75.0	194	110	4904929	203407
EC41	735416	61.7	170	114	5282179	170883
SA189	835856	72.7	140	73	5120014	271777
SA191	788170	69.0	164	70	5075641	279153
Ent. faecalis						
*Ent*32	607320	83.1	99	34	2849658	328077
*Ent*39	611334	78.1	103	47	2980325	150904
*Ent*108	1579445	226.7	133	56	3006768	195279
*Ent*242	481557	65.6	96	42	2965419	326890
*Ent*257	420526	55.6	80	41	2991394	310573
*Ent*271	1118291	148.4	108	49	3040406	294313
*Ent*273	571083	71.0	76	35	3068881	257025
*Ent*283	583863	73.1	107	48	3183337	254955
*Ent*313	1102517	157.0	116	42	2951515	201246
*Ent*314	839443	109.8	106	56	3167824	222580
*Ent*335	622459	76.7	87	34	3139463	398537
*Ent*372	901921	105.8	106	61	3421177	183261
*Ent*376	827839	108.3	299	52	2985627	236868
*Ent*380	758594	101.6	89	33	3151559	327345
*Ent*408	372922	44.8	136	80	3083559	243872
*Ent*440	653296	87.0	95	38	3049983	284382
*Ent*442	390500	50.1	71	40	3119657	252674
*Ent*459	709822	89.9	129	49	3034864	284394

Although there are no national or international standards for QC thresholds to assess NGS data quality, sequences and assemblies were based on the following quality control data and interpretations (Ellington *et al.*, 2017): 'Number of reads' refers to the sequence yield (the amount sequenced); 'Depth of coverage' describes the average number a base pair has been sequenced on an individual read, often expressed as x coverage, a minimum depth of >30 is generally acceptable; 'Total number of contigs' generally <1000 indicates good

quality but is dependent on the size of the genome, <200 for 5-6Mb is acceptable; 'Number of contigs >=500bp' should correspond well to the total number of contigs; 'Size of assembled genome' can indicate contamination during specimen preparation if genome is larger than expected; 'N50' statistical interpretation based on the contig lengths, which can be thought of as half of the mass of the contig distribution, a minimum size of 30,000 is preferred. The raw data and assembly metrics were all within acceptable ranges for both the *E. coli* and *Ent. faecalis* genomes.

3.3.2 MLST results for UPEC and *Ent. faecalis* isolates, serotyping, *fimH* typing and identification of antibiotic resistance factors in ST127 and ST73 Derriford isolates using WGS data.

Sanger sequencing MLST results for all Derriford hospital isolates (from Chapter 2) were confirmed correct from the WGS data, as were the additional eight UPEC genomes downloaded from NCBI. MLST results determined from the WGS data for the *Ent. faecalis* isolates obtained from Derriford hospital and the NCBI database are displayed in Figure 17 and 18.

All ST127 isolates were of the O6:H31 serotype except for specimen 124L and EC18, which did not possess any O-antigen genes but did carry the H31 serotype. Both of the ST73 isolates found in specimen 263 (strains 263AR and 263TR) possessed the O6:H1 serotype. The majority of ST127 isolates carried the *fimH*2 type gene with a handful of exceptions: isolate 39 carried the *fimH*5, strain 263AS carried the *fimH*224, isolate 264 carried *fimH*462 and isolates 316

and SA191 carried *fimH*180. The two ST73 isolates from specimen 263 both carried the *fimH* type 30.

Antimicrobial susceptibility showed that all ST127 isolates were sensitive to Trimethoprim except the 124L isolate. One of the ST73 isolates was also resistant to Trimethoprim (263TR). An initial genome search for the 42 known Trimethoprim resistant Dihydrofolate reductase (DHFR) genes in the CGE database revealed no hits for isolate 124L. Manual investigations of the DHFR genes (artemis annotation *folA*) and the DHFR flanking regions found that all ST127 isolates had an identical 615bp DHFR gene with identical flanking regions between the Glutathione-regulated potassium-efflux system KefC and the Antitoxin encoding gene CcdA. The 124L isolate was the only exception showing variation from the consensus sequence ST127 DHFR gene (Figure 7). Point mutations were identified at 48bp (A to G) and 88bp (T to G) causing two non-synonymous amino acid substitutions (Figure 7). Additionally one further point mutation and a Thymine dinucleotide deletion was present upstream of the DHFR gene (data not shown) but the impact upon gene function was unknown. The consensus sequence for ST127 received 100% identity match for the DHFR gene of *E. coli* K12 in the Swiss-prot protein database.

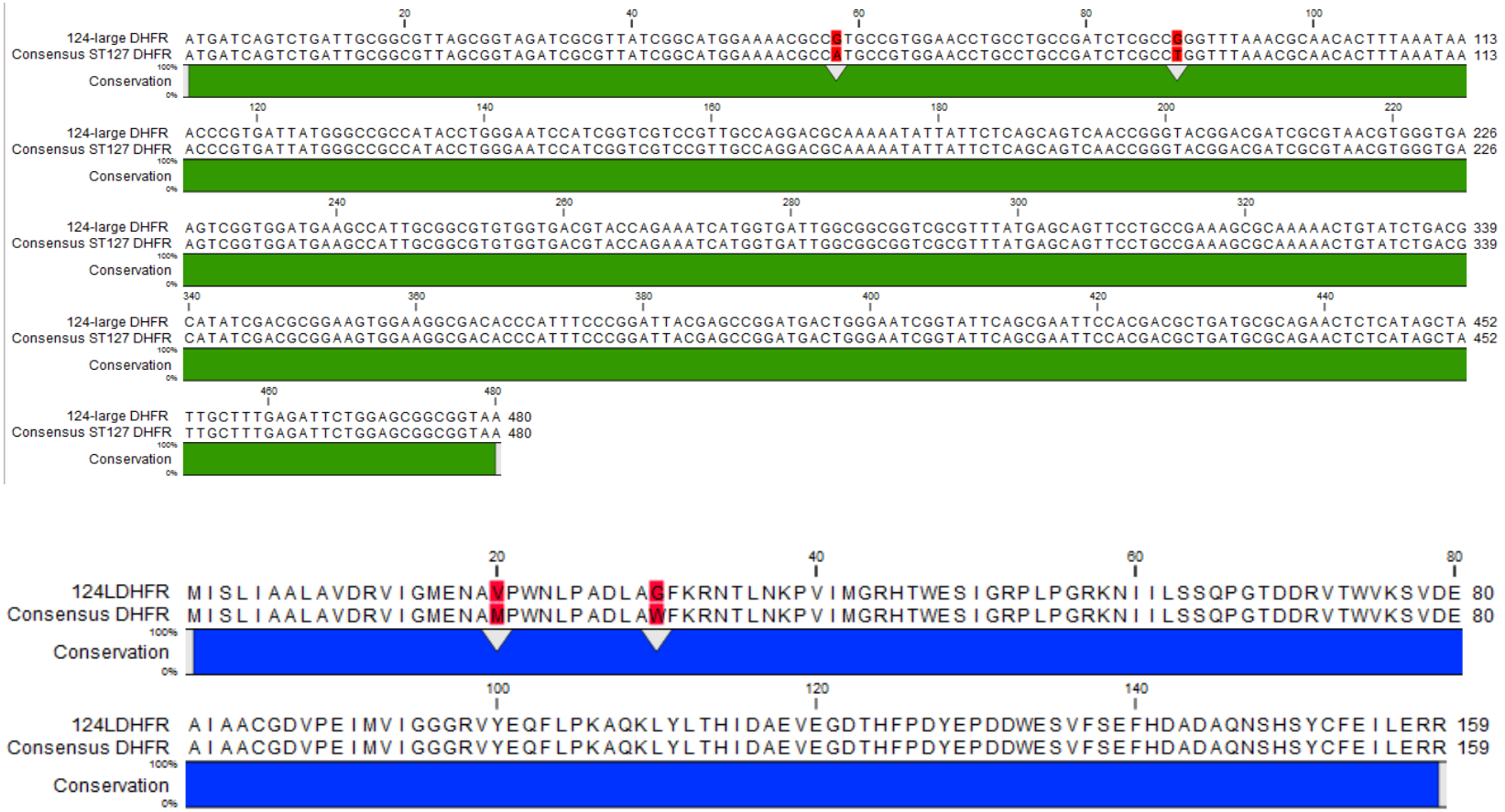

Figure 7. Sequence and amino acid alignments of the consensus ST127 DHFR gene and the 124L DHFR gene. Point mutations and subsequence amino acid substitutions are highlighted in red. Alignment was created using CLC workbench 7.5.1.

Unlike the Trimethoprim resistance observed in the 124L isolate, resistance in the ST73 263TR isolate was not due to mutations within the DHFR genes as the sequence for all five ST73 isolates used in this study were identical. Trimethoprim resistance for the ST73 isolate was due to the presence of the Tn7 type I DHFR transposon containing the drfA1 (ResFinder 3.0 annotation) variant (Fling & Richards, 1983) and the resistance to the penicillin antibiotics in ST73 263AR isolate was attributed to the presence of the blaTEM-1B gene (NCBI accession number JF910132). The blaTEM-1B gene was also responsible for penicillin resistance in both isolate 39 and 264 and the blaTEM-1A gene was found in isolate 9 (Bailey *et al.*, 2011). Resistance in *Ent. faecalis* isolates using WGS data or plate disc diffusion, was not recorded.

3.3.3 Clinical relevance of additional bacterial isolates that underwent WGS.

Table 18 lists the additional organisms sequenced by MicrobesNG along with pertinent information regarding their clinical relevance, location of isolation and patient details. Specific information on bacterial growth or patient details for the additionally sequenced *E. coli* isolates was not available. All but three of the *Ent. facalis* were isolated as the secondary organism in respect to bacterial concentration and the three specimens in which *Ent facalis* was in abundance or equal concentration, were considered negative culture for UTI.

Table 18. Additional sequenced organisms and their clinical relevance. Abbreviations: NK, Not Known; F, Female; M, male; Sc, Scanty; SA, Saudi Arabia.

Isolate	Species	Location isolated	Clinical relevance				
			Growth quantity	Isolated *E.coli* /quantity	Patient age	Patient gender	UTI result
EC18	*E. coli*	Manchester	NK	NK	NK	NK	Positive
EC41	*E. coli*	Manchester	NK	NK	NK	NK	Positive
SA189	*E. coli*	Riyadh, SA	NK	NK	NK	NK	Positive
SA191	*E. coli*	Riyadh,SA	NK	NK	NK	NK	Positive
Ent32	*Ent. faecalis*	Plymouth	Sc	3+ *E. coli*	73	F	Positive
Ent39	*Ent. faecalis*	Plymouth	Sc	3+ *E. coli*	35	F	Positive
Ent108	*Ent. faecalis*	Plymouth	Sc	3+ *E. coli*	35	F	Positive
Ent242	*Ent. faecalis*	Plymouth	Sc	2+ *E. coli*	75	F	Positive
Ent257	*Ent. faecalis*	Plymouth	Sc	3+ *E. coli*	42	F	Positive
Ent271	*Ent. faecalis*	Plymouth	2++	3+ *E. coli*	75	F	Positive
Ent273	*Ent. faecalis*	Plymouth	Sc	3+ *E. coli*	30	F	Positive
Ent283	*Ent. faecalis*	Plymouth	1+	3+ *E. coli*	80	F	Positive
Ent313	*Ent. faecalis*	Plymouth	Sc	3+ *E. coli*	42	M	Positive
Ent314	*Ent. faecalis*	Plymouth	Sc	3+ *E. coli*	78	F	Positive
Ent335	*Ent. faecalis*	Plymouth	1+	Sc *E. coli*	74	F	Negative
Ent372	*Ent. faecalis*	Plymouth	Sc	3+ *E. coli*	76	F	Positive
Ent376	*Ent. faecalis*	Plymouth	2+	1+ *E. coli*	58	F	Negative
Ent380	*Ent. faecalis*	Plymouth	3+	3+ *E. coli*	70	F	Negative
Ent408	*Ent. faecalis*	Plymouth	2+	3+ *E. coli*	3	F	Negative
Ent440	*Ent. faecalis*	Plymouth	1+	3+ *E. coli*	84	M	Positive
Ent442	*Ent. faecalis*	Plymouth	Sc	3+ *E. coli*	59	F	Positive
Ent459	*Ent. faecalis*	Plymouth	3+	3+ *E. coli*	79	F	Negative

3.3.4 Identification of gene regions specific to UPEC ST127 and the development and validation of an ST127 specific multiplex PCR.

3.3.4.1 Identification of UPEC ST127 specific genomic sites.

Using BRIG comparisons, several regions on the ST127 genome were identified as having low similarity to non-ST127 genomes (Figure 8). Each locus identified within the UPEC ST127 genomes with ≤70% similarity in comparison to the other common non-ST127 UPEC genomes, underwent further investigation using blastn and blastp database comparisons, culminating with the identification of two gene variants highly specific to ST127, namely the *fliC*

gene and a putative *upaG* gene. Each of the 14 sequenced ST127 genomes and the genome of reference ST127 strain 536 carried an identical 1668bp *fliC* gene, with the single exception in isolate SA189, which exhibited a C to A substitution at position 699, producing only a synonymous mutation in a valine codon. The second locus identified was a large 4875bp gene sequence that shared 73% identity with the *upaG* trimeric autotransporter (AT) protein found in *E. coli* CFT073 (Valle *et al.*, 2008). The putative ST127 *upaG* gene shared 100% similarity across 12 of the 14 sequenced ST127 isolates. The remaining two sequenced isolates shared at least 99.96% similarity; isolate 264 had two nucleotide changes at position 2971 (G to A resulting in a Alanine to Theronine codon) and position 3147 (C-T producing only a synonymous Aspartic acid codon); isolate 298 shared the same point mutation at position 2971. The reference ST127 536 isolate also had one differing nucleotide from the consensus *upaG* at position 3265 (A-G producing a Glycine to Serine codon).

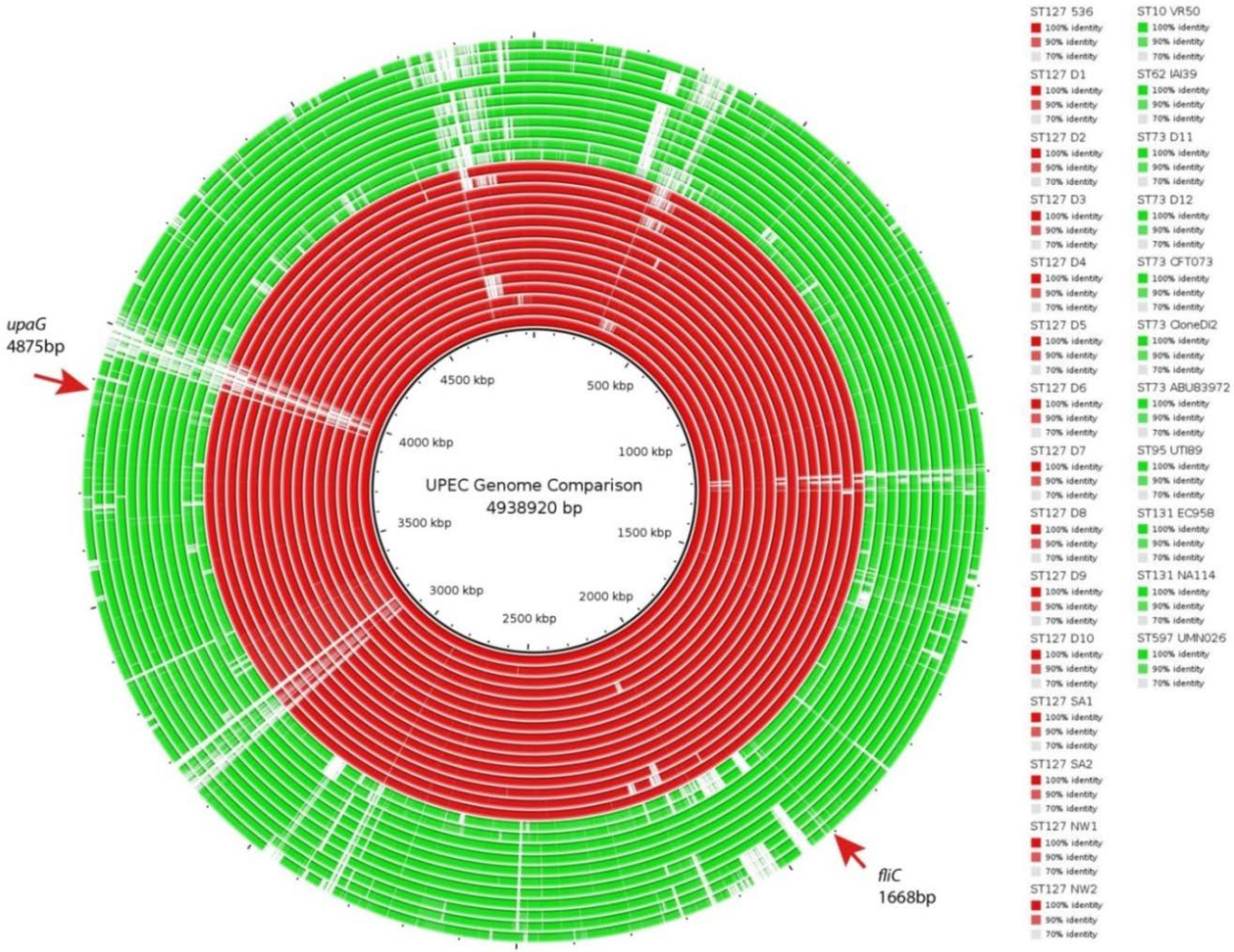

Figure 8. Comparison of 15 UPEC ST127 (red) with 11 non-ST127 common UPEC lineages (green). Arrows indicate two areas of interest with sequences present in ST127 genomes but absent in other tested UPEC genomes. Lightly shaded regions represent areas in the genome with ⩽90% similarity with the reference genome 536, areas void of colour represent ⩽70% similarity. Abbreviations: D, Derriford Hospital Plymouth; SA, Saudi Arabia; NW, North West England.

3.3.4.2 Primer Design.

Blast searches identified *upaG* and *fliC* as highly specific (May 2017). All genomes in the NCBI database with closely related *fliC* or *upaG* sequences were downloaded and run with the MLST 1.8 programme at the CGE website to determine the organism's MLST profile. The 1668bp *fliC* gene recorded only 3 blast hits with exact homology. The three matching bacterial genomes were 536 (reference ST127), K-15WO1 and ECONIH2 with another isolate, 317, recording 99% similarity. These four *fliC* homologous Blast hits were all in ST127 strains. Other genomes that had high similarity were FH140, Ecol 224 and AR0118 (97% identity). MLST results for these genomes identified Ecol 224 and AR0118 as ST101 and FH140 as an unknown (faecal isolate, possible new ST or poor sequence data). Other hits were either individual *fliC* gene sequences submitted to the Blast database or genomes with approximately only 50% of the ST127 *fliC* coverage. The forward primer focused on a *fliC* region conserved within all *E. coli* strains located early in the gene sequence (position 12-30bp), whereas the reverse primer focused on the central variable region, specifically at a variable 4 base nucleotide sequence observed in the closest matching ST variant ST101 (position 531 to 534).

The consensus ST127 *upaG* sequence also identified strains K-15WO1 and ECONIH2 as sharing 100% identity and coverage. Genomes 536 and 317 both recorded 100% and 99% coverage, respectively. Other blast hits for the ST127 *upaG* sequence were in strains PAR (ST1858) and FHI72 (Unknown, faecal isolate) at 97 and 96% identity, respectively. Genomes FHI_NMBU_03, PSU02 and NGF1 were all at 95% similarity, these strains being ST28, ST141 and ST998, respectively. The remaining Blast hits only recorded coverage of 69%.

Analysis of the presumptive *upaG* genes in the closest matching non-ST127 genomes revealed variable regions between 150bp to 1100bp and 1790bp to 1840bp, the forward and reverse *upaG* primers exploited those regions of variability, respectively.

3.3.4.3 UPEC ST127 specific multiplex PCR assay results and validation.

Each primer pair underwent individual validation, all were 100% sensitive but neither pair was 100% specific. Acceptable sensitivity and specificity results were obtained by optimising the two primer pairs into a multiplex PCR incorporating both *fliC* and *upaG* primers and *gyrB* primers as an extraction/PCR control. A positive ST127 isolate was identified by the presence of 3 distinct DNA bands (Figure 9).

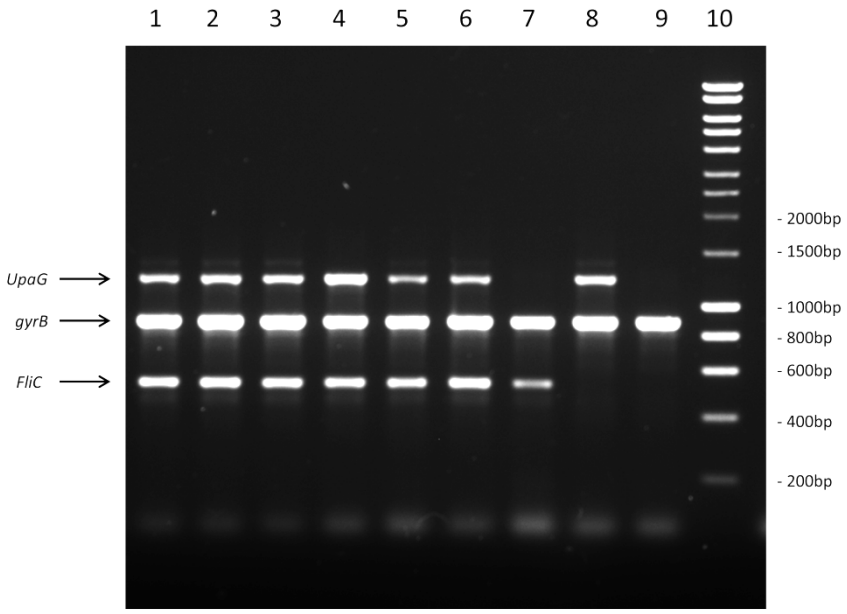

Figure 9. PCR detection for members of the ST127 clone. The *gyrB* band (911bp) corresponds to the extraction/PCR control amplified in all isolates. The *upaG* and *fliC* bands both amplified in the same reaction correspond only to isolates from the ST127 lineage. Lanes 1-6 are ST127 isolates, lanes 7-9 are non-ST127 isolates. Lane 1, NW41; lane 2, D3; lane 3, D4; lane 4, NW72; lane 5, NW112; lane 6, NW154; lane 7, NW153 (ST1529 *fliC*+); lane 8, SA027 (ST537 *upaG*+), lane 9, EC958 (ST131) and lane 10, Molecular marker-1kb (Bioline, London, UK).

The *gyrB* extraction/PCR control was amplified in all validation reactions and amplification of both ST127 PCR targets occurred only with the 29 isolates of UPEC ST127. With reference to the individual PCR targets, FliC flagellin PCR primers produced the best results proving to be highly sensitive and specific for the UPEC isolates producing only three false positive results for ST372, ST420 and ST1529 with a sensitivity and specificity result of 100% and 97.3%, respectively. The three non-ST127 strains that produced an amplicon with the fliC primers were not closely related to the ST127 clone as ST372 contained only three of the seven matching ST127 MLST alleles (*gyrB*, *icd* and *mdh*), ST420 contained only one matching allele (*adk*) and ST1529 contained no matching alleles. Amplification from just the two gyrB and upaG primers occurred in 9 non-ST127 isolates (ST14, ST80, ST141, ST537, ST540, ST550, ST785, ST807 and ST998). Unlike the *fliC* locus, three of *upaG* false positive results (ST14, ST550 and ST807) were easily distinguishable as negative as they produced a shortened amplicon in the range of ~1000bp (Figure 10, lane 23). Of the isolates that recorded a positive result with the UpaG primers, none were particularly closely related to ST127. ST80 had 4 matching MLST alleles (*adk*, *gyrB*, *mdh* and *recA*), ST537 3 matching alleles (*fumC*, *purA* and *recA*) and the remaining STs with only 2 or fewer matching alleles.

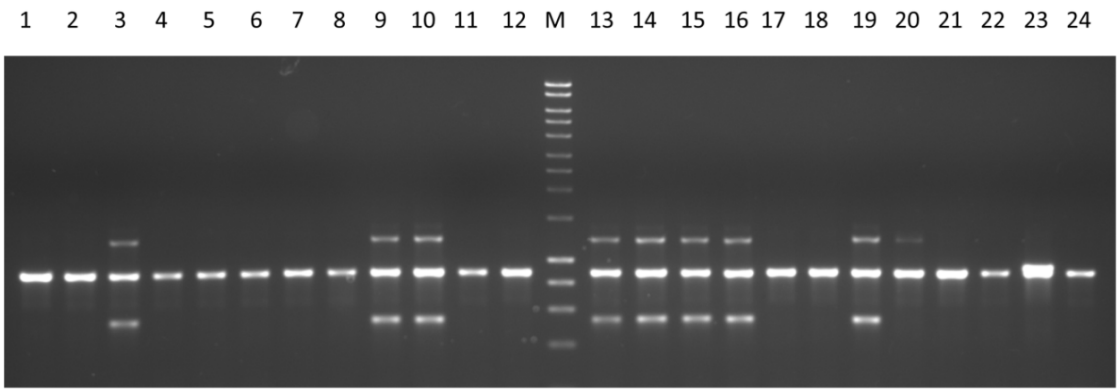

Figure 10. Routine testing of upaG-gyrB-fliC ST127 specific PCR. Lanes 3, 9, 10, 13-16 and 19 were ST127 isolates, positive for the 3 gene loci. Lanes 1-2, 4-8, 11, 12, 17, 18 and 20-24 were non-ST127 isolates. Lane 20 and 23 (ST80 and ST550, respectively) are examples of a weak *upaG* positive (lane 20) and a *upaG* positive (lane 23) of reduced amplicon length causing the *upaG* to merge with the *gyrB* amplicon. M, Molecular marker-1kb (Bioline, London, UK).

3.3.5 Examination of different UPEC variants within the same urine culture.

Genomic comparisons between the two ST127 isolates found in urine specimen 124 (Figure 11) and the two ST73 isolates found in specimen 263 (Figure 12) were performed using the BRIG analysis software. Employing the 124S genome as the reference genome (Figure 11a), a large variance (≤70% identity) in genomic content was observed (red arrow) in the 124L genome at ~2.2mbp (million base pars). The mirror comparison using genome 124L as the reference (Figure 11b) did not present with any major genomic variances. Using genome 124S as the reference, a comparison of both 124 genomes and the O-antigen negative EC18 was performed (Figure 11c). An identity of ≤70% genomic content, of varying size, was observed at ~2.2mbp in both EC18 and 124L genomes.

Further analysis of the two ST127 isolated from specimen 124 and ST127 EC18 was performed using the Artemis Comparison Tool (ACT). The gap represented on both genome 124L and EC18 at ~2.2mbp using BRIG analysis was identified

as being involved with O-antigen Biosynthesis (Figure 13). The genome assembly of 124L possessed an 18,231bp deletion between the *algC* phosphomannomutase gene and a *manC* mannose-1-phosphate guanylytransferase gene (Figure 13). The O-antigen deletion in the genome assembly of EC18 was much larger comprising 28,212bp. Numerous genes were missing from the 124L O-antigen region including the liposaccharide biosynthesis gene *wzxC* and the *rfbX* O-antigen transporter gene (Figure 13).

In addition to the missing O-antigen in isolate 124L and EC18, both isolates possessed two point mutations in the *ompC* gene causing non-synonymous amino acid translational changes in both isolates. The subsequent amino acid substitutions were at four different locations and individual to the two isolates but not present in any of the other O-antigen positive ST127 isolates.

Comparison of the two ST73 isolated from specimen 263, using alternate genomes as a reference (Figure 12a and 12b), displayed numerous regions on each genome that varied with the other (examples highlighted with black arrows) providing evidence that the two ST73 isolated from specimen 263 were different strains of the ST73 lineage. Further differences in genomic content were observed (Figure 12c) when both of the ST73 isolates from sample 263 were compared to the reference genome CFT073 (red and blue arrows).

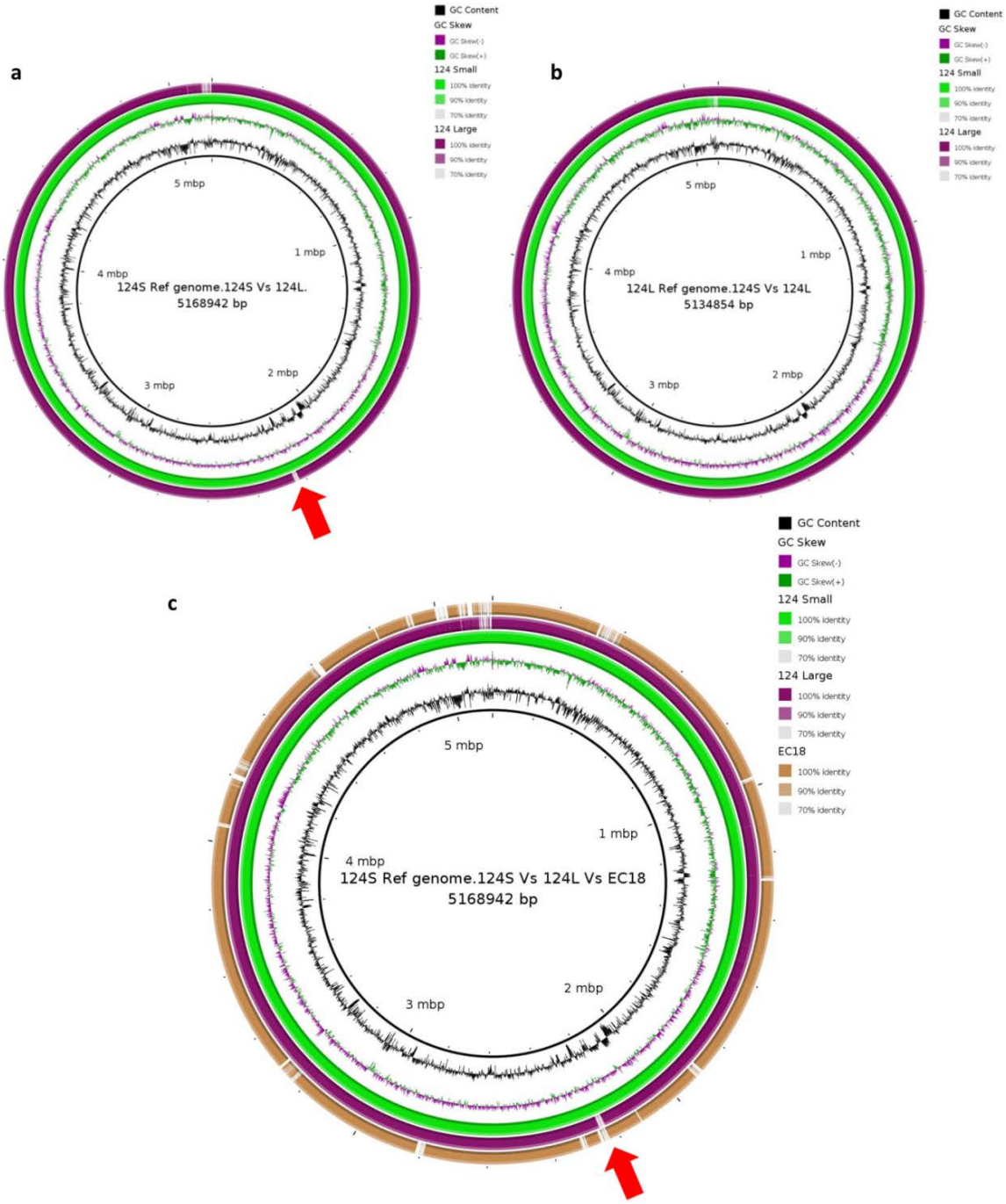

Figure 11. Comparison between three ST127 genomes. Ring a, 124S as reference genome (green) and genome 124L (purple); Ring b, mirror comparison with 124L as reference genome; Ring c, comparison of the two 124 isolates including the O-antigen negative EC18 (brown) with the124S genome as reference. The red arrows indicate regions of DNA absent in genomes 124L and EC18 in comparison to genome 124S. All comparisons were completed using Blast Ring Image Generator.

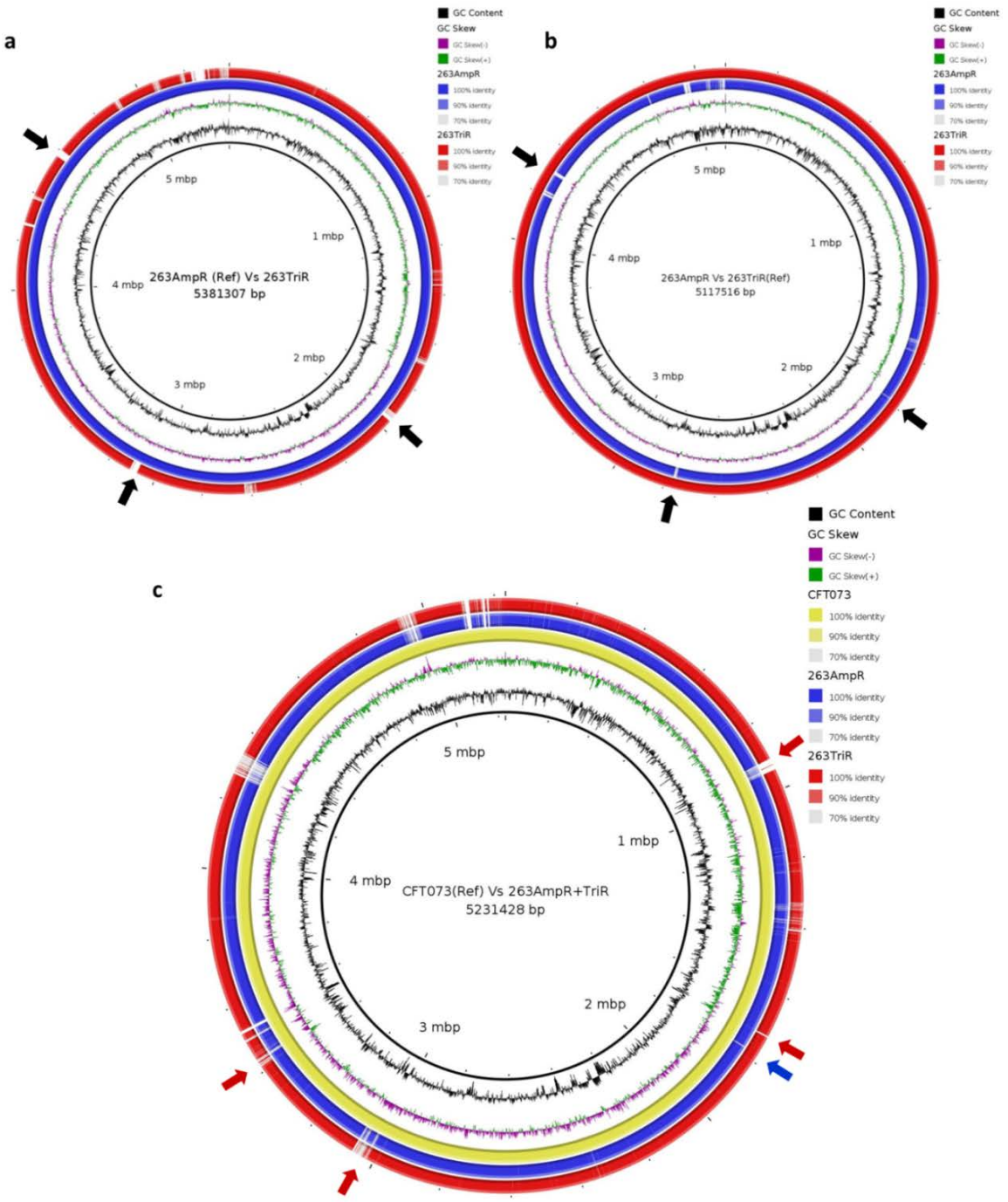

Figure 12. Comparisons between three ST73 genomes. Ring a, 263AR as reference genome (blue) and genome 263TR (red); Ring b, mirror comparison with 263TR as reference genome; Ring c, comparison of the two 263 isolates including CFT073 (yellow) as the reference genome. The black arrows indicate regions of DNA present in only one genome of ST73 isolated from urine specimen 263. The red and blue arrows represent regions within the 263 ST73 genomes at variance with the reference CFT073 genome. All comparisons were completed using Blast Ring Image Generator.

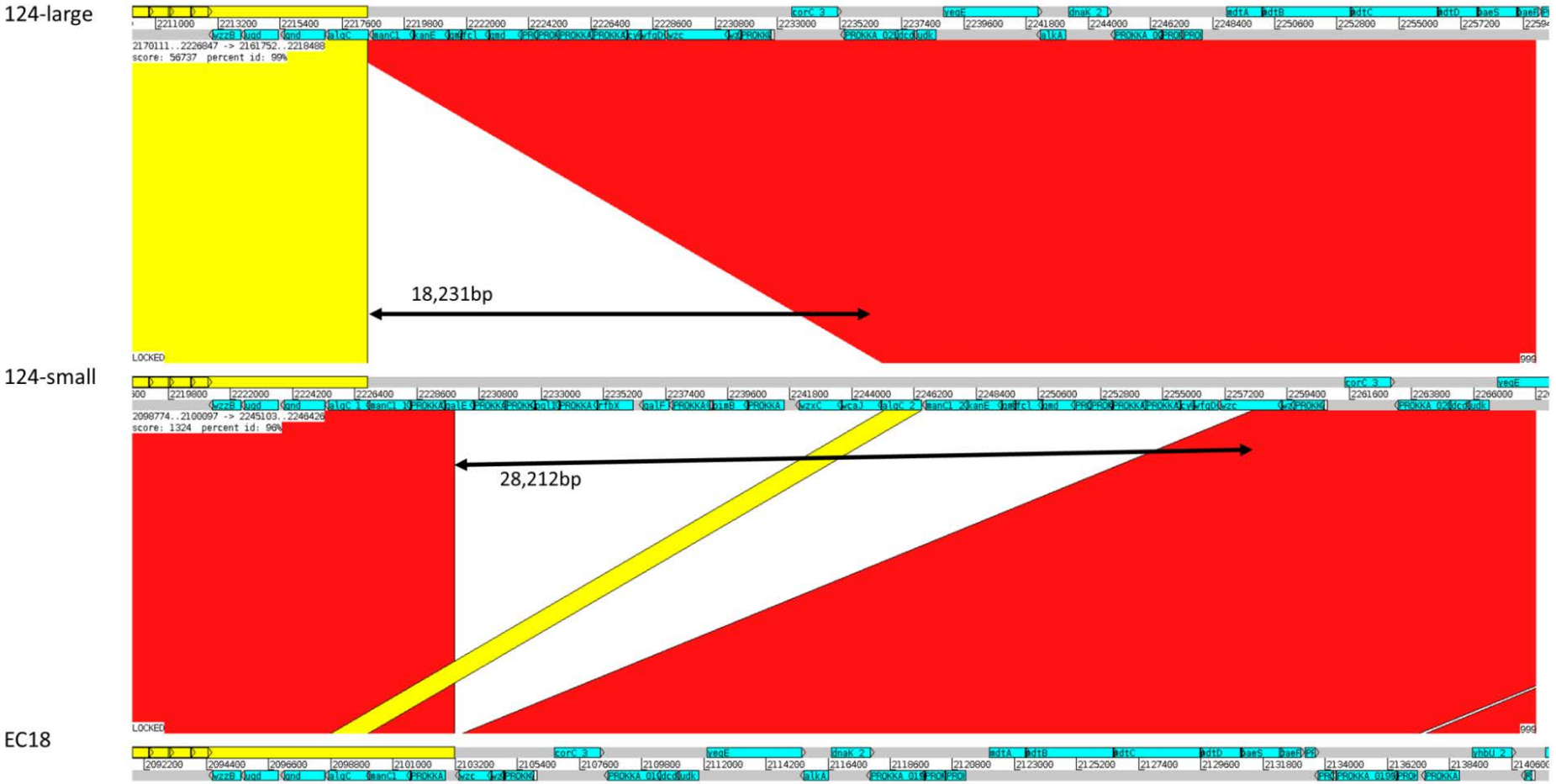

Figure 13. Comparison of ST127 strains 124L, 124S and EC18 using ACT. The large deletion of 18,231bp in 124L and 28,212bp in EC18 compared to 124S genome.

A search for insertion sequences using ISfinder (Zhang *et al.*, 2000) revealed a 225bp region in the 124L genome adjacent to the *manC* gene with 98% similarity to the insertion sequence (IS) IS*1X3* found in *Escherichia fergusonii*, providing some evidence that the O-antigen deletion may be due to an IS, similar to that seen in EC18 (Alghoribi *et al.*, 2014). The same exact 225bp sequence region was found at seven different sites across the 124L genome.

Additionally, a ST127 genome became publically available in December 2017 on the NCBI website that also contained an O-antigen deletion. The genome belonging to isolate BH100N_MG2017 (https://www.ncbi.nlm.nih.gov/nuccore/-1337727359) had a 1432bp region between the *hisE* and the *wzxC* genes belonging to the IS*3* family (IS*150* group, IS*1397*), disrupting the O-antigen.

3.3.6 Phylogenic relatedness of UPEC ST127 isolates and urine associated *Ent. faecalis* as determined using SNP core analysis phylogenies.

SNP core analysis of the genome was performed on all UPEC ST127 genomes individually (91% genome coverage - Figure 14) and alongside the publically available UPEC genomes (73% genome coverage - Figure 15). Reference genome ST127 563 was used for both the ST127 phylogeny and the phylogeny involving all UPEC isolates.

The SNP core analysis phylogeny for the ST127 isolates (Figure 14) shows the reference EC536 as the most divergent isolate. The two isolates obtained from the same urine specimen (sample 124) revealed a low number of SNP variances suggesting a small and very recent divergence. The SNP core analysis inferred phylogenies for the multiple ST127, ST73 and ST131 used in

this study were clustered together into individual clades, revealing their genetic relatedness in agreement and supporting the MLST method for the identification of different *E. coli* genotypes (Figure 15).

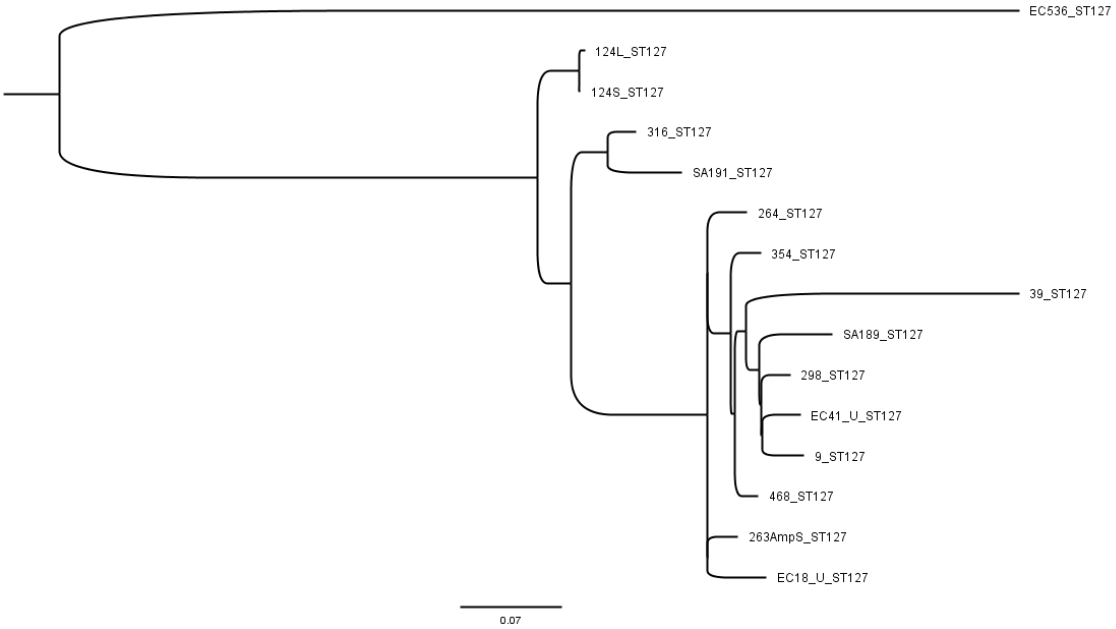

Figure 14. Maximum likelihood phylogeny inferred from SNP core analysis on all ST127 isolates. Isolates 124S and 124L are very closely related but not identical. Labels represent specimen isolate and assigned ST. UPEC isolate 536 ST127 was used as the reference genome. Scale bar represents substitutions per site.

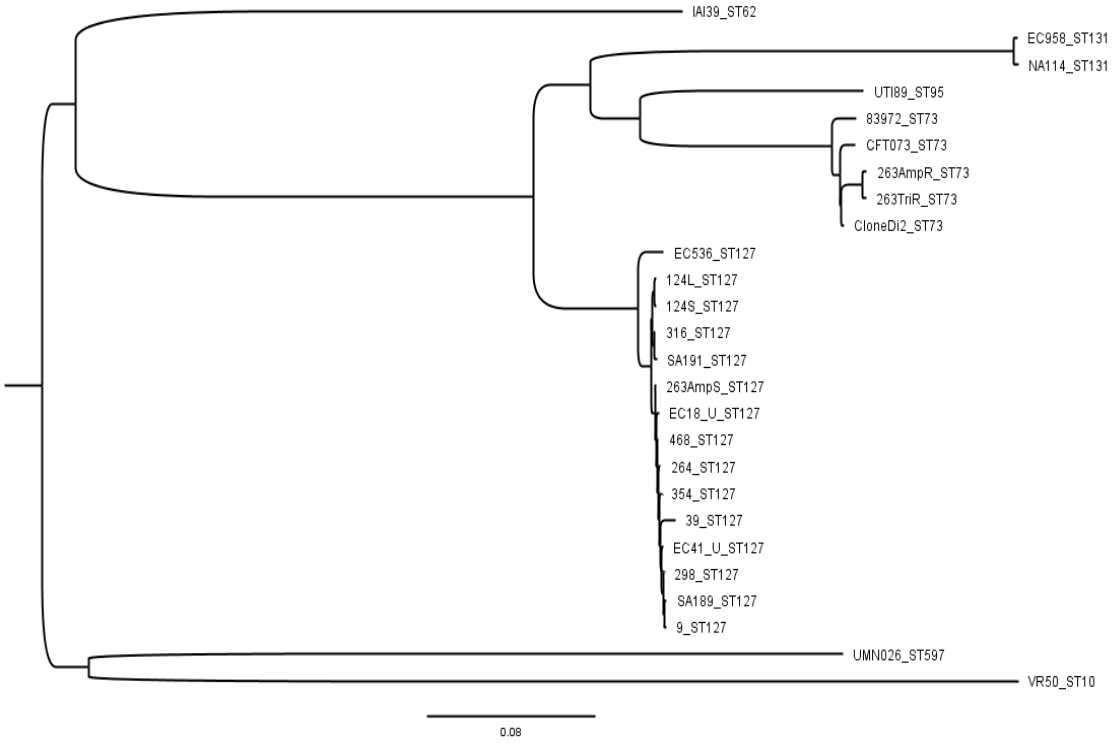

Figure 15. Maximum likelihood phylogeny inferred from SNP core analysis on all ST127 genomes and additional UPEC genomes from NCBI database. Labels represent specimen isolate and assigned ST. All ST127, ST73 and ST131 were present in their own individual clade. UPEC isolate EC536 was used as the reference genome. Scale bar represents substitutions per site.

All ST73 isolates used in this study were also placed in an individual phylogeny (91% genome coverage – Figure 16).

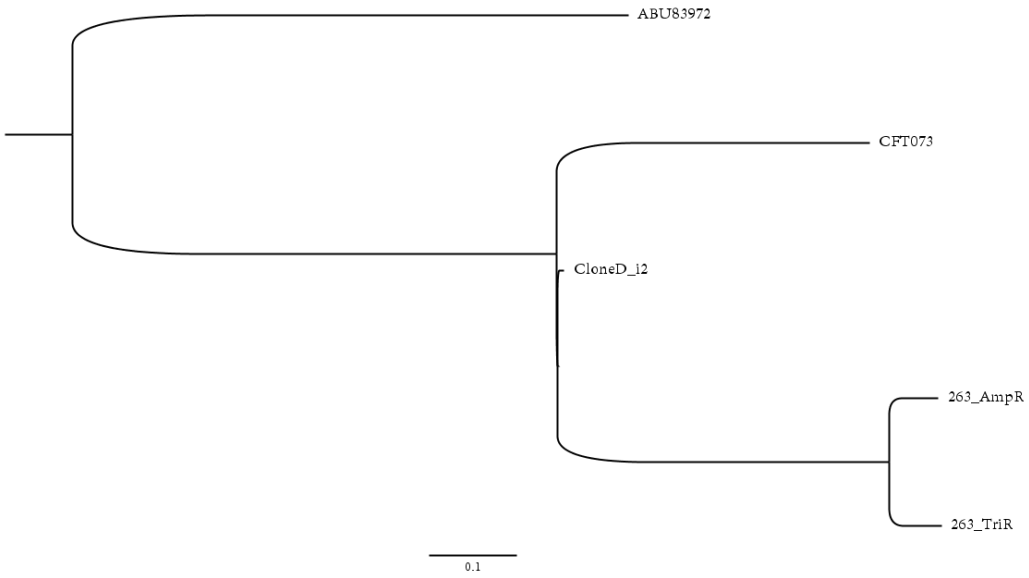

Figure 16. Maximum likelihood phylogeny inferred from SNP core analysis on all ST73 isolates used in this study. CFT073 was used as the reference genome. Scale bar represents substitutions per site.

The two ST73 isolated from specimen 263 (Figure 16) displayed a recent but much larger divergence than the ST127 124 isolates. The ABU strain had a large divergence from the pathogenic isolates of ST73.

The urine associated *Ent. faecalis* genomes isolated from Derriford hospital were also subject to individual SNV calling (86.5% genome coverage – Figure 17) and alongside the 33 publically available genomes of the same species (81% genome coverage - Figure 18).

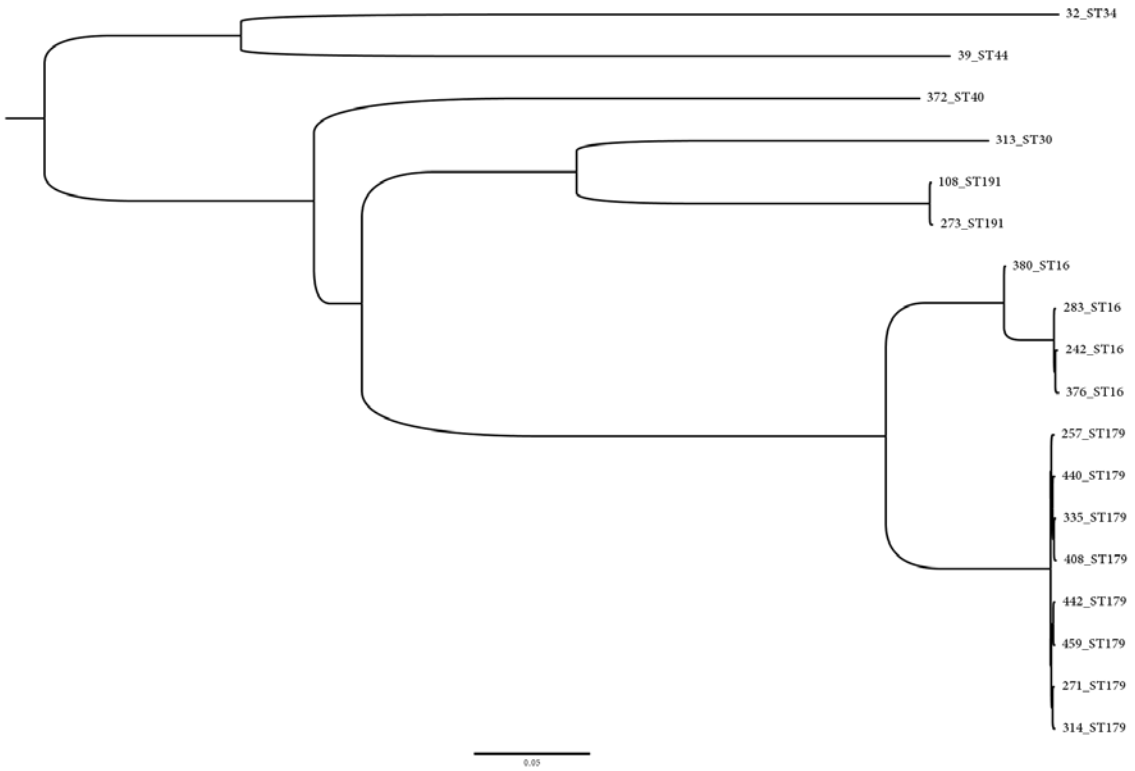

Figure 17. Maximum likelihood phylogeny inferred from SNP core analysis on all urine associated *Ent. faecalis* isolated from Derriford hospital. Labels represent specimen isolate and assigned ST. Isolate 257 ST179 was used as the reference genome for SNP calling. Scale bar represents substitutions per site.

Ten individual reference genomes were used for the *Ent. faecalis* phylogenies (Figure 17 and 18) in an effort to obtain the highest genome coverage. The best agreement and highest genome coverage was seen using a genome of *Ent. faecalis* isolated from a water purification plant (ST 21).

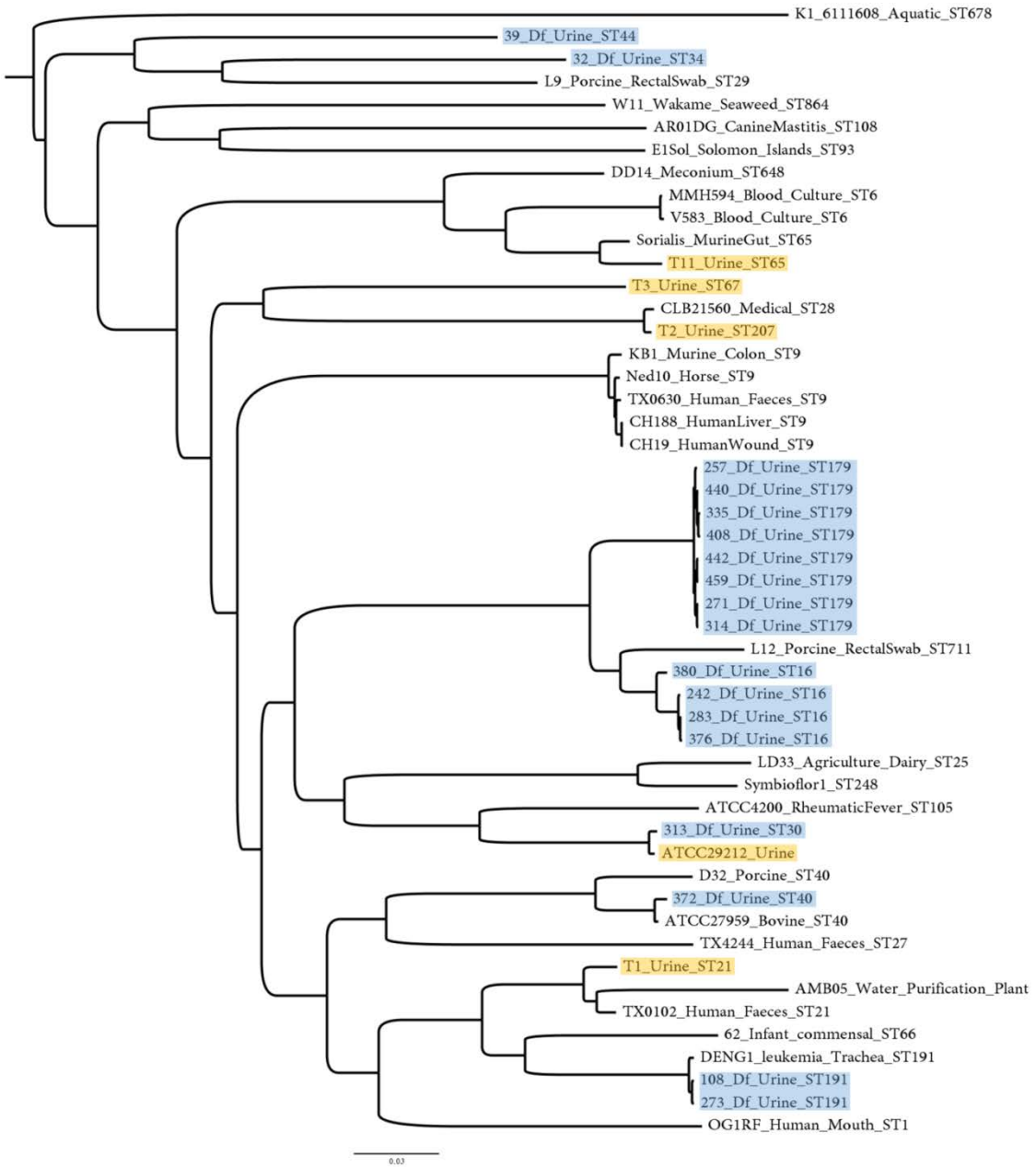

Figure 18. Maximum likelihood phylogeny inferred from the SNP core analysis on all urine associated *Ent. faecalis* isolated from Derriford hospital and publically available genomes available from NCBI. Labels represent specimen isolate and assigned ST. The AMB05 Water Purification Plant *Ent. faecalis* was used as the reference genome for SNP calling as this organism gave the highest core genome coverage of 82%. Specimens highlighted in blue are the *Ent. faecalis* isolated from Derriford hospital. Highlighted in yellow are additional urine *Ent. faecalis* from the NCBI database. All identical STs were grouped in the same clade. Scale bar represents substitutions per site.

Similarly to the UPEC, the *Ent. faecalis* phylogenies grouped the strains from the same STs together in the same clades (Figure 17 and 18). All specimens

originally cultured from urine either from Derriford hospital or Japan (T1, T2, T3 and T11) were not localised in one part of the phylogeny but a large proportion of the Derriford *Ent. faecalis* isolates belonged to two ST types (ST179 and ST16) which both clustered on one major branch of the phylogeny. However, six of the Derriford *Ent. faecalis* and the five NCBI urine associated isolates were scattered across the whole phylogeny.

3.3.7 SNP core analysis

The SNP core analysis was completed using separate SNP core alignments involving two test organisms and one reference, obtained from CSI phylogeny. A conservative 10bp minimum distance between SNP positions was used for the core SNP comparison; a necessary requirement for closely related organisms. The number of potential SNP positions varied between different comparisons, therefore the percentage SNP variation was calculated in an attempt to analyse the comparison set as a whole allowing a prediction of a 'cut-off' value by which the level of core SNP variation can reliably determine if two bacteria should or should not be considered as the same organism (see Table 19).

Table 19. SNP core genome analysis of ST127 and ST73 isolates

Isolate comparison	Reference strain	Number of SNPs	Potential SNP Positions	% core SNP variation	Isolates origins
			ST127		
9 Vs 39	536	968	3783	25.5	Derriford Hospital in-patient and GP Liskeard, Cornwall
124S Vs 124L	536	24	2537	0.95	GP Callington, East Cornwall
263AS Vs 264	536	245	2986	8.2	GP South Brent, Devon and GP St Judes, Plymouth
354 Vs 468	536	205	3025	6.7	GP Stoke, Plymouth and Derriford hospital in-patient
			ST73		
263AR Vs 263TR	CFT073	451	2451	18.4	GP South Brent, South Devon
ABU83972 Vs CloneiD2	CFT073	1960	2894	67.7	Sweden and USA
263AR Vs ABU83972	CFT073	2834	3932	72	GP South Brent, South Devon and Sweden
263TR Vs CloneiD2	CFT073	1424	2407	59	USA and GP South Brent, South Devon

The core SNP analysis for the 124S and 124L specimens had less than 1% variation strongly suggesting the two UPEC isolated from specimen 124 should be considered as the same organism. Comparisons of the SNP variation with other UPEC ST127 isolated in the study ranged from 6.7% to 25%. Table 19 includes the origins of all isolates. The UPEC ST127 all originated from the south west of England, within 20 miles of Derriford Hospital, from different patients and data indicate that the strains bear no relationship to one another.

Core SNP comparisons performed on the ST73 isolates in this study presented a much higher percentage SNP core variation. As only two ST73 were isolated and sequenced, the same local-level comparisons were not possible. However, comparisons were made between the Derriford Hospital ST73 and the genomes downloaded from the NCBI website. As expected, a much larger range of

variation was observed using these genomes, which ranged from 18.4% to 72% SNP core variation. The 18.4% core SNP variation was observed between the two UPEC ST73 from specimen 263, which when compared to the percentage SNP core variation between the Derriford UPEC ST127 isolates (0.95-8.2% predicted cut-off value), seems quite high; suggesting these two organisms are different isolates possessing distinct genomic variation.

3.3.8 Detection of virulence factors within the ST127 genome.

The well-established virulence factor PCR assay (Johnson & Stell, 2000), VirulenceFinder 1.5 and Virulence searcher software was used to determine the virulence complement of ST127 and other UPEC. Examples of PCR findings are shown in Figures 19 and 20.

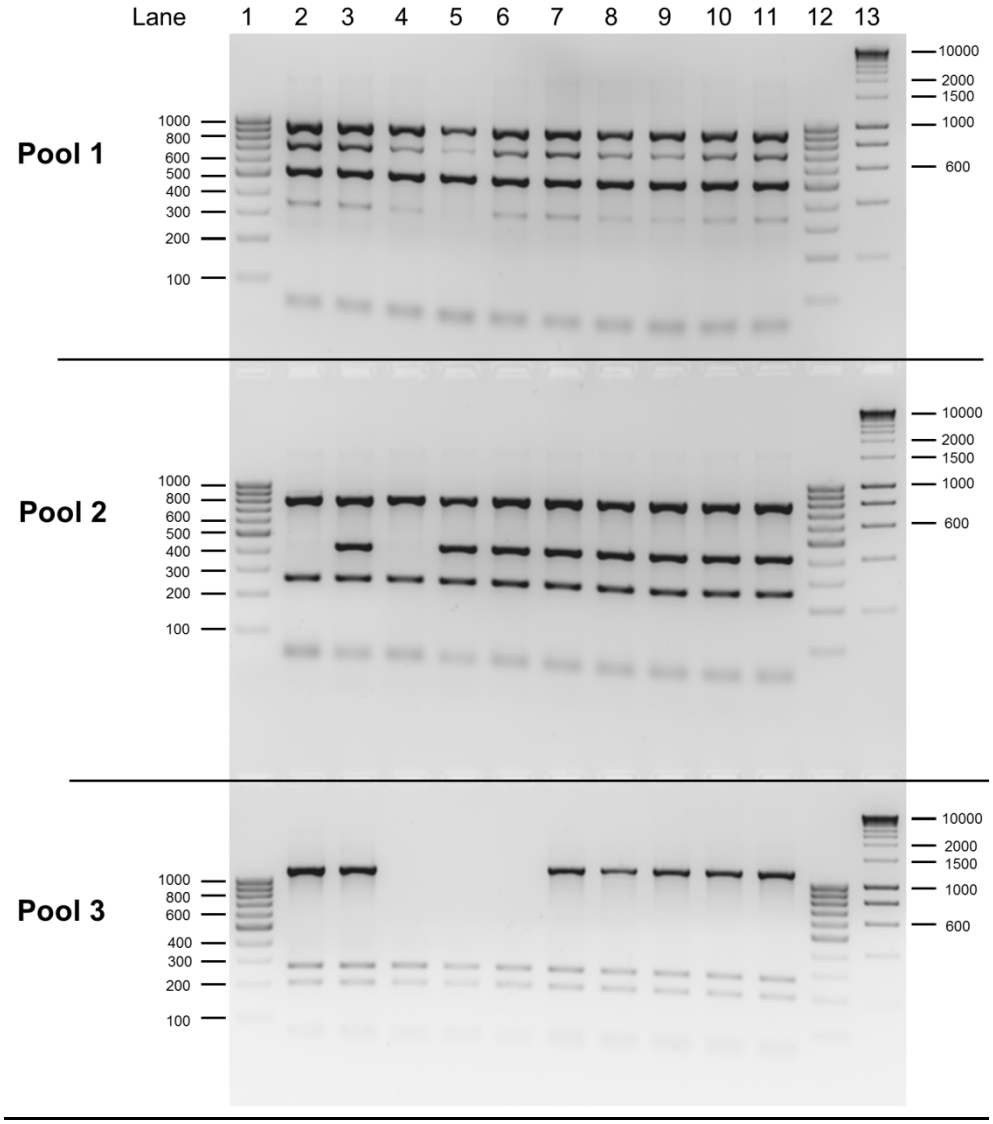

Figure 19. Virulence PCR assay results for the 10 Derriford ST127 isolates, pools 1-3. Lane 1, 100kb molecular marker; lane 2, specimen 9; lane 3, 39; lane 4, 124S; lane 5, 124L; lane 6, 263AS; lane 7, 264; lane 8, 298; lane 9, 316; lane 10, 354; lane 11, 468; lane 12, 100kb molecular marker; lane 13, 1kb molecular marker. Amplicon sizes – refer to table 16 p112.

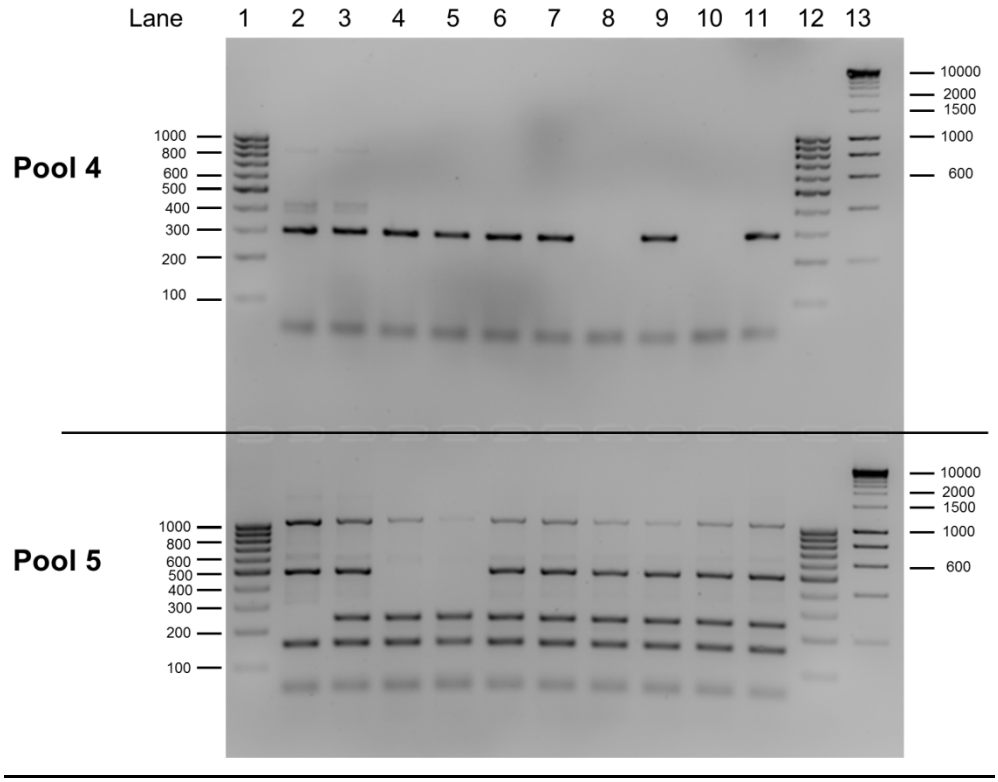

Figure 20. Virulence PCR assay results for the 10 Derriford ST127 isolates, pools 4 and 5. Lane 1, 100kb molecular marker; lane 2, specimen 9; lane 3, 39; lane 4, 124S; lane 5, 124L; lane 6, 263AS; lane 7, 264; lane 8, 298; lane 9, 316; lane 10, 354; lane 11, 468; lane 12, 100kb molecular marker; lane 13, 1kb molecular marker. Amplicon sizes – refer to table 16 p112.

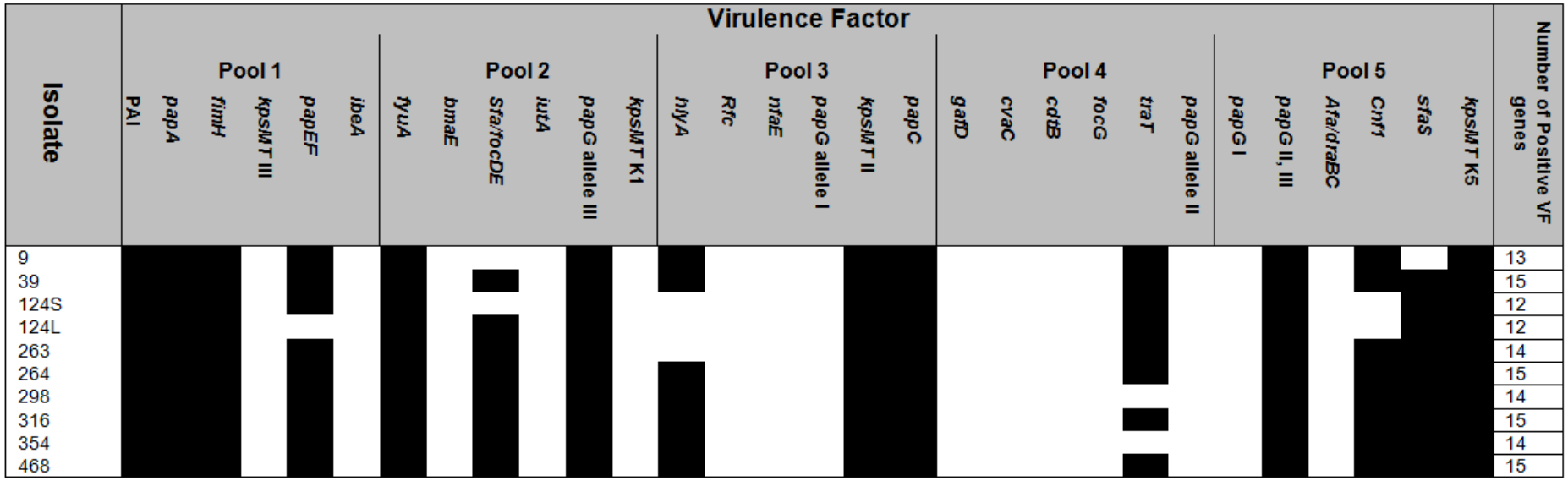

Figure 21. Virulence profile of Derriford ST127 UPEC isolates based on PCR Virulence assay. Black boxes represent positive PCR results

The virulence PCR assay performed well for the most part, however interpretation was subjective for a few targets (Figure 19-21). In pool 1, the *papEF* and *KpsMT III* have only 56bp difference between the two products and as such, interpretation was not completely clear. Several PCR products produced a faint band (faint but defined bands were interpreted as positive) such as *papA* for isolate 124L, *papG* II, III for 124L and *papEF* for 124S. The number of virulence factors for all Derriford UPEC ST127 isolates, using the VF PCR, ranged between 11-15 with 124L and 124S possessing the least number of VFs and strains 39, 264, 316 and 458 possessing the most. Commonly identified VFs in all ST127 isolates were *fimH* (Type 1 fimbrial adhesin), *fyuA* (siderophore), *pap* genes (P-pili), PAI (generic PAI from CFT073) and both the *kpsMT II* and *kpsMT* K5 (both group II capsule synthesis). Non-ST127 isolates were not tested using the VF PCR assay.

Discrepancies in results did occur between the 124 isolates. Manual investigation of genome sequence data confirmed that both carried identical sequences related to *papEF* and *sfa/focDE* genes and primer sites. The reason for the discrepancy is unknown, however, due to time constraints the assay was not repeated.

The VirulenceFinder and Virulence Searcher software recorded different results from each other and the PCR assay due to their specificity and number of potential targets in their database. Results from both programmes are displayed in Table 21 and 22. The VirulenceFinder software gave 263AR the highest virulence score and NA114 the lowest. The lowest ST127 score was held by strains EC18 and EC41, both with a score of 3. Virulence Searcher identified isolate UMN026 with the highest VF gene count of 189 and VR50 with the

lowest with a gene count of 165. In relation to ST127 isolates, 39 and 124L recorded the highest and lowest scores recorded as 187 and 171, respectively.

Individual genes sequences and their protein translation were identified manually using prokka, artemis, NCBI Blast, Uniprot (Swiss-prot) and interpro. Two such groups of genes were the pap/prs (pap related sequence) and the *sfaS* (S fimbrial tip adhesin) gene. VirulenceFinder did not recognise any of the pap or prs VFs but did recognise the presence of *sfaS*. The PCR assay detected the presence of pap genes in all isolates as did the Virulence Searcher software, however, VirulenceFinder did not record the presence of any pap genes in any specimens due to the lack of targets in their database (https://bitbucket.org/-genomicepidemiology/virulencefinder_db). Results for both the VF PCR assay and Virulence Finder were in agreement for the *sfaS* gene, however, the Virulence Searcher software did not identify the *sfaS* adhesion marker, again due to inadequate database entries. Results for the *sfaS* gene were confirmed manually as the entire operon was absent in isolate 9, and although the VF PCR was not performed on isolates SA189, EC41 and EC18, the *sfaS* operon (Figure 22) was also missing in these isolates, as per VirulenceFinder results. The presence of the siderophore gene *fyuA* was recorded in all ST127 isolates using the VF PCR assay (confirmed manually) and numerous iron related protein signature motifs were identified using Virulence Searcher but no siderophore genes were detected using the Virulence Finder software for specimen 9.

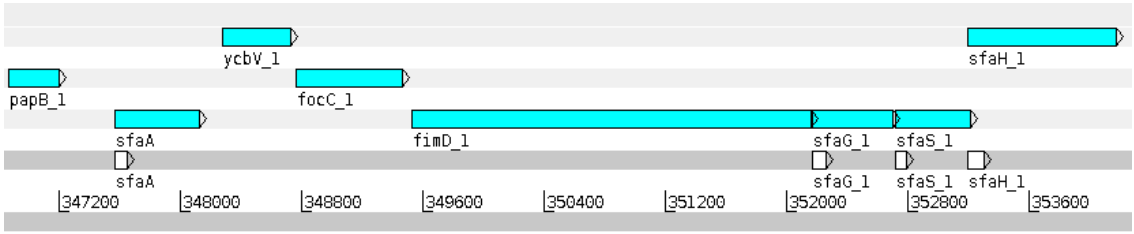

Figure 22. Missing *sfa* operon in ST127 isolates 9, SA191 and EC18. Image produced with Artemis 16.0 and annotation by Prokka 1.11.

Table 20. Number of virulence genes found using Virulence searcher. Isolate UMN026 recorded the most virulence factors whereas VR50 recorded the least. The presence of VFs were based on putative protein motifs present on the PRINTS database from gene predictions within unannotated genomes.

Isolate	Number of VFs	Isolate	Number of VFs	Isolate	Number of VFs
UMN026	189	354	180	EC536	176
39	187	ABU83972	180	UTI89	176
EC131	184	316	179	263TR	175
SA189	184	263AS	178	CloneiD2	174
NA114	183	EC41	178	124S	173
9	182	264	177	124L	171
CFT073	182	298	177	IAI39	170
263AR	181	EC18	177	VR50	165
468	181	SA191	176		

Table 21. Gene hits from VirulenceFinder. Numbers represent percentage identity to database virulence genes. Minimum identity was 90% and minimum sequence coverage was 60%.

Isolate	Virulence Factor																					Number of VFs
	iss	*vat*	*gad*	*cnf1*	*pic*	*sfaS*	*iroN*	*senB*	*celb*	*mcmA*	*mchB*	*mchC*	*mchF*	*iha*	*sat*	*nfaE*	*ireA*	*ellA*	*air*	*ipfA*	*capU*	
9	100	99.9	100	100																		**4**
39	100	99.9		99.9		99.8	99.5	99.7		100												**7**
124S	100	99.9				99.8	99.5	100	98.6	100												**7**
124L	100	99.9	100			99.8	99.5	100	98.6	100												**8**
263AS	100	99.9	100	100		99.8	99.5			100												**7**
263AR	100	100	100	100	99.9		100	100		100	100	100	100	99.9	100							**13**
263TR	100	100	99.9		100		100			100	100	100	100	99.9	100							**11**
264	100	99.9	100	99.9		99.8	99.5	100		100												**8**
298	100	99.9	100	99.9		99.8	99.5			100												**7**
316	100	99.9		100		99.8	99.5			100												**6**
354	100	99.9	100	99.9		99.8	99.5			100												**7**
468	99.7	99.9		100		99.8	99.5	100		100												**7**
EC18	100	99.9	100																			**3**
EC41	100	99.9						99.9														**3**
SA189	100	99.9	100	100				99.7														**5**
SA191	100	99.9	100	100		99.8	99.5	99.9		100												**8**
EC536	100	100	100			99.8	99.5			100												**6**
EC131	100		100											100	100	99.8						**5**
CFT073	100		100				100			100	100	100	100				100					**8**
UTI89	100	99.9	100	100		100	100															**5**
VR50	100		100											99.9	99.9	98.9						**5**
UMN026	100		100											100	100			100	100	100		**7**
NA114														100			100					**2**
IAI39			100															100	98.7	100	100	**5**
CloneiD2	100	99.9	100		100		100			100	100	99.9	100	100	100							**11**
ABU83972	100	100	100	100	100		99.9			100	100	100	100	100	100							**12**

Gene annotations: *iss*, increased serum survival; *vat*, vacuolating autotransporter toxin; *gad*, glutamase decarboxylase; *Cnf1*, cytotoxic necrotizing factor; *pic*, serine protease autotransporters of *enterobacteriacae*; *sfaS*, S-fimbrial subunit; *iroN*, Enterobactin siderophore receptor protein; *senB*, plasmid encoding enterotoxin; *celb*, Endonuclease colicin E2; *mcmA*, Microcin M part of colicin H; *mchB*, Microcin H47 part of colicin H; *mchC*, mchC protein; *mchF*, ABC autotransporter protein mchF; *iha*, adherence protein; *sat*, secreted autotransporter toxin; *NfaE*, Diffuse adherence fibrillary adhesin gene; *ireA*, iron-regulated outer membrane protein; *ellA*, *Salmonella* HilA homolog; *air*, enteroaggregative immunoglobulin repeat protein; *IpfA*, long polar fimbriae; *capU*, Hexosyltransferase homolog.

3.3.8.1 Detection of virulence factors present in urine associated *Enterococcus faecalis*.

VirulenceFinder 1.5 was also used to determine the virulence potential of the urine associated *Ent. faecalis* isolates. Only one of the 18 isolates cultured from Derriford hospital displayed any virulence factors. Isolate 32 (ST34) produced an array of virulence factors involved in adhesion, invasion and toxin production (Figure 23). Isolate 32 was cultured from a 73 year old female patient who had a 10^5 *E. coli* positive UTI along with the *Ent. faecalis* (scanty) in question, and an additional *Ent. faecium* (scanty) isolate. Both Enterococcus isolates were considered as contamination during the original diagnostic assessment. The closest organism phylogenetically was a commensal isolated from a porcine rectal swab (ST29).

Virulence genes for Enterococcus						
Virulence factor	%Identity	Query/HSP length	Contig	Position in contig	Protein function	Accession number
cOB1	99.76	819 / 819	32_ST34	1093092..1093910		CP002621.1
ElrA	99.72	2172 / 2172	32_ST34	1241656..1243827		CP003726.1
tpx	99.80	510 / 510	32_ST34	1420014..1420523		CP003726.1
hylA	99.08	3264 / 3266	32_ST34	1500749..1504014		CP002491.1
SrtA	99.05	735 / 735	32_ST34	1523772..1524506		AE016830.1
cad	99.78	930 / 930	32_ST34	1714018..1714947		CP002621.1
cCF10	99.88	828 / 828	32_ST34	1788553..1789380		CP002621.1
hylB	99.24	3015 / 3015	32_ST34	2423006..2426020		CP002621.1
cylL	100.00	207 / 207	32_ST34	2667312..2667518		AY032999.1
cylM	99.30	2982 / 2982	32_ST34	2667804..2670785		AD1CLYL
cylB	99.86	2145 / 2145	32_ST34	2670797..2672941		AD1CLYL
cylA	100.00	1239 / 1239	32_ST34	2672938..2674176		AD1CLYL
camE	97.60	501 / 501	32_ST34	282136..282636	sex pheromone cAM373 precursor	AF435442.1
ebpA	99.09	3312 / 3312	32_ST34	32233..35544		CP003726.1
ebpB	99.23	1431 / 1431	32_ST34	35548..36978		CP003726.1
ebpC	99.36	1884 / 1884	32_ST34	36975..38858		CP002491.1
ace	97.68	2025 / 2025	32_ST34	45066..47090	collagen adhesin precursor	AE016830.1
efaAfs	99.68	927 / 927	32_ST34	813195..814121		FP929058.1

Figure 23. Output from VirulenceFinder software for the ST34 urine associated isolate 32.

Background colour: Dark green, perfect match 100% identity; light green, <100% identity – sequence equals virulence gene length; grey, warning due to non-perfect match – sequence shorter than the virulence gene length. Gene *cOB1, camE*, and *cad,* Sex pheromones; *ElrA,* Enterococcal leucine rich protein A; *tpx*, Thiol peroxidase; *hylA* and *hylB*, Hyaluronidase; *SrtA*, Sortase A; *cCF10*, Cell-associated pheromone peptide; *cyl*, *cylM*, *cylB* and *cylA*, Cytolysin proteins; *ebpA, ebpB and ebpC*, endocarditis and biofilm associated pilus genes; *ace,* Collagen-binding MSCRAMM protein; *efaAfs*, Cell wall adhesin.

3.4 Discussion

3.4.1 Development and validation of an ST127 specific PCR

As previously mentioned, the majority of ST127 isolates are fully susceptible to antibiotics commonly used for the empirical treatment of UTI and, as a result, are likely to be under-represented in most published prevalence surveys. To enable further study of the ST127 clone and attempt to gain a better idea of the prevalence in UTI, a rapid, reliable and inexpensive PCR method was required for detection of this lineage, as has been designed for other STs (Clermont *et al.*, 2009; Doumith *et al.*, 2015; Li *et al.*, 2010). To that end, the design and validation of this 3-gene multiplex PCR was successful and accomplished the stated goal for this piece of work. The ST127 specific PCR assay, targeting two specific genes namely *fliC* and *upaG*, achieved excellent results with the cohort of isolates representing 99 different commonly isolated UPEC ST.

Although *E. coli* is the model organism for experimental molecular microbiology and probably the best understood bacterium genetically, a large percentage of the genome still consists of putative open reading frames with unknown functions (hypothetical proteins). It may have added unnecessary limitations to the study, but whilst searching for gene targets for specific PCR, hypothetical proteins were avoided as it was felt that distinct variations in known or putative gene sequences with known functions would serve as a better and more unambiguous target for PCR.

The *fliC* gene codes for the subunit protein flagellin, the major constituent of the flagellar filament. The flagellin proteins are conserved at terminal regions while the central region is variable and often carries an H-serotype specific epitope

(Wang *et al.*, 2003). The protein is also implicated in pathogen-associated interactions, stimulating the Toll-like 5 receptor (Smith *et al.*, 2003) which, in turn, has given rise to the prospect of the more conserved regions of the flagellin protein becoming a potent adjuvant in the design of new vaccines for UTI (Bennett *et al.*, 2015; Savar *et al.*, 2014). Using SerotypeFinder 1.1 the ST127 *fliC* gene was seen to have 100% identity to the predicted serotype H31 variant, in agreement with previous reports for carriage of this serotype in ST127 strains (Johnson *et al.*, 2008). The conservation within UPEC ST127 isolates, coupled with the reported variability within the *E. coli* species and previously published studies employing *fliC* as a discriminatory marker with enteropathogenic *E. coli* (Beutin, Delannoy & Fach, 2015) justified selection of *fliC* as a worthy candidate for ST127 specific PCR.

The translated protein sequence for the putative *upaG* gene in ST127 was found to share many structural features with *E. coli* CFT073, the *Yersinia yadA* and *Haemophilus influenzae Hia* AT genes, including specific homologies with the Hia and YadA proteins at the C-terminal region and the Left-handed Beta-roll of YadA at the hydrophobic N-terminal region (Nummelin *et al.*, 2004; Szczesny & Lupas, 2008). The 73% identity with the CFT073 *upaG* (Totsika *et al.*, 2012; Valle *et al.*, 2008) is not surprising as variability within genera and species for the AT family of adhesins is particularly high. The membrane anchor is the only domain that remains conserved throughout the AT and, as such, defines the family (Linke *et al.*, 2006). The *yadA* gene was the first of this family identified in *Yersinia* species and originally named P1 (Bolin, Norlander & Wolf-Watz, 1982) or autoagglutination protein (Bolin & Wolf-Watz, 1984) and to date remains the best characterised AT family of adhesins (El Tahir & Skurnik, 2001;

Muhlenkamp *et al.*, 2015). The AT adhesins are important virulence factors for many Gram-negative pathogens and, although they are universally associated with adherence to epithelial cells and extracellular matrix (ECM) proteins (Valle *et al.*, 2008), their functions appear extensive with reported roles in biofilm formation (Allsopp *et al.*, 2012), invasion into host cells (Eitel & Dersch, 2002) and serum resistance (Biedzka-Sarek, Venho & Skurnik, 2005).

Ideally, one PCR target would be used to identify this particular ST but considering the size and variability within the *E. coli* pan genome, such a precise single PCR target was thought to be over optimistic. However, a PCR assay targeting a hypothetical protein for the detection of ST127 has recently been published by Ciesielczuk and colleagues (Ciesielczuk *et al.*, 2016). Their assay focused on a single locus to identify ST127 but the study did not provide any clinical validation for the assay. Performing *in silico* analysis, as part of the work for this thesis, predicted that the single locus primers had high specificity, but with no laboratory validation of performance, the utility of this assay has not been confirmed. The three false positive results found in the current study with primers for *fliC* indicate that even primers which appear highly specific *in silico* may perform less well when used in practice. A significant finding of this study was that no single primer pair was able to reliably identify ST127 and a combination of PCR targets was required to attain 100% specificity. Additionally, from the collection of ST127 used in the study by Ciesielczuk and colleagues, five different O-antigen serotypes were recorded. From our collection of 29 ST127 isolates, 27 belonged to the 06 serogroup and the remaining two had an O-Antigen deletion (EC18 and 124L). A search for extra O-antigens genes within the EC18 and 124L genomes using SerotypeFinder revealed no

additional O-antigen regions within these genomes. An additional search for ST127 genomes in the NCBI database (June 2018) returned 7 extra genomes that matched the ST127 MLST alleles, each serotyped as O6:H31 except for one isolate (BH100N_MG2017) that also had an O-antigen deletion caused by a 1342bp region belonging to the IS3 family. Furthermore, how, when and by whom the tested specimens in this study were originally sequenced typed is not clear (Ciesielczuk *et al.*, 2013; Ciesielczuk *et al.*, 2016; Doumith *et al.*, 2012; Reynolds, Hope & Williams, 2008). As most of the specimens were collected pre 2012 or 2008, it must be assumed the MLST type was determined by a Sanger sequencing PCR method. The technique of reading and, possibly when necessary, manually editing abi sequence chromatograms is a difficult procedure that should only be performed by well-trained individuals as one incorrectly placed nucleotide can lead to a mis-assigned ST result. So much so, that the Warwick MLST database (Enterobase) no longer accepts Sanger sequence results for MLST assignment as one third of all new alleles received were sequencing errors rather than real sequence variations (http://enterobase.readthedocs.io/en/latest/faq.html#how-do-i-get-new-allele-st-with-sanger-abi-sequencing-traces).

As a final note on the technical aspects of PCR specific assays, any PCR method without the presence of an extraction/PCR control will always introduce an element of doubt upon obtaining a negative result and will reduce the practicality for use in any diagnostic setting.

To greater understand the true genetic background of aetiological agents of UTI it is paramount that future CA-UTI studies focus on specimens collected from all patients at the point of care, prior to empiric treatment. The investigations

performed by Yamaji and colleagues (Yamaji *et al.*, 2018) go some way to emphasise the importance of such studies. In their work, ST127 was found to be the second most common lineage in a presumably young and healthy Californian student cohort, in contrast to reports of low prevalence from other studies involving some selection bias (Adams-Sapper *et al.*, 2013; Alghoribi *et al.*, 2015; Croxall *et al.*, 2011a; Gibreel *et al.*, 2012b).

It is understood that the presence of antimicrobial resistance in a pathogen is a prerequisite for increased prevalence; however, in the case of ExPEC, resistance may not be the dominant driver towards increased prevalence. Recent studies show that drug resistant and drug susceptible strains have both remained equally prevalent in UTI and BSI over the last 17 and 11 years, respectively (Kallonen *et al.*, 2017; Yamaji *et al.*, 2018). Furthermore, in the case of the globally disseminated ST131 clone, acquisition of specific virulence determinants predates the mutations in the *gyrA* and *parC* genes that led to the development of fluoroquinolone resistance in Clade C2 strains carrying the CTX-M-15 ESBL gene (Ben Zakour *et al.*, 2016). This suggests that the presence or acquisition of virulence genes in ExPEC may be a necessary precursor towards the future success of a pathogen. The majority of UPEC ST127 remain relatively susceptible to antibiotics but with an already high virulence potential, increase surveillance for UPEC ST127 isolates would be advisable, on a prospective basis, to reduce the potential impact from this virulent clone.

The ST127 specific PCR reported here is the first validated multiplex PCR for detection of *E. coli* multi-locus Sequence Type 127. The assay is simple, yet highly discriminatory, rapid, reliable and inexpensive. The multiplex PCR can

also be performed directly from individual colonies removing the need for any extraction or DNA purification protocols.

3.4.2 Comparative genomics and pathoadaptive mutations

The use of comparative genomics provided good evidence to suggest the presence of three UPEC in the same urine specimen 263. Phylogenies using SNP analysis show a minor divergence between the two ST127 isolates in specimen 124 (Figure 14) and a distinct but recent evolutionary divergence between the two ST73 isolates in specimen 263. Additional evidence was supplied by BRIG analysis to add support for the hypothesis that two distinct ST73 isolates were present in the 263 specimen. The divergence between the 124 isolates seems to be very recent and although the 124L isolate has some very clear and distinctive phenotypic traits (O-antigen deletion and Trimethoprim resistance) separating it from the 124S isolate, further examination of previous studies that also used SNP core analysis was required to compare results and determine if each should be classified as separate entities.

Genomic studies by McNally and colleagues (McNally *et al.*, 2013) looking into urosepsis isolates found a very low number of SNPs between the blood and urine isolates (total of seven SNPs in four sets of paired isolates) collected within 48 hours from the same patient (McNally *et al.*, 2013). A study by Zdziarski and colleagues (Zdziarski *et al.*, 2010) described inoculation of the ABU83972 *E. coli* strain into six human hosts who suffered from urinary tract dysfunctions and rUTI, as a proposed asymptomatic bacteriuria model to study the mechanisms underlying the development of commensalism. They found the

ABU83972 strain to be relatively stable with only 34 mutations after bladder colonisation for 423 days and observed the number of genomic alterations increased with colonisation time. It is known that mutation rates vary with the environmental conditions and increase under stressful conditions (Lee *et al.*, 2012), but interestingly, in the Zdziarski study, the type of genomic adaptation observed in resequenced *E. coli* ABU83972 isolates collected from patients post human incubation, was dependent on the host, with unique genomic adaptations targeting regulators of bacterial metabolism, specific to each patient (Zdziarski *et al.*, 2010), emphasising the immense complexity in understanding the genomic variation and *in vivo* adaptation of infecting or commensal organisms. Kram and colleagues (Kram *et al.*, 2017) studied the adaptation of *E. coli* K12 during serial passage in aerated LB medium through different phases of bacterial growth. After ~30 passages using triplicate populations, a total of 213 unique point mutations were identified in all populations. Understandably, estimation of mutation rates vary. Ochman and colleagues predict one of the lowest mutation rates for *E. coli* at 0.1-0.2× 10^{-3} per genome per generation (Ochman, Elwyn & Moran, 1999) whereas Lee and colleagues predict ~1×10^{-3} per genome per generation but this is not taking into account mismatch repair, the primary pathway for correcting replication errors (Lee *et al.*, 2012).

It must be appreciated that each of the SNP core analyses discussed were performed using different methods with differing parameters, therefore like for like comparisons may not be fair. To mitigate the possible variation between studies, core SNP comparisons were performed between several of the ST127 strains to get a general idea of the level of variation between isolates.

The results from the ST127 phylogeny using the 536 genome as reference, detected only 24 SNPs difference between the two sample 124 ST127 isolates in a total of 2537 SNP positions (0.95% SNP variation). Using CFT073 as the reference, the same comparison for the two ST73 isolates found in specimen 263 revealed a total of 462 SNPs in 2451 potential SNP positions (18.8% SNP variation). The evidence indicates the two ST73 isolates found in specimen 263 should be classified as different variants; however, it is not known how long the pathogens resided within the patient or in what capacity, commensal and/or pathogen. As previously stated, the longer the bacteria remain in the patient, the more SNPs occur but without a defined taxonomic criterion to classify closely related isolates as individual variants based on SNP core analysis, no definitive decision can be made. The same uncertainty is not present for the 124 isolates. Although the 124L isolate has gone through two distinct phenotypic alterations, the low number of SNPs between their core genomes strongly suggests a recent divergence from the same parent organism.

As previously discussed, specimen 124L possessed two distinct phenotypic traits separating it from the 124S isolate. Trimethoprim resistance in the 124L isolate was due to alterations in the Dihydrofolate reductase (DHFR) gene. DHFR is an essential enzyme in all living cells necessary for the biosynthesis of several amino acids and nucleotides (Huovinen, 1987). By its structural similarity to dihydrofolate, Trimethoprim becomes a competitive inhibitor to this enzyme in bacteria. The human DHFR is intrinsically resistant to Trimethoprim, which is the basis for its selectivity and its clinical use (Huovinen *et al.*, 1995). Different mechanisms mediate bacterial resistance to Trimethoprim as resistance can be intrinsic (cell wall impermeability/efflux pumps), transferable

(numerous transposons), from naturally insensitive target enzymes, from alternative metabolic pathways or by mutation of the enzyme promotor region (leading to over production) or modulation of the active site, or both (Huovinen, 2001). The Centre for Genomic Epidemiology ResFinder database contains 42 DHFR Trimethoprim resistance genes from various sources of which none share homology with the DHFR sequence in 124L trimethoprim resistant isolate. Although none of the resistance genes in this database matched the mutations seen in specimen 124L, it seems very likely that the two point mutations seen only in the DHFR gene of specimen 124L, coupled with the lack of any plasmid mediated resistance, are the cause for the Trimethoprim resistance in this bacteria.

The second distinctive trait observed in the 124L specimen was the lack of O6 antigen production. The bacterial lipopolysaccharides (LPS) are present as part of the outer monolayer of an asymmetrical outer membrane and generally consists of three structural units; lipid A heat-stable endotoxin (embedded within outer monolayer), core oligosaccharide and the O-antigen, which is composed of an polyoligosaccharide chain that can vary in length with up to 40 repeated units of dideoxyhexoses (Lerouge & Vanderleyden, 2002; Rosa Eugenia Reyes, 2012). The LPS is estimated to cover approximately 75% of the total membrane surface and the largely lipid A outer monolayer membrane is protected from the actions of complement by the O-antigen and capsular polysaccharide structures (Alexander & Rietschel, 2001; Suerbaum *et al.*, 1994). The O-antigens are key targets for the action of complement, so it is thought that the greater the length of the O-antigen, the better the protection as the lytic actions of complement

take place further away from the cell surface, preventing cell lysis (Lerouge & Vanderleyden, 2002).

The archetypal phenotypic colony description of mutants lacking the O-antigen in Gram-negative bacteria is that they are denoted as ‘rough-forms’, as opposed to ‘smooth-forms’ for non-mutants with functional O-antigen structures. This transition from smooth to rough colonies (or possibly vice versa) is a concept commonly understood since the beginning of microbiology as a science. However, as most bacteria that have lost the O-antigen still produce smooth colonies (as does 124L), which are indistinguishable from O-antigen positive colonies, the term ‘semi-rough’ has been adopted for these colony types (Bettelheim & Taylor, 1969; Webb, Goodwin & Green, 1982), even though the term remains somewhat of a misnomer.

Alexander and colleagues (Alexander & Rietschel, 2001) state that bacteria can only persist and survive in tissue or body fluids if they express an O-antigen chain to protect from phagocytosis and complement. Ample evidence exists in support of this hypothesis (Burns & Hull, 1998; Osawa *et al.*, 2013; Phan *et al.*, 2013; Pluschke *et al.*, 1983) with some reports of the O6-antigen in UPEC being one of the most resistant O-antigens to human serum (Falkenhagen, Zingler & Naumann, 1991). However, the 124L from this study and EC18 ST127 from a previous study (Alghoribi *et al.*, 2014) both seemed to have O-antigen deletions but did not conform to this generalisation. As both strains were isolated from urine (EC18 also isolated from blood), they both must have reached a high concentration in urine to be detected, suggesting that both strains have not only survived but thrived in the urinary tract. The urinary tract possesses highly organised and effective innate and adaptive immune responses to protect

against invading bacteria. The family of Toll-like receptors (TLRs) present on bladder and kidney epithelia (Samuelsson *et al.*, 2004), upon LPS mediated stimulation, promote a vigorous cytokine response (Song & Abraham, 2008). Although serum resistance is an established marker for how well an organism can survive *in vivo*, the bladder is a very different environment to blood or tissues. Factors including physiology, the host immune system and the adaptability of the pathogen to its environment are pertinent in relation to the host response and rate of clearance. It is known that alternating periods of rapid phenotypic evolution can occur in *E. coli* (Gould & Eldredge, 1977) with evidence of surface antigen alteration, loss of long chain LPS, capsule alteration and loss of virulence factors attributed to host immune pressure (Bielaszewska *et al.*, 2007; Olesen *et al.*, 1998; Zdziarski *et al.*, 2010).

The evidence from this study suggests that the two ST127 124 isolates were the product of an extremely recent divergence. Therefore, it is reasonable to suggest that the SNP core variances, along with the mutations in the DHFR gene and the suspected insertion of an IS region into the O-antigen cluster, occurred within the patient during infection as a result of positive selection.

It is known that *E. coli* are subject to alterations of its genetic mechanisms *in vivo* that can lead to enhance bacterial virulence or persistence; such functions have been designated pathoadaptive (Sokurenko, 2016). An example of this phenomenon has been well-studied in the *fimH* gene that codes for the adhesin tip subunit of the type 1 fimbriae. (Sokurenko, Hasty & Dykhuizen, 1999; Weissman *et al.*, 2007; Weissman *et al.*, 2003). A single SNP in the *fimH* gene can greatly increase the binding properties of the type 1 adhesin to bladder epithelia. These types of mutations have been detected from paired *E. coli*

specimens isolated from urine and rectal swabs from patients with acute cystitis (Weissman *et al.*, 2007). However, in contrast, single mutations in the *fim* operon have been suggested to alter phase variation, reduce binding and inhibit the formation of intracellular IBCs (Chen *et al.*, 2009). Obviously, genetic mutations and recombinations events can be a double edged sword in terms of bacterial persistence, an observation that would, at first glance, seem to be exemplified by the 124L specimen in this study, which enhanced its potential for survival by gaining resistance to Trimethoprim but theoretically reduced its resistance to immunological pressures with the loss of the O-antigen. However, with patients suffering from recurrent UTI or asymptomatic bacteriuria, it has been observed that a large proportion of *E. coli* isolated from repeat specimens tend to lose their O-antigen over time (Bettelheim & Taylor, 1969; Lindberg *et al.*, 1975; Webb, Goodwin & Green, 1982). If the O-antigen loss is an adaptive advantage to UPEC it is yet to be fully understood but it may facilitate evasion of host immune defences, such as anti-LPS antibody or innate immunity (Sokurenko, 2016).

If we follow the notion or premise that the O-antigen deletion increases the pathogenicity of the bacterium, it may be feasible to suggest that the loss of the O-antigen may results in a greater release of lipid A or increase the accessibility of the bound lipid A to the host tissues (Russo *et al.*, 1995a). The identification of a shiga-toxigenic *E. coli* (STEC) strain in a haemorrhagic colitis patient with a similar deletion in the O-antigen caused by an IS has been reported by Rump and colleagues (Rump *et al.*, 2010a; Rump *et al.*, 2010b). However, the author suggested that, in this case, this type of deletion did not have any detrimental effects on the pathogenicity and may not be as rare as anticipated.

To explore the contrary point of view, Lerouge and colleagues suggest the loss of the O-antigen may attenuate the pathogenicity of an organism (Lerouge & Vanderleyden, 2002). The endotoxin portion of the LPS (hydrophobic lipid A) can dissociate from the surface of Gram negative bacteria and elicit a severe inflammatory response. The O-polysaccharide is hydrophilic and may allow the diffusion or delivery of the endotoxin in the hydrophilic environment (Lerouge & Vanderleyden, 2002), therefore, the lack of an O-polysaccharide may coincide with an inhibition of endotoxin function.

There are also studies that conflict with the hypothesis with reference to the importance of the O-antigen in serum resistance. Russo and colleagues showed that the O4-antigen only played a minor role in protecting against complement mediated killing and presented results indicating the capsular polysaccharide was the primary bacterial structure that impeded complement attack (Russo *et al.*, 1995a). Furthermore, studies have shown the importance of the group II family capsule (K5 present on ST127 is part of the group II family) in the protection from complement mediated killing and neutrophil association (Buckles *et al.*, 2009; Burns & Hull, 1999). And finally, a deletion in the w*baV* gene responsible for the transfer of sugars to the O-antigen in one isolate of *Salmonella* Enteritidis, resulted in increased resistance to human serum and also positively affected the strains ability to bind and invade tissues, compared to other gene mutants (Jaiswal *et al.*, 2015).

The O-antigen was not the only membrane apparatus that was affected by pathoadaptive mutations in the 124L and EC18 isolates. The *ompC* gene has been previously shown to be positively selected for mutation during the adaptation of *E. coli* (Petersen *et al.*, 2007). The *ompC* gene codes for beta

barrel porin on the outer membrane of Gram-negative bacteria and seems to have multiple functions: it allows the passage of small molecules through the membrane; has a role in pathogenesis; interaction with the host immune system; and can serve as receptors for antibiotics, colicins and phages (Andersen *et al.*, 1999; Fourel *et al.*, 1993; Lazdunski *et al.*, 1998; Petersen *et al.*, 2007; Schirmer, 1998). Two point mutations in the *ompC* gene in both the O-antigen negative ST127 isolates (EC18 and 124L) caused non-synonymous amino acid translational changes in both isolates. The effect of these amino acid substitutions are unknown, however, as these changes were only present in the two isolates missing the O-antigen, it is reasonable to suggest this adaptation will bear some influence, directly or indirectly, on the functionality surrounding the O-antigen deletion.

How a strain of UPEC adapts to the urinary tract is clearly complicated as the pathogen and host will both have influence on the pathoadaptations that take place within the bacterium. That being said, the urinary tract is probably one of the best sites to study such a phenomenon as there is always a very large sample size within the bladder that can persist for years (as in asymptomatic bacteriuria) and whose population is periodically refreshed upon voiding, positively selecting the better adapted isolates to remain and expand (Sokurenko, 2016). It is fair to say that host dependant-selection of specific mutants (genomic imprinting) verses random selection, is an issue of immense complexity involving numerous pathogenic and host variables (Zdziarski *et al.*, 2010). The influence of bacterial within-host micro-evolution is an area of study that is anticipated to expand in the forthcoming years with the increasing accessibility of WGS data.

3.4.3 Uropathogenic virulence factors

Three assays were used to determine the virulence complement for each of the ST127 isolates. The assays consisted of one PCR method and two WGS analysis methods. Each produced comparable results with some VFs but very different results with others, due to the availability and the quantity of primer targets/database entries. The PCR assay performed well, although a primer concentration reduction (x10) from the original study was required for good amplification, the authors opinion is that this is probably due to the enhanced functionality of improved commercial PCR mastermix products that were not available at the time the original assay was designed.

The VirulenceFinder software was originally created to detect VFs in verotoxigenic *E. coli,* but the database has now been expanded to include VFs of generic *E. coli*, *Listeria*, *S. aureus* and *Enterococcus* (Joensen *et al.*, 2014). The VirulenceFinder database lacked some of the well-documented VFs in UPEC such as *pap/prs* genes and type 1 adhesin genes, but does however, identify genes such as *gadA*, which codes for glutamate decarboxylase, a protein involved in acid resistance in *E. coli* and is suggested to be important for EPEC to resist low gastric pH (Clarke *et al.*, 2003). The *mcm* set of genes, responsible for the production of antimicrobial peptides (Microcins), was also identified by VirulenceFinder. However, these antimicrobial compounds seem to be as prevalent in commensal strains as they are in pathogenic strains of *E. coli* (Joensen *et al.*, 2014; Zschuttig *et al.*, 2012). Although many UTI isolates seem to be clonal, there is no individual trait or set of virulence genes that is able to define an isolate as being a member of the UPEC group. The VF PCR assay remains to be the most relevant and specific VF assay in relation to UPEC, with

primers able to detect a variety of adherence and siderophore genes known to contribute to bladder colonisation and iron acquisition. Other sequence based assays are either lacking databases entries or, as is the case with the Virulence Searcher software, present results from predicted virulence factors from putative protein motifs derived from the PRINTS database (http://130.88.97.239/PRINTS/index.php), which are generalised, challenging to interpret and difficult to assign to specific VFs.

Adherence is thought to be the fundamental virulence mechanism of UPEC and specific adhesins including type 1, P, S and other fimbriae certainly aid in the colonisation of the urinary tract, but to what extent each influence urovirulence is debatable as these fimbriae and other VFs are found in different combinations at differing percentages across various subgroups of UPEC (Kaper, Nataro & Mobley, 2004). The presence of P-fimbriae within the *E. coli* genome was thought to be essential for pyelonephritis, a prerequisite for urosepsis. However, when McNally and colleagues performed genomic studies on paired blood and urine isolates from five urosepsis patients, two of the five isolates did not carry the *pap* genes encoding P fimbriae (McNally *et al.*, 2013) suggesting that other adhesins may be just as important for attachment to kidney epithelia.

Although the VF PCR assay designed by Johnson and Snell is probably the most relevant UPEC VF assay, in certain areas it lacks specificity. To use the *fimH* adhesin as an example, almost every *E. coli* strain possesses type 1 fimbriae and only minor mutations in the *fimH* gene modify the affinity of the lectin to bind to monomannose receptors (high concentration on bladder epithelia) that are present in ~70% of UPEC, as opposed to the trimannosyl

receptors, present in ~80% of commensal strains (Bower, Eto & Mulvey, 2005; Sokurenko, Hasty & Dykhuizen, 1999). Identification of the type 1 fimbriae or its monomannose binding subunit does not seem to be specific for the UPEC VF PCR assay. A quick search on the NCBI/Primer-BLAST website using the *fimH* primers shows that the primer site is conserved across *E. coli* ETEC strain 2265, UPEC CFT073 and the laboratory strain K12. As the vast majority of *E. coli* possess *fimH*, the usefulness of this primer pair is debatable. Incidentally, this search may also have exposed a primer misprint in the original paper with no genomes receiving 100% specificity for the forward primer as the G and the C in positions 2 and 3, respectively, are in opposite positions in all other *E. coli* genomes. However, as this putative error occurs at the 5' end of the primer, the effects on the *fimH* PCR seem to be minimal.

The number of VFs identified was also extremely variable across the three assays with Virulence Finder generally identifying <10 VF per isolate, the PCR assay identifying 10-20 VFs per isolate and Virulence Searcher identifying approximately 180 VFs per isolate. It was also interesting to note that the strain that is known to cause asymptomatic bacteriuria (ABU83972) had one of the highest scores in both sequenced based assays. It seems evident that scoring how virulent an UPEC isolate is by the number of VFs detected is an inadequate and simplistic view that assumes a pathogen can be understood merely by the number of VFs it possesses. Previously it was thought that acquisition of virulence genes via horizontal gene transfer was a primary vector in enabling a commensal strain to become a uropathogen. However, the availability of WGS data seems to have blurred the distinctions between

pathogen and commensal and between virulence factor and colonisation factor (Rump *et al.*, 2010a; Rump *et al.*, 2010b).

The VF assays used to compare the different ST127 genomes found that some possessed distinct virulence factors whilst others were lacking. Both isolates 124S and 124L lacked the gene *cnf1*, a known potent toxin involved in apoptotic death of urinary epithelia and invasion (Bien, Sokolova & Bozko, 2012; Landraud *et al.*, 2004). These two isolates along with the 263AS isolate were also negative for another toxin gene *hlyA* (Haemolysin A). Interestingly, the 263AS isolate possessed a copy of the *hlyA* gene but it was assembled in two parts with a ~350bp region between the two. A nucleotide search of the 350bp region using IS finder, found two separate regions within this sequence (123bp and 116bp- IS1A Accession number X52534) matching an IS found in several strains of *Escherichia*, *Salmonella* and *Shigella*, once again demonstrating the affect IS can have on the prokaryotic chromosome.

Many of the important adhesins in UPEC (Type-I fimbriae, P-pilus, S-pilus) are transcribed within the bacteria and transported through the cell membrane via process known as the chaperone usher (CU) pathway. The CU systems consist of, at least an usher protein, a chaperone and fimbrial subunits (Busch & Waksman, 2012). Genes that encode the CU pathway are found in gene clusters scattered throughout the bacterial genome. Transport of the pilus structures to the outer cell surface relies on the pilus subunits first passing into the peri-plasmic space via the general secretory pathway SecYEG and then binding to a chaperone that assists with the folding of the protein and transportation to the usher, situated in the outer membrane. Here the subunits

polymerise and are a transported through the outer membrane to form a pilus (Busch & Waksman, 2012).

The *sfa* operon codes for the S-fimbriae and within the operon lies the *sfaH* (designated *sfaS* with the VF assays – varied nomenclature discussed later in this chapter) gene, which codes for the minor fimbrial subunit adhesin necessary for the full expression of S-specific binding to glycoproteins, terminating with alpha-sialic acid-$_{2\text{-}3}$-beta-gal moiety (Chahales & Thanassi, 2015). The *sfa* fimbrial gene cluster proteins are highly homologous to another CU set of adhesins, the *foc* gene cluster, so much so that the genes can be exchanged without loss of function (Mol & Oudega, 1996; Ott *et al.*, 1988; Riegman *et al.*, 1990). This fimbrial adhesin has been associated with both neonatal meningitis and UTI (Korhonen *et al.*, 1986; Saukkonen, Nowicki & Leinonen, 1988). The operon possessing the *sfaH* binding protein was absent in specimen 9 (Figure 22), SA189, EC41 and EC18 but what effect this loss will have on the organisms' pathogenicity is unknown and requires further study.

Visualisation of this operon in Figure 22 goes some way to demonstrate the homology between some of the CU pathway subunits present within *E. coli* and highlights the problem encountered with the annotation and classification of such genes. There may be up to 12 CU gene clusters in UPEC and the functions of many are still unknown (Korea *et al.*, 2010; Wurpel *et al.*, 2013). The variation in nomenclature with the fimbrial gene clusters is enormous and confusing. For example, the annotation obtained using prokka named the first regulatory protein of the *sfa* operon, *papB*, but a Uniprot search of the DNA sequence gave a 100% hit with an unreviewed putative F1C/S-fimbrial regulatory switch. However, the top reviewed (Swiss-prot) hit was with the

regulatory protein PapB at 82% identity. In other areas of the operon, the usher protein was annotated *fimD* by prokka but *focD* by Uniprot and the binding protein *sfaH* (100% identity Swiss-Prot database) is named *sfaS* in the VF assays, yet the protein named *sfaS* with the prokka annotation gave a 98% similarity to the Swiss-Prot entry *fimG*.

The number of proteins coded per CU pathway combined with the number of CU operons within the *E. coli* genome, their similar homology and their ability to exchange genes with other CU pathways, makes their assignment to specific classes of fimbrial structures and adhesins very difficult. Several of the binding sites and some of the pharmacological responses of fimbrial adhesins have been determined, but in combination with the effects of other CU fimbriae, curli and autotransporter adhesin responses, quantification of pathogenesis by the presence of single fimbriae, again, is an over simplification. Finally, as an additional complication, the number of genes present on the operon containing *sfaH* was determined to be seven by Wurpel and colleagues (Wurpel *et al.*, 2013) however, the ST127 operon seems to have eight with the previously mentioned PapB (Siss-Prot identification- 82% identity) fulfilling a role as a S-fimbriae transcriptional regulator. An identical transcriptional regulator is also present at the beginning of the P-fimbriae operon in UPEC ST127.

3.4.4 UTI Associated *Enterococcus faecalis* isolates

Enterococcus faecalis are ubiquitous in nature and have successfully adapted to a range of habitats due to their ability to survive environmental pressures such as broad pH ranges, increased salinity and the presence of harmful

compounds like disinfectants (Gawryszewska *et al.*, 2017). They are a common in the gastrointestinal tract of humans and other animals including birds, fish reptiles and insects (Beukers *et al.*, 2017). *Ent. faecalis* are, in many respects, considered non-virulent but they are often clinically significant pathogens due to their opportunistic nature and high intrinsic resistance to commonly used antibiotics (Gawryszewska *et al.*, 2017). Although UTIs caused by *Ent. faecalis* in young and otherwise healthy patients are rare, they remain a significant etiological agent of UTI due to an increased prevalence in the elderly and their ability to form biofilms aiding the colonisation of catheters (Kart *et al.*, 2017). High numbers of *Ent. faecalis* were isolated in this study and it was hypothesised that members of a particular ST isolates carrying a group of specific virulence determinants could be attributed to these prevalence levels.

The *Ent. faecalis* specimens collected in this project that underwent WGS were originally the focus of a separate PhD project and were selected for WGS on the basis of production of antimicrobial peptides. As such, this selection bias must be recognised, but the data have been included in the work presented here as they offered an opportunity to explore the potential that certain VFs or pan-genome phylogeny may reveal 'UTI specific' traits. However, it is significant to note that nearly half (8 of 18) were ST179 and nearly a quarter (4) were ST16. Strains from both of these ST are commonly isolated from urine (Weng *et al.*, 2013; Zheng *et al.*, 2017). Numerous genomic comparisons made between the 18 *Ent. faecalis* strains isolated in this study and the 32 genomes downloaded from the NCBI database (comparisons not shown) revealed no obvious genomic regions specific to the urine associated *Ent. faecalis* isolates. This is understandable as previous studies on *Ent. faecalis* using a pan genomic

approach have found that *Ent. faecalis* lacks clear demarcation between pathogenic and non-pathogenic strains at the genome level (Bakshi *et al.*, 2016). It has also been suggested that there is a very high level of horizontal gene transfer within the *Enterococcus* genus suggesting exchange of genomic material within this genus is commonplace making it difficult to attribute specific virulence determinants to a normally avirulent organism (Eaton & Gasson, 2001; Soheili *et al.*, 2014).

The *Ent. faecalis* core genome phylogenies correspond with the MLST system, grouping each ST into individual clades. One major branch of the large *Ent. faecalis* phylogeny contained both the ST179 and ST16 (sharing 6/7 MLST alleles) isolates suggesting there may be a certain genomic distinction between *Ent. faecalis* isolated from urine and those from other medical conditions or commensal strains. However, the cohort studied was a small selection of *Ent. faecalis* chosen on the basis of their ability to produce antimicrobial substances and they may not be typical of the entire collection, although, as previously mentioned, these two ST types are commonly isolated from urine.

Bakshi and colleagues state there is no apparent distinction at the genome level between pathogenic and non-pathogenic *Ent. faecalis* isolates and suggest the urine isolates may not be evolutionary distinct from the commensal ones (Bakshi *et al.*, 2016). The large *Ent. faecalis* phylogeny constructed in the current study supplies some evidence to support that opinion with the non-ST179 or ST16 isolates (11 strains) from Derriford Hospital and Japan, scattered across the entire phylogeny.

Analysis of the VFs using the VirulenceFinder database determined that 17 of the 18 *Ent. faecalis* isolated from urine possessed no known virulence factors. The lack of VFs in the *Ent. faecalis* specimens coupled with their low urine concentration strongly suggests that most, if not all of these isolates were contamination from the peri-urethral area during micturition.

The one exception to the above observation was isolate 32 (ST34) that contained numerous known virulence genes including the endocarditis and biofilm associated pilus (*Ebp*) and the *ace* MSCRAMM (microbial surface components recognizing adhesive matrix molecules) adhesin. This urine specimen was taken from a 73 year old female who presented with a 3+++ *E. coli* and a high WBC but was relatively unremarkable with respect to the three *Ent. faecalis* colonies isolated alongside the UPEC. Compared to the other isolated strains of *Ent. faecalis*, isolate 32 was highly virulent and further patient information may reveal a more clinical role for this bacteria, however, the only conclusion that can be made from the available information is that this strain was a contaminant.

There were 17 occasions in the original polymicrobial UTI study (Chapter 2) where *Ent. faecalis* was isolated alongside *E. coli,* at a comparable concentration. These 17 *Ent. faecalis* isolates did not undergo WGS but with this information, additional investigations may reveal increased numbers of *Enterococcus* VFs and may go some way to supporting the notion that the two organisms display some type of symbiotic relationship with each other within the urinary tract.

3.4.5 Concluding remarks

The ease with which microbial WGS data are obtained has allowed molecular microbiologists the opportunity to quickly determine molecular intricacies that previously would have taken weeks, if not months to determine. The comparative genomics performed in this study have provided the foundation for design of a successful PCR for the specific detection of UPEC ST127. The additional laboratory validation of this assay against 99 ST has proved this assay to be a useful tool for future epidemiological investigations allowing the true prevalence of ST127 to be determined quickly and accurately.

The large volume of genomic data obtained in this study has also allowed us to identify the mechanism by which three ST127 isolates have lost their O-antigen. The insertion of an IS may be the common method by which all UPEC lose their O-antigen whilst inhabiting the urinary tract. Obviously, further work is required to fully elucidate this process, but if this phenomenon is seen in other UPEC genomes and is reversible, as suggested by some authors (Webb, Goodwin & Green, 1982), then particular aspects of IS dynamics may soon be seen as a common form of UPEC genetic regulation; a simple way to temporarily deactivate a gene. The IS detected in both the O-antigen regions and in the *hlyA* gene may be evidence of the adaption towards commensalism and the evolution of a less pathogenic organism, similar to the evolution of the ST73 ABU strain (Zdziarski *et al.*, 2010).

As we move towards WGS data becoming commonplace in the microbiology laboratory and eventually the clinical laboratory, we must concentrate our efforts on an improved and standardised form of annotation along with better information regarding isolate origins. The *sfa* operon serves as a good example

to show the variations in annotation designated from different sources. Through numerous nucleotide and protein searches conducted in this study, the author is inclined to agree with the comments made by Ellington and colleagues that a standard BLAST analysis will retrieve plenty of hits that will be inconsistently annotated even when the actual sequences are identical (Ellington *et al.*, 2017). To date, the majority of publically available genomes are draft sequences that vary in quality and coverage (Lo *et al.*, 2017), a shortcoming that will hopefully be resolved with the advent of 3rd generation sequencing. The quality of WGS data was an issue when gathering genomes of *Ent. faecalis* for comparison but additionally there was a lack of strain information pertaining to the original isolate. Several descriptions of *Enterococcus* isolate origins were labelled as just ‘medical’ and many, not labelled at all, limiting the value of these genomes in relation to epidemiological studies and in association with pathogenesis.

Chapter Four

4. Prevalence of UPEC ST127 in community acquired urinary tract Infections, as determined by type specific PCR

4.1 Introduction

The UPEC or EXPEC pathotype designation is not easily defined. A range of virulence factors can influence the pathogenicity of a UPEC isolate, but none have been proven as absolutely essential for urinary disease. Specific virulence factors have been associated with different types of UTI such as the pap (pyelonephritis) and fim (cystitis) fimbriae, but urinary associated disease has still occurred in the absence of these specific virulence factors (McNally *et al.*, 2013). It is apparent that there are many ways *E. coli* can cause disease in the urinary tract and investigators often focus on the initial binding mechanism of UPEC to the uroepithelia. There can be up to 12 CU pathway adhesive fimbriae in UPEC isolates (Wurpel *et al.*, 2013), and an array of other uroepithelial binding proteins such as the AT adhesins and curli that are present within the *E. coli* chromosome. The large number of CU fimbriae can also be regulated by phase variation; the expression of one fimbriae suppressing the expression of another (Chahales & Thanassi, 2015). This arsenal of binding fimbriae may ensure that the chromosomal absence of just one fimbrial gene may be suitably substituted by the presence of another.

The ability of UPEC isolates to survive *in vivo* is also deemed to be a measure of virulence, and the same scenario of available auxiliary genes may be said for the iron scavenging mechanisms, with strain CFT073 containing at least 10 confirmed ferric uptake systems and several putative ones (Alteri & Mobley,

2015; Welch *et al.*, 2002). To complicate matters, some of the genes deemed virulence determinants are present in commensal strains.

It seems apparent that the ability of UPEC isolates to cause disease is best observed and measured by a scale of virulence whereby several additional factors such as motility, growth rate and metabolic fitness may also be taken into account. The predicted results would be that some UPEC isolates would be considered highly adapted towards urovirulence, some moderately and others poorly and opportunistic, of which the latter may be designated as commensals dependant on the host and circumstance.

As discussed in earlier chapters, UPEC ST127 has been shown to be highly virulent in comparison to other common UPEC isolates (Alghoribi *et al.*, 2014; Beyrouthy *et al.*, 2013; Croxall *et al.*, 2011a; Salipante *et al.*, 2015); a conclusion derived mainly from results obtained using the well-established VF PCR designed by Johnson and Snell (Johnson & Stell, 2000) with additional evidence of heightened virulence in insect models of infection (*Galleria mellonella* infection assay) (Alghoribi *et al.*, 2014). Logic would suggest that the most virulent UPEC strains would have the highest prevalence. However, this and previous studies have shown the prevalence of the highly virulent ST127 UPEC to be low compared to other lineages such as ST131. Whereas, the leading UPEC lineage, ST131, has repeatedly been reported as a low virulence UPEC using the VF PCR assay (Croxall *et al.*, 2011a; Gibreel *et al.*, 2012a), a contradiction inadequately explained.

It is accepted that antimicrobial resistance can influence the prevalence of UPEC STs in facilities with high antimicrobial use; typified by the predominance

of antimicrobial resistant ST131 in many clinical studies (Alghoribi *et al.*, 2015; Cagnacci *et al.*, 2008; Platell *et al.*, 2010). However, other authors have suggested that bacterial traits such as high metabolic capacity may be a vital towards the persistence of a pathogen with improved fitness leading to an increased prevalence (Gibreel *et al.*, 2012a). To truly determine the most prominent influences on UPEC virulence, patient characteristics must be taken into account, therefore it is reasonable to suggest that the most virulent and, therefore, predominant UPEC pathogens will cause disease in normally healthy men or premenopausal adult women. It is also important that the specimen collection is not tainted by empiric treatment guidelines; i.e. samples taken at the point of care prior to empiric treatment.

Taking the diversity of the *E. coli* pathogen into account, the expectation is that the lineage of UPEC isolates predominant in a retirement facility will not be the same as the type found in neonatal UTI; similarly, the UPEC types predominant in hospitalised patients will not be the same as the community acquired lineages. We have argued that the low prevalence of ST127 may be due to specimen bias within collections originating from clinical laboratories. A recent publication has supported this hypothesis (Yamaji *et al.*, 2018) reporting a high rate of ST127 isolates recovered from specimens taken from the point of care, prior to empiric treatment.

4.1.1 Study aims and objectives

The purpose of this brief study was to use the ST127 specific PCR designed and validated in Chapter 2, to determine the prevalence of the ST127 lineage in

a collection of community acquired UTI specimens. The development of the ST127 specific PCR assay will allow the use and evaluation of the assay in practice, and test our hypothesis that ST127 is underrepresented in most prevalence surveys. The specimens included in this study were taken from the point of care prior to antibiotic treatment. It was hypothesised that this strategy would remove the selection bias present in clinical laboratory specimens and would reveal a higher prevalence of UPEC ST127 than the level recorded from the Derriford Hospital collection.

4.2 Methods

4.2.1 Specimen collection

A total of 218 *E. coli* isolates collected originally for the POETIC study (Bates *et al.*, 2014; Hullegie *et al.*, 2017) were supplied by the Specialist Antimicrobial Chemotherapy Unit, Public Health Wales, University Hospital of Wales, Heath Park, Cardiff. The specimens were collected from female patients between 18-65 years of age, at the point of care on the patients' first visit to the GP surgery and prior to empiric antibiotic treatment (see appendix II). All received isolates were anonymized with a four digit number before arrival at the University of Plymouth.

4.2.2 ST127 specific PCR assay

Colony DNA extraction, ST127 specific PCR and the interpretation of PCR products was performed as described in Chapters 2 and 3 (section 2.2.2.3, 3.2.2.3 and 3.3.3.4). The only variations from the method used for validation was the use of SYBR safe™ (10µl of a x10,000 concentrate per 100ml of agarose gel) (Invitrogen, ThermoFisher, California, USA) instead of Ethidium bromide for the visualisation of amplicons and the GeneAmp® 9700 thermal cycler (Applied Biosystems, California, USA) was used for the PCR amplification.

4.2.3 Antimicrobial susceptibility of PCR positive isolates.

All UPEC isolates that were positive for the ST127 specific PCR assay were tested for antimicrobial susceptibility using the EUCAST disc diffusion method (http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/).

Antibiotics tested included Ampicillin (AMP-10), Co-amoxiclav (AMC-30 20 Amoxicillin/10 Clavulanate), Cefpodoxime (CPO-10), Levofloxicin (LEV-5), Cephalexin (CLE-30), Mecillinam (MEC-10), Nitrofurantoin (NIT-100), Trimethoprim (TRI-5) and Fosfomycin/Trometamol (FOT-200). The choice of antibiotic susceptibility testing was in line with current clinical practice and protocol of University Hospitals Plymouth Teaching Hospitals, 2018.

4.3 Results

4.3.1 ST127 specific PCR results

Limited clinical information was supplied with the specimens received from the POETIC study. Of the 218 specimens received, all were diagnosed as positive UTI by Cardiff laboratory staff but the patient age for specific isolates was not available. A total of 143 samples were reported as containing a pure *E. coli*, 72 with *E. coli* as the predominant organism and three samples reported as *E. coli* mixed with two organisms. Clear ST127 PCR positive isolates, putative positive isolates and specimens with a weak production of the *gyrB* extraction control were repeated with both the 3 primer pair PCR and without the extraction control added to the PCR mixture (Figure 24).

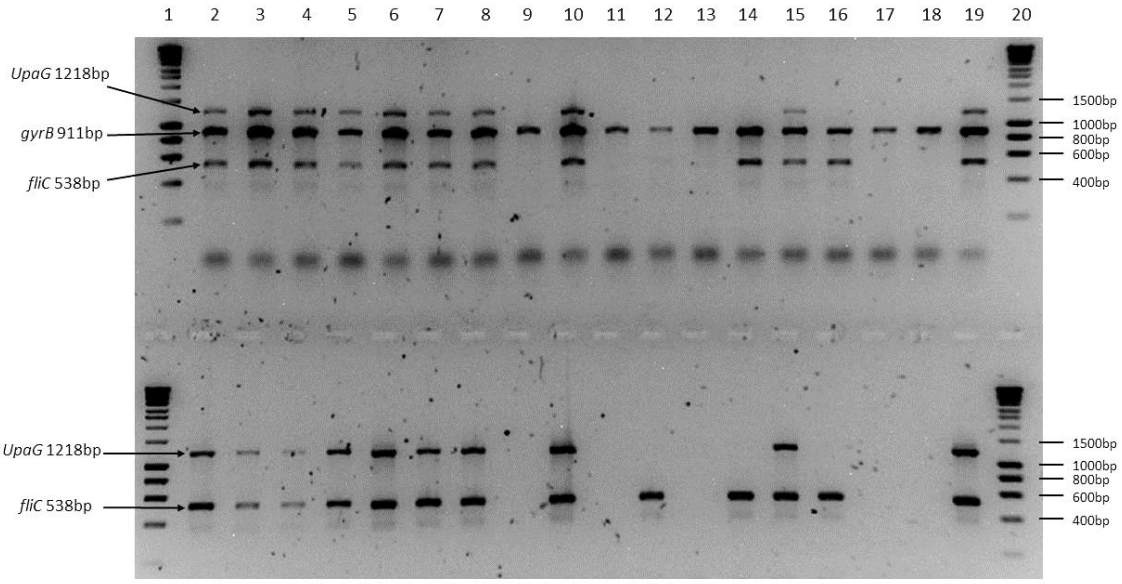

Figure 24. Detection of the ST127 clone using specimens collected from the CA-UTI. Gel displays eight of the nine positive specimens from the Cardiff POETIC collection. *E. coli* isolates were confirmed positive by retesting with both the 3 primer pair PCR assay (top) and with just the two specific targets (bottom). Lanes 1 and 20, Molecular marker-1kb (Bioline, London, UK); lanes 9 and 18, ST131 negative control; lanes 10 and 19, positive ST127 control; lanes 11-14 and 16-17, negative isolates; lanes 2-8 and 15, positive ST127 isolates.

A total of 9 specimens were positive for the ST127 specific PCR assay equating to a prevalence of 4.1%, which is over double the prevalence recorded from the Derriford hospital collection. The prevalence of ST127 isolates obtained from specimens considered as monomicrobial was 5.6% whereas ST127 isolated as the predominant organism in a mixed culture was very low with only one isolate found in 75 mixed cultures.

Variability in respect to the intensity of PCR amplicons was displayed in some of the reactions (Figure 24 - lane 5 and 15 top row, and 3 and 4 bottom row). Positive reactions were still easily distinguishable but intensities varied. This is likely to be due to a change in the thermal cycler and variations in well temperature as there was no record of regular maintenance on this piece of equipment. Each PCR reaction in Figure 24 was performed on the same PCR run and on the same plate.

4.3.2 Antibiotic sensitivity results of PCR positive specimens

All positive PCR ST127 isolates were fully sensitive to all antibiotics tested except isolate 9345 and 9717, which were both resistant to Ampicillin (Table 23).

Table 22. Antibiotic sensitivity results of the 9 positive ST127 isolates using the EUCAST disc diffusion method. NIT, Nitrofurantoin; TRI, Trimethoprim; LEV, Levofloxacin; AMP, Ampicillin; MEC, Mecillinam; CPO, Cephpodoxime; AMC, Co-Amoxiclav; CL, Cephalexin; FOT, Fosfomycin/Trometamol.

Positive Isolates	Antibiotic								
	NIT	TRI	LEV	AMP	MEC	CPO	AMC	CL	FOT
8095	S	S	S	S	S	S	S	S	S
8128	S	S	S	S	S	S	S	S	S
9088	S	S	S	S	S	S	S	S	S
9173	S	S	S	S	S	S	S	S	S
9182	S	S	S	S	S	S	S	S	S
9190	S	S	S	S	S	S	S	S	S
9345	S	S	S	R	S	S	S	S	S
9684	S	S	S	S	S	S	S	S	S
9717	S	S	S	R	S	S	S	S	S

4.4 Discussion

The prevalence of ST127 was higher with CA-UTI specimens collected from GP surgeries in Cardiff than the isolates originating from the Derriford hospital clinical laboratory (1.75%). As expected, the ST127 specimens isolated from this study were all very sensitive to empiric antibiotics with only two isolates resistant to Ampicillin. It is believed that these data provide reasonable evidence in support of our hypothesis that the antimicrobial susceptibility of ST127 isolates leads to an under-representation of this lineage in most prevalence surveys. The prevalence was still well below the levels reported by Yamaji and colleagues (Yamaji *et al.*, 2018) but more than double in respect to the isolates obtained from Derriford. Additionally, the prevalence of ST127 reported in monomicrobial culture, was even higher at 5.6% compared to 1.3% from the Derriford hospital isolates; more than 4x the prevalence. In contrast, the Derriford polymicrobial occurrence of ST127 was recorded at 2.5% whereas the CA-UTI specimens from this study were at 1.3%. The results from both Derriford hospital (clinical laboratory specimens) and from the POETIC study (point of care specimens) providing evidence to reject the hypothesis that ST127 is observed at a higher prevalence in polymicrobial infections. As both studies reported low levels of UPEC ST127, no statistical relevance can be attributed to these data. Although the evidence presented by this small study is useful, a much larger study with specimens obtained from several regions in the UK is required to determine not only the prevalence of ST127 in CA-UTI, but to also determine the regional variability of UPEC.

Although these results provide good evidence in support of our hypothesis relating to an increased prevalence of ST127 within CA-UTI, a direct

comparison between the two sets of specimens may be, understandably, viewed with a slight scepticism as the two sets of specimens originated from two distinct areas of the UK approximately 150 miles apart.

The ST127 specific PCR assay designed in this study was completed quickly and easily. This brief study using 218 isolates was completed in just a few days and involved five PCR runs (~2.5 hours each) and eight electrophoresis runs (~1 hour each). The speed at which these results can be obtained using the specific PCR demonstrates the usefulness of this assay in determining the prevalence of ST127 in different specimen collections.

Chapter Five

5. Virulence determinants and the effect of motility on the ability of UPEC ST127 to bind and invade uroepithelial tissues.

5.1 Introduction

A significant proportion of patients will suffer from recurrent urinary tract infections (rUTI) (Butler *et al.*, 2015) with approximately one fourth of women experiencing a second infection within six months and several experiencing multiple recurrences (Mulvey, Schilling & Hultgren, 2001). Originally, UPEC were regarded as strictly extracellular pathogens with rUTI attributed to the reinfection of the uroepithelia by organisms inhabiting the gastrointestinal tract. However, studies suggest a large proportion of rUTI are caused by the same organism (Stapleton & Stamm, 1997) and coupled with the knowledge that UPEC are able to invade uroepithelial tissue, avoid destruction by degradative lysosomes and enter a latent state or proliferate intracellularly forming intracellular bacterial communities (IBC), the focus of rUTI has been on UPEC with a propensity to invade uroepithelial tissue (Fukushi, Orikasa & Kagayama, 1979; Martinez *et al.*, 2000; McTaggart, Rigby & Elliott, 1990; Mulvey *et al.*, 1998; Mulvey, Schilling & Hultgren, 2001; Wakefield & Hicks, 1974).

Bacterial pathogens are often classified as intracellular and extracellular, depending on their aetiology and survival within host cell tissues. It has been known for decades that certain bacteria such as *Mycobacterium tuberculosis* (Bermudez & Goodman, 1996) and Enterohaemorrhagic *E. coli* (Oelschlaeger, Barrett & Kopecko, 1994) invade host cells as part of their infection cycle, whereas UPEC were never previously associated with tissue invasion. This invasive characteristic provided evidence of how UPEC are able to cause

recurrent infections. The ability to thrive or simply survive within uroepithelial cells in a quiescent state for several weeks explains how UPEC are able to avoid the actions of antimicrobials and circumvent the influx of immune cells to the bladder upon initiation of infection (Mulvey, Schilling & Hultgren, 2001; Schilling & Hultgren, 2002).

The method by which UPEC bind and invade uroepithelial tissues seems to be mainly attributed to the actions of the monomannose FimH adhesin present at the end of type 1 fimbriae (Martinez *et al.*, 2000), although there is evidence to suggest that the Dr/Afa adhesins and cytotoxic necrotizing factor 1 (CNF1) can also mediate bacterial invasion by *E. coli* (Bower, Eto & Mulvey, 2005). The FimH adhesin is present on the end of type 1 fimbriae expressed by the majority of UPEC isolates (Bower, Eto & Mulvey, 2005; Sokurenko, Hasty & Dykhuizen, 1999) and has a highly conserved N-terminal carbohydrate binding pocket perfectly designed to engage in tight interactions with a monosaccharide receptor (Chia-Suei *et al.*, 2002). The essential nature of this interaction has been demonstrated by the action of mannosides (FimH Inhibitors) in patients with chronic and recurrent urinary tract infections (Cusumano *et al.*, 2011; Mydock-McGrane, Hannan & Janetka, 2017; Totsika *et al.*, 2013), as well as research into type 1 fimbriae as a potent adjuvant for potential vaccine development (Habibi, Asadi Karam & Bouzari, 2015; Habibi, Asadi Karam & Bouzari, 2016; Imani Fooladi *et al.*, 2014).

Type 1 fimbriae are approximately 1-2µm long surface anchored adhesive organelles. They consist of a 7nm thick helical rod composed of repeating FimA subunits joined to a 3nm wide tip containing two adaptor proteins (FimF and FimG) and the FimH adhesin. The uroepithelial binding site for the FimH

adhesin has been identified as the Uroplankin 1a (UPIa) (Zhou *et al.*, 2001), however, numerous other FimH interactions with several glycosylated and non-glycosylated host factors have been reported including the Tamm-Horsfall protein (Pak *et al.*, 2001), α3 and β1 integrin subunits (Eto *et al.*, 2007) matrix associated type I and IV collagens (Pouttu *et al.*, 1999), the glycosylphosphatidylinositol (GPI) anchored protein CD48 (Khan *et al.*, 2007), laminin (Kukkonen *et al.*, 1993) and fibronectin (Schembri, Sokurenko & Klemm, 2000).

5.1.1 Physiology of the Uroepithelial Umbrella cells

The uroepithelia covers the surfaces of the bladder, ureters, renal pelvis and prostatic urethra and consists of at least three cell layers, the umbrella, intermediate and basal (Wu *et al.*, 2009). The umbrella cells are the outermost layer of the epithelia and possess specialised characteristics to function as a high resistance permanent barrier protecting the blood from the toxic effects of urine. Paradoxically, damaged uroepithelia can be restored extremely quickly yet a healthy urinary tract has one of the slowest cell turnover rates in the body (approximately 200 days) (Kreft *et al.*, 2005; Veranic *et al.*, 2009). The uroepithelium must also be extremely flexible to accommodate the constant filling and emptying of the bladder whilst remaining a functional barrier. This flexible impenetrable barrier is achieved by the production of uroplankins (UPs) on the outermost surface of the umbrella cells. There are five reported UPs particles, UPIa, UPIb, UPII, UPIIIa and UPIIIb (the latter considered a minor component), which combine to form a 16nm uroplankin particle that in turn bind together in hexagonally packed units forming a uroepithelial plaque (Figure 25) (Deng *et al.*, 2002; Khandelwal, Abraham & Apodaca, 2009). The epithelial

plaques connect with other plaques via a specialised tight hinge region (85-kDa glycoprotein urohingin) to form the uroepithelial barrier, which covers the majority of the bladder lumen (Liang *et al.*, 1999; Wu *et al.*, 2009). The layer of uroplankins embedded in the outer layer of the uroepithelia has led to the accepted terminology of the asymmetric unit membrane (AUM) because of its appearance in cross section electron micrographs (Figure 25c) (Mulvey *et al.*, 1998).

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Figure 25. Structure of the uroplankin conformational unit forming the atypical unit membrane (AUM) of the uroepithelia. (a) Conformational structure of the five uroplankin subunits of a single AUM particle; (b) Conformational structure of the multiple hexagonally packed units as part of an AUM plaque; (c) Scanning electron micrograph of a transverse section of the AUM, note the outer leaflet is twice as thick as the inner leaflet; (d) Transmission electron micrograph of an umbrella cell highlighting the Discoid fusiform vesicles (DFV) beneath the AUM; (e) Ultrastructure of an uroepithelial plaque (P) and its tight hinge region (H). Figure adapted from a, b, e: Wu *et al* 2009; c, Khandelwal et al 2009; d, Derganc *et al* 2011.

5.1.2 FimH mediated uroepithelial cell invasion

The internalisation of UPEC into uroepithelial cells is reported to involve the binding of the FimH type 1 adhesin to the bladder surface via the proposed binding sites, including but not limited to, the UPIa monosylated glycoprotein and/or the α3 and β1 intergrin subunits. The binding of FimH initiates the activation of host signal transduction cascades leading to actin rearrangements that may involve over 40 host cell factors including GTPases (Rho, Cdc42 and Rac1), kinases (tyrosine kinase, FAK, MAP kinases and PI 3-kinase), actin binding proteins and nucleators (Arp2/3, WAVE2, α-actin and vinculin) (Lewis, Richards & Mulvey, 2016; Martinez & Hultgren, 2002; Martinez *et al.*, 2000). The actin rearrangement results with the UPEC being engulfed by, what is described as, a zipper-like mechanism (discussed later in chapter), a process observed in several invasive pathogens, e.g. *Yersinia enterocolitica* (Wiedemann *et al.*, 2001). The natural expansion and reduction of the bladder surface area may also facilitate the internalisation of UPEC. Discoid/fusiform vesicles (DFV) containing sections of uroplankin particles (Figure 25d) are present just below the apical AUM surface. During bladder expansion and subsequent cell elongation, the DFV migrate and bind with the apical membrane increasing the surface area of the AUM. Upon voiding, sections of the AUM retreat back into the umbrella cell in a process referred to as compensatory endo/exocytosis (Duncan *et al.*, 2004; Khandelwal, Ruiz & Apodaca, 2010). UPEC may take advantage of this process with the association of FimH to the UPIa protein inducing conformational changes in the UP particle triggering downstream signalling pathways resulting in the internalisation of UPEC in an uroplankin enclosed DFV (Wang *et al.*, 2009). From observations in

the murine model, Justice and colleagues (Justice *et al.*, 2004) proposed that once internalised, UPEC intracellular proliferation involves four distinct developmental stages: Early IBC formation (~6-8 hours post inoculation) was observed as a loose collection of non-motile rod shaped UPEC just below the membrane surface with a doubling time of ~30 minutes. Middle IBC formation occurred between 6 and 8 hours post inoculation and consisted of a very dense UPEC community. During this stage, the morphology of the daughter cells simultaneously changed to a more coccoid morphology, presumably due to quorum signalling from a result of bacterial density. Observations of bacteria filled pods on the umbrella surface are typical at this stage in the process and the speed of growth suggests that intracellular nutrients are in abundance. The late IBC process took place as early as 12 hours post inoculation and was identified by the coccoid UPEC returning to their prototypical morphology, becoming highly motile and dissociating from the pod into the bladder lumen resulting in bacteriuria and a second round of invasion. The final stage consisted of the remaining pod bacteria continuing to grow but not separate (Figure 26). The filamentous bacteria grew up to 70µm in length and produced rod shaped daughter cells. The stimulation of TLR4 receptors in response to UPEC LPS recruits an influx of polymorphonuclear leukocytes (PMNs) to the bladder epithelia (Bower, Eto & Mulvey, 2005) where they gorge themselves with invading surface bound rod shaped bacteria. Video microscopy conducted by Justice and colleagues provides some evidence to support the suggestion that filamentous forms of UPEC were not phagocytosed by PMN. This observation implies a distinct survival advantage for infecting UPEC and has been proposed to involve the cell division inhibitor SOS DNA damage repair

system SulA (Horvath *et al.*, 2011; Justice *et al.*, 2006), but as yet the specific mechanism by which filamentous organisms avoid PMN uptake is unknown.

Figure 26 has been removed due to Copyright restrictions

Figure 26. Scanning electron micrographs of invading UPEC. (a) Bacterial intracellular pod bulging from the AUM surface; (b) Eruption of a bacterial intracellular pod and resulting fluxing of bacteria into the bladder lumen; (c) Filamentous forms of UPEC protruding from the AUM surface. Images adapted from (a) Anderson *et al* 2009; (b) and (c) Mulvey *et al* 2001.

5.1.3 Alternative methods of cell invasion

Bacterial pathogens have evolved numerous ways to incorporate themselves into host tissues and proliferate and have even developed mechanisms to disseminate directly into neighbouring cells (e.g. *Listeria* and *Shigella*) (Kuehl *et al.*, 2015). An attempt to assess or quantify the pathogenicity of an organism often focuses on the attachment apparatus, as adherence to host tissues is a prerequisite to invasion. As demonstrated with UPEC, the action of bacterial attachment alone can lead to internalisation but alternatively other, better understood, mechanisms exist which often share common strategies with UPEC invasion. Similar to UPEC, *Yersinia pseudotuberculosis* and *Listeria monocytogenes* both invade host cells via a zipper like mechanism (Cossart & Sansonetti, 2004). This involves actin polymerisation resulting in the formation of a phagocytic cup on the host membrane surface (Wang, Liang & Kong, 2008). The phagocytic cup forms around the bacteria in response to ligand binding,

eventually enclosing around the bacterium with actin depolymerisation retracting the vesicle into the host cytoplasm. *Y. pseudotuberculosis* encodes two outer membrane proteins, invasin and YadA, that both promote intimate host cell contact by interactions with the β1 integrin, which in turn activates the PI 3-kinase through the protein kinases FAK and Src (Uliczka *et al.*, 2009). Similarly, *L. monocytogenes* produces two outer membrane proteins named internalin A and B (InlA and InlB), which bind to E-cadherin and the hepatocyte growth factor receptor Met/HGF-R, respectively. Both receptors can induce bacterial invasion and, as with UPEC, employ PI 3-kinase and Rac1 activation (Cossart & Sansonetti, 2004; Seveau *et al.*, 2007) to induce cytoskeleton rearrangements.

Another well-studied mechanism of invasion is via the Type III secretion system (T3SS). This type of secretory system is commonly used by enteric pathogens such as *Salmonella*, *Shigella* and EHEC (Coburn, Sekirov & Finlay, 2007). The T3SS is only triggered when the bacterium is in close contact with the host and involves the delivery of specialised proteins, known as bacterial effectors, directly into the host cell cytoplasm via a needle-like structure that punctures the host cell membrane and injects the effector protein directly into the cytoplasm. Numerous effector proteins have been identified such as Sop group in *Salmonella* and the Yop group in *Yersinia* (Coburn, Sekirov & Finlay, 2007; Matsumoto & Young, 2009). Once present in the host cytoplasm the effector proteins initiate a rearrangement of the host cytoskeleton leading to the internalisation of the bacterium (Minamino, Imada & Namba, 2008). The T3SS export injectisome shares distinct sequence/protein similarities with the export apparatus of the flagellar complex (Figure 28) suggesting an evolutionary

relationship and a possible exaptation of the flagella secretory complex into the injectisome function, although which evolved first is still a matter for debate (Abby & Rocha, 2012; Pallen, Beatson & Bailey, 2005; Saier, 2004).

T3SS consist of four sets of proteins, transcriptional regulators, chaperones, the secretion apparatus and the extracellular filamentous organelle. The effector protein travels through the lumen of the filamentous organelle into the cell host cytoplasm, which leads to the formation of actin-rich membrane ruffles that engulf the infecting bacteria. This distinctive ruffling is caused by the formation of intricate filopodial and lamellipodial structures on the host cell membrane and is characteristic of the trigger mechanism (Figure 27) of bacterial invasion (Cossart & Sansonetti, 2004). There are many variations by which bacteria are internalised and the two major methods described here cannot be typical for every type of invasive bacteria. Many bacterial effector proteins have been identified, with estimates of nearly 40 experimentally confirmed effector proteins present in the Sakai strain of EHEC O157:H7 alone (Tobe *et al.*, 2006). To date, no reports of functional injectisome assemblies and their effector proteins have been reported in UPEC (Puhar & Sansonetti, 2014).

Figure 27 has been removed due to Copyright restrictions

Figure 27. Two described methods of bacterial invasion. Zipper mechanism (a): Effect of bacterial surface proteins that cause membranes to curve up and engulf bacteria closing in a zipper-like manner to internalise the bacterium; Trigger mechanism (b): bacterial effector proteins are secreted that activate signalling pathways leading to a modification in the sub-membrane cytoskeleton creating membrane ruffles that eventually engulf the pathogen. Image adapted from MBINFO website www.mechanobio.info.

5.1.4 Flagella structure and the role of motility in bacterial adhesion

The role of flagella in UPEC virulence seems to be generally overlooked with the focus of researchers concentrating upon the type 1-adhesin, FimH. The fact that the majority of UTIs are acquired by ascending infection leads to the obvious conclusion that a motile organism would possess a distinct advantage over a non-motile organism. But locomotion is where the focus of UPEC flagella function generally ends in UPEC research. It is known that non-motile organisms such as *S. saprophyticus*, *Enterococcus* and non-motile strains of *E. coli* are still able to cause urinary infection but at an assumed lower prevalence (non-motile *E. coli*) or gain access to the urinary epithelia opportunistically (increasing age with *Enterococcus* spp.) or by urethral massage (*S. saprophyticus*). Some studies have attempted to assess the importance of flagella mediated motility in murine models and generally suggest that flagella mutants hold a distinct disadvantage to wild type UPEC, especially with disseminating disease such as pyelonephritis (Lane *et al.*, 2007; Lane *et al.*, 2005; Wright, Seed & Hultgren, 2005). However, with reference to UPEC, much more research is required in this field of study.

The bacterial flagellum is a long helical structure that is 20nm in diameter and 15-20µm in length. It is composed of a of a basal body that spans the bacterial envelope and functions as a motor driven by a proton or sodium ion-motive force; a torsion hook that functions as a flexible joint and a helical hollow tube that consists of several thousand flagellin (*fliC*) subunits. At the end of the flagellum appendage there is a filament cap coded by the highly conserved *fliD* gene (Figure 28) (Evans, Hughes & Fraser, 2014; Paradis *et al.*, 2017; Ramos, Rumbo & Sirard, 2004). Some of the most interesting aspects of flagella

construction are the self-assembling nature of the flagellin subunits, their unique process of migration through the flagellum lumen and their regrowth upon mechanical breakage (Asakura, Eguchi & Iino, 1964; Evans *et al.*, 2013; Paradis *et al.*, 2017). The first stage of the flagellin export across the cell membrane is performed by the T3SS export machinery and is ATP driven. The flagellin subunits must then travel up the narrow 2nm lumen of the flagellum filament across the entire length of the flagellum to self-assemble at the distal end beneath the capping structure (Evans, Hughes & Fraser, 2014). In the absence of an apparent energy mechanism to facilitate flagellin transport, this was once thought to be achieved by passive diffusion theorised from the observations that the speed of flagella growth reduced in direct proportion to the flagellum length (Aizawa & Kubori, 1998). However, more recent studies by Turner and colleagues (Turner, Stern & Berg, 2012) have supplied compelling evidence to indicate that the growth of the flagella filament is independent of filament length. This has led to a simple model of flagellin transportation by which the unfolded flagellin subunits form a chain structure within the flagellum lumen and the energy for transportation derived from the folding crystallisation of the flagellin subunit beneath the flagellum cap. The conformational change of the folding flagellin subunit pulling the structural proteins through the shaft towards the flagellum tip (Evans *et al.*, 2013).

Figure 28 has been removed due to Copyright restrictions

Figure 28. Schematic diagram of the *Salmonella* flagella complex (left) and the injectisome (right) along with the genes responsible for the protein sub-structures. Image from Minamino *et* al, 2008.

The speed at which flagella can rotate is incredible with some estimates of speeds in excess of 15,000rpm (DeRosier, 1998; Magariyama *et al.*, 1994; McCarter, 2005), a rotational speed that is capable of propelling the bacteria several body lengths per second. This not only ensures the bacterial flagella filament comes into contact with a host cell surface, but does so at force, with the peritrichous flagella of UPEC likely to be the first point of contact with host cells. However, flagella speeds vary dependent on many factors including reserves of energy and chemotaxic signalling. To increase the chances of adherence to host tissues, and with the understanding that the flagella apparatus is the most protuberant cellular structure, it would be advantageous for the flagella to retain some adhesive qualities.

The role of flagella in adhesion to host tissues and extracellular matrix has been well studied for decades (Haiko & Westerlund-Wikstrom, 2013) with organisms such as *Clostridium difficile* (Tasteyre *et al.*, 2001), *Pseudomonas aeruginosa* (Lillehoj, Kim & Kim, 2002), *Salmonella* Enterica (Allen-Vercoe & Woodward, 1999) and EPEC (Giron *et al.*, 2002) all providing evidence towards the role flagella plays in adherence. The role of flagella in biofilm formation (Pratt & Kolter, 1998; Serra *et al.*, 2013) and even their ability to act as probes on uneven surfaces and attach into crevices (Friedlander *et al.*, 2013) gives some indication of how important flagella are to the virulence of an organism and how non-specific the flagella binding can be. That being said, several studies have reported tropism for particular cell types demonstrated by increased adherence of purified flagella of the H2 and H6 serotypes to HeLa cells (Giron *et al.*, 2002) and the H7 of EHEC to bovine intestinal epithelia (Mahajan *et al.*, 2009), demonstrating the importance of using appropriate cell lines for infection models.

Although numerous studies have identified the important role in adherence and colonisation, the precise mechanism and specific interaction that allows flagella to bind to a variety of host tissues and artificial surfaces has until recently, remained elusive.

Rossez and colleagues suggest that the very nature of the flagellin filament structure may be the critical factor required for adhesion (Rossez *et al.*, 2015). The several thousand FliC proteins present in the flagellum filament form a polymeric structure comprising of repeating epitopes. These repeating epitopes provide high avidity by a consolidation of the many low affinity ionic interactions which may take place on an epithelial surface, especially if the binding substrate is also repetitive.

A novel role of flagella adhesion in Enterotoxigenic *E. coli* (ETEC) has also been defined by Roy and colleagues (Roy *et al.*, 2009) who demonstrated the role of the two-partner secretion (TPS) exoprotein EtpA, a putative adhesin reported to bind to the conserved regions of the flagellin protein. These conserved regions of the FliC protein lie on the interior shaft of the flagellum filament and are not generally open to the extracellular milieu, however after many failed attempts to purify the EtpA protein due to the co-isolation of the flagellin protein, Roy and colleagues theorised the potential of a protein-protein interaction *in vivo*, serendipitously identifying a mode of action for EtpA. Recombinant EtpA labelled protein localised to mucin-producing regions of the small intestines and Immunogold labelling revealed that the putative EtpA adhesin also bound largely to the tips of intact and fragmented flagella. The observation supported the concept that EtpA interacts with usually inaccessible

FliC regions and suggests that EtpA could promote ETEC interaction with intestinal mucosal surfaces.

5.1.5 The role of flagella in bacterial invasion

The role of flagella in bacterial invasion has been studied intensely dating back to the 1980's. A large focus of this research was based on *Salmonella* species with *S.* Typhi requiring intact functional motility to invade (Liu *et al.*, 1988), *S.* Typhimurium demonstrating a significant decrease in invasion with motility mutants (Jones, Richardson & Uhlman, 1981) and data for *S.* Enteritidis emphasising the importance of flagella over fimbriae when a paralysed flagellated mutant (wild type transformed via mutation in the flagella motor gene, *motAB*) was recovered from the liver and spleens in similar numbers to the wild type, using a day old chick model of infection (Allen-Vercoe, Sayers & Woodward, 1999). More recent work has further strengthened the case that the actions of motility, or the presence of the actual flagella filaments themselves, are required for invasion in some pathogens. Duan and colleagues (Duan *et al.*, 2013) observed that non-motile flagellated strains of Shiga-toxin producing *E. coli* (STEC- commonly referred to as EHEC) invaded piglet epithelial cells more efficiently than motile strains. Whilst studying the effect of *fliC* inactivation, Luck and colleagues (Luck *et al.*, 2006) found that adherence to Human ileocecal adenocarcinoma cells (HCT-8) was not affected by the absence of flagella but an eightfold decrease in invasion was observed.

The role of flagella in relation to UPEC invasion is less well studied in comparison to the enteric pathogens. However, the research that is available

describes a similar role for flagella in the invasion of uroepithelial cells. Pichon and colleagues (Pichon *et al.*, 2009) studied the invasive properties of several motility and adhesin mutants on the pyelonephritis isolate AL511 and the ability to invade mouse collecting duct cells (mpkCCD) and human embryonic kidney cells (HEK293). Mutations in the *fliC* (flagellin) and *fliD* (capping protein) genes drastically reduced the rate of internalisation by 94% and 82%, respectively, whereas mutations in the *fimH* and *papC* genes had no effect on invasion levels. Similar results were also confirmed using an anti-H12 flagellin serum. Interestingly, via the use of scanning electron microscopy, a distinct interaction between the flagellum filament and the membrane ruffles was observed.

The most compelling evidence of flagella involvement in UPEC invasion was conducted by Kakkanat and colleagues (Kakkanat *et al.*, 2015) who used strains of the globally dominant multidrug resistant clone ST131 to study the role of adhesion and invasion in T24 bladder epithelia and macrophages. Using mutants with enhanced flagella expression, interchanged H flagella serotypes and diminished type 1-fimbriae expression, the enhanced flagellated mutants displayed significantly increased rates of bacterial uptake into the T24 cells and macrophages, independent of type 1 fimbriae expression.

5.1.6 Study aims and objectives

Numerous studies have reported UPEC ST127 to possess a high pathogenic potential; a conclusion generally based on the virulence factor PCR assay designed by Johnson and Stell in the year 2000 (Johnson & Stell, 2000). It is reasonable to suggest that the ability to adhere to and invade uroepithelial cells

are two of the most important pathological features when assessing the virulence of UPEC strains. To determine whether the VF PCR assay results correlate with experimental models, we sought to challenge the UPEC ST127 organisms using cell culture based adherence and invasion assays.

UPEC ST127 has previously been reported as a non-invasive strain of UPEC (Alkeskas *et al.*, 2015). This seemed contrary to the results of the virulence PCR assay with all UPEC ST127 strains possessing the *fimH* gene and most containing the CNF-1 gene. However, it was noted that the adhesion and invasion assay using UPEC ST127 was performed using non-uroepithelial cells (Alkeskas *et al.*, 2015). In light of the logical conclusion that bacterial invasion is tissue specific (Pichon *et al.*, 2009; Rossez *et al.*, 2015), it was decided to retest the adherence and invasive characteristics of ST127 strains in uroepithelial cells and use the genomic information acquired from the VF PCR assay and bioinformatic analysis to determine which genomic characteristics were pertinent in relation to cell attachment and invasion.

As the motility of bacteria in a limited number of UPEC, and other genera, have been shown to be important or even critical in both adherence and invasion of mammalian tissues, the motility of ST127 prior to tissue inoculation will also be evaluated using motility assays, the gentamicin invasion assay and electron microscopy.

Additionally, the published methods to determine the extent of bacterial invasion by UPEC in uroepithelial tissue are extremely varied. The original method was devised by Elsinghorst in 1994 (Elsinghorst, 1994) and has become the most widely used method to identify the invasive phenotype due to its reported

simplicity and sensitivity in comparison to techniques such as giemsa staining and direct observation (Elsinghorst, 1994). The original publication by Elsinghorst indicates that the protocol can only serve only as a guideline as the conditions for optimal invasiveness by a particular pathogen must be empirically determined. However, those statements seem to be taken to excess in published invasion studies involving UPEC and uroepithelial cells as large variations are observed in the bacterial pre-treatment, inoculation procedure, incubation times, gentamicin concentration and lysis solution composition (Berry, Klumpp & Schaeffer, 2009; Duncan *et al.*, 2004; Eto *et al.*, 2007; Kakkanat *et al.*, 2015; Madelung *et al.*, 2017; Martinez *et al.*, 2000; Thumbikat *et al.*, 2009). Therefore, it was decided that a thorough assessment of the published methods should take place to ascertain the best procedure for use.

5.2 Methods

5.2.1 Bacterial strains

The ten UPEC ST127 strains isolated from Derriford hospital clinical laboratory were used as the test organisms for all experiments described in this chapter. Two ST127 isolates obtained from Saudi Arabia and Manchester (SA191 and EC18, respectively) were also tested for their ability to invade cultured bladder cells. The majority of control organisms for each experiment were obtained from the University of Plymouth bacterial stock collection, unless otherwise stated. UPEC ST131 specimen EC958 were used as a positive control for the uroepithelia cell invasion assay (Kakkanat *et al.*, 2015). The laboratory strain *E. coli* K12 C600 was used as the positive adhesion/negative invasion control organism (Boudeau *et al.*, 1999; Elsinghorst, 1994) and a K12 *fimH* negative mutant N2880 strain (obtained from the Coli Genetic Stock Centre at Yale University, New Haven, CT, USA) was used as the negative adherence/negative invasion control organism (Berry, Klumpp & Schaeffer, 2009). A smooth swimming laboratory strain of *Salmonella* Typhimurium and UPEC ST131 EC958 was used as the positive control for the motility assay along with a laboratory strain of *Klebsiella pneumoniae* as the negative control. Additionally, a laboratory strain of *E. coli* K12 was used as a control to demonstrate how a small change in the concentration of agar can affect motility results.

The subset of bacterial strains used for the following experiments were stored at minus 20°C in BHI broth supplemented with 15% (v/v) glycerol to prevent bacterial cell lysis. Defrosted cultures were streaked on to LB agar and grown at 37°C in aerobic conditions. After 18-24 hours, a single bacterial colony was

used to inoculate into Lysogeny Broth (LB) (Oxoid). Liquid cultures for inoculation on to uroepithelial culture cells lines or stab inoculation into motility media were grown in 5ml of LB in successive rounds of static growth to enrich for type 1 fimbriae (Kakkanat *et al.*, 2015). After 20-24 hours of static growth at 37°C, 100µl of culture was inoculated in to a new 5ml of sterile LB. The inoculation sub-culture into sterile LB was repeated at 48 hours to conclude in 3 days of static growth. The 72 hour culture was enumerated by Miles and Misra technique (Miles, Misra & Irwin, 1938) using Mueller-Hinton agar (Melford laboratories, Suffolk, UK). The expression of type 1 pili was confirmed by agglutination of baker's yeast (*Saccharomyces cerevisiae*) (Martinez *et al.*, 2000) suspended in phosphate-buffered saline (PBS) (Life Technologies, Paisley, UK) and the presence of agglutination observed under low power microscopic objective.

5.2.2 Bacterial motility assay

A simple yet definitive motility medium incorporating Triphenyltetrazolium chloride (TTC) was used to determine the motility of test bacteria (Kelly & Fulton, 1953). The formula was adjusted to test bacterial motility in both 0.3% and 0.4% (w/v) agar. Tests were performed using 7ml polystyrene bijou containers incorporating 5ml of media. Test bacteria were inoculated with a straight wire loop to 1cm below the agar surface. Isolates from the three day static growth culture were used as the inoculum and incubated aerobically at 25°C, 30°C and 37°C. Cultures were monitored over 42 hours. Motility media formula is listed in Table 24.

Table 23. Formula for motility media incorporating TTC with 0.3 and 0.4% agar. Recipe adapted from the Handbook of Microbiological media Second edition (Atlas, 1996).

Formula per litre of motility medium (pH 7.3 ± 0.2)	
Pancreatic digest of gelatin	10.0g
Sodium chloride	5.0g
Beef extract	3.0g
Agar	3.0/4.0g
TTC	0.05g

The motility of each organism was recorded using a semi-quantitative scale with negative and positive observations; where negative (-) was no motility and 3+ positive (+++) depicted the presence of motile bacteria that had traversed the entire medium.

5.2.3 Genomic analysis of chemotaxis and motility genes

Genomic analysis and comparison of all known chemotaxis and motility genes was performed on all ten isolates of UPEC ST127 isolated from Derriford hospital. Analysis was performed using several bioinformatics programmes, as described in section 3.2. Mutations/differences between isolates were identified from the consensus sequence of all 10 Derriford isolates. DNA sequences were translated using CLC genomics and amino acid reference sequences for comparisons and gene standardisations were acquired from the closest matching DNA sequence in the Swiss-prot database. Amino acid sequences were collected from *E. coli* isolates K12, UTI89, CFT073 and ATCC 700928.

5.2.4 Publically available ST127 scaffold genomes used for analysis with SeqFindR

Two hundred and forty six ST127 scaffold genomes were downloaded from the enterobase (https://enterobase.warwick.ac.uk/species/index/ecoli) website (see appendix III for list of scaffold files) and used to identify specific sequences of interest originally identified from genome analysis of the ST127 Derriford isolates. Most genomes were unassembled and sequenced using a variety of methods. The quality of each genome was not assessed prior to use. A 60bp region was used as the template to determine the likely presence of an intact *flhB* gene using the SeqFindR 0.31.1 (https://github.com/mscook/SeqFindR) programme for analysis at a percentage identity of 1.0. The output of the SeqFindR analysis was checked manually to ensure result quality.

5.2.5 HTB-9 cell culture

The human bladder epithelial cell line 5637 ATCC HTB-9 (LGC, Middlesex, UK) was grown in Minimum Essential Media (MEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 2mM L-glutamine (Life Technologies). The HTB-9 cell line was grown in 75cm^2 tissue culture flasks (Grenier, Gloucestershire, UK) at 37°C with 5% CO_2 and utilised for the gentamicin invasion assay between 11 and 24 passages.

HTB-9 cells were seeded into flasks and grown to confluency. Monolayers were harvested by washing two times with PBS and addition of 0.5ml of Trypsin-EDTA (0.25%) (Life Technologies), which was spread across the surface of the monolayer and incubated at 37°C for 5-15 minutes until all cells detached from

the flask inner surface and were present in solution. The HTB-9 cells were seeded into 24-well (surface area per well 1.9cm^2) flat bottom tissue culture plates (Grenier) and grown to 95-100% confluency. HTB-9 cell seeding concentrations in to the 24-well plate was adjusted dependant on several variables pertaining to factors such as availability of confluent stock monolayers and availability of laboratory equipment (lamina flow cabinet). Each 24-well plate was given at least three days growth and only healthy monolayers with >95% confluency were used for experimentation. Enumeration of the average number of HTB-9 cells present in a single test well was performed by harvesting six confluent single well monolayers from three different passages and calculating the average total volume using a haemocytometer.

5.2.4.1 Bacterial cell binding to monolayers and the gentamicin invasion assay

As there are numerous published methods available for the gentamicin/binding assay using UPEC and bladder tissue culture cells, the original procedure by Elsinghorst (Elsinghorst, 1994) was used as a guide for variations on the assay in an attempt to determine the best method for the HTB-9 cell line and UPEC. All monolayers were washed two times with sterile PBS prior to the inoculation of test bacteria and all incubations were performed at 37°C in 5% CO_2.

Both the gentamicin invasion assay and the binding assay were performed in parallel using triplicate wells for each bacterial specimen. The same bacterial inoculation suspension was used for both assays. All parallel test monolayers were also a product of the same passage number and the same seeding

density. For the inoculation, a standardised solution containing 175µl - 350µl of 10^{-1} LB bacterial culture (25 to 50µl per specimen/0.5ml MEM) was added to pre warmed MEM with a reduced concentration of FBS (2% (v/v) - to discourage bacterial and cell line growth) to a total volume of 3.5ml. The standardised inoculation mixture for each isolate was pulse vortexed prior to inoculation to ensure an homogenous bacterial solution with a multiplicity of infection (MOI) of ~10 (Ellis & Delbruck, 1939). Washed monolayers were inoculated with 0.5ml of homogenised bacterial/MEM solution. Tissue culture plates were then either incubated for one hour or centrifuged at 600 x*g* for five minutes using a Beckman Coulter Allegra X-22R Centrifuge S2096 (Beckman Coulter, Indiana, USA) to expedite bacterial binding. Infected monolayers were then incubated for one hour.

The triplicate wells used to determine the level of bacterial binding were washed 6 times with PBS and monolayers were lysed and enumerated. Two lysis solutions were used for different experiments, 0.4% (v/v) Triton X-100 (Sigma-Aldrich, Gillingham, Dorset) in 1ml of PBS, and 0.4% (v/v) Triton X-100 with 0.25% (v/v) of Trypsin-EDTA in 1ml of PBS. Monolayers were incubated ~15 minutes in lysis solution and the bacterial counts enumerated from the lysate.

The triplicate monolayers used to determine invasion frequencies were washed x3 with 1ml of PBS then incubated for 1 hour in MEM (2% FBS) containing 100 or 200µg/ml gentamicin to kill any extracellular bacteria. Monolayers were then washed three times with PBS, lysed and enumerated as described above.

5.2.4.2 Statistical analysis

Bacterial adhesion was calculated with the average bacterial recovery from the triplicate wells expressed as a percentage of the total number of bacteria inoculated (Elsinghorst, 1994). The level of invasion was calculated by the percentage recovery of the inoculum after gentamicin treatment and using the invasion index; the number of invaded organisms taken as a percentage of the number of adhered organisms (Elsinghorst, 1994; Elsinghorst & Kopecko, 1992). Analysis was performed using Microsoft Excel® (Redmond, Washington, USA) and the standard error mean (SEM) and graphical representations were performed GraphPad Prism® (GraphPad, CA, USA).

5.2.4.3 Effect of centrifugation and gentamicin concentration on bacterial recovery

Centrifugation of inoculated cell monolayers was performed at 600 x*g* on a Beckman Coulter Allegra X-22R Centrifuge S2096 (Beckman Coulter, Indiana, USA). Assessment of bacterial recovery using different centrifugation times and gentamicin concentration was performed using triplicate wells and four bacterial isolates; ST127 Derriford specimen 9, K12 N2 (*fimH* negative mutant), K12 C600 (positive binding/negative invasion) and ST131 EC958 (positive invasion). Centrifugation times of 0, 2, 5 and 10 minutes was performed on the four *E. coli* strains. Each set of inoculated monolayers were incubated for up to one hour post inoculation. Monolayers were washed three times with PBS and incubated for one hour in MEM (2% FBS) supplemented with gentamicin at concentrations of 100 and 200µg/ml to determine if the concentration of gentamicin had any effect on intracellular/base line bacterial counts. HTB-9 cell lysis and bacterial

counts were performed using 0.4% Triton X100 as per instructions in section 5.2.4.1.

5.2.4.4 Effect of Triton X100 and trypsin concentration on bacterial recovery

A HTB-9 cell concentration of 5 x 10^6 was placed in 1ml of MEM (2% FBS v/v) containing 0.1% and 0.4% Triton X100 (v/v) with and without 0.25% Trypsin-EDTA. For the control, 1ml of MEM (2% v/v) without supplements was inoculated. Eight specimens were tested and incubated for one hour. Bacterial concentrations were determined by plate dilution.

5.2.5 Transmission Electron Microscopy of UPEC

To observe the presence of flagella and fimbriae on the bacterial surface, a sample of the test specimen cohort were subject to Transmission Electron Microscopy (TEM). Specimens 9, 39, 124L, 124S, 316 and EC958 were cultured for three days in static growth conditions as described in section 5.2.1. Cultures were centrifuged at 600 x*g* for 5 minutes and the supernatant removed. Loose bacterial pellets were gently washed with PBS and fixed in a solution of distilled H_2O (0.5ml) with 2.5% (v/v) glutaraldehyde (Agar scientific, Stansted, UK) and incubated for at least 1 hour at room temperature. Specimens were gently washed twice using 0.1M sodium cacodylate buffer (pH 7.2) (Agar scientific, Stansted, UK) then placed in 0.5ml distilled H_2O and homogenised by gentle rocking of the pellet/H_2O solution to minimise breakage of surface appendages. Fifteen microliters of specimen solution was placed on a

Formvar/Carbon 200 mesh Copper grid (Agar scientific, Stansted, UK) for ten minutes. The bacterial solution was removed via contact with filter paper. The specimens were washed by placing the inoculated side of the Copper grid on to a drop of distilled water for three seconds then stained by placing on a drop of saturated solution of uranyl acetate (Agar scientific, Stansted, UK) for five minutes. Grids were washed by gentle immersion in distilled water, allowed to dry and visualised on a JEOL 1400 TEM (JEOL Ltd, Hertford, UK) and Gatan computer software (Pleasanton, CA, USA).

5.2.5.1 Transmission Electron Microscopy of HTB-9 bladder cells infected with bacteria

To conclusively determine the presence of bacteria within the HTB-9 cell cytoplasm, TEM was performed on HTB-9 cells infected with a selection of test cultures including three ST127 specimens (263AS, 124L and 124S), one ST73 (263TR), the negative invasive control K12 C600 and the positive invasive control EC958 (ST131). Specimens chosen for TEM were based on preliminary results from the gentamicin invasion assay. For the experiment, entire 75cm^2 flasks were used to enable a larger yield of cellular mass necessary for sectioning in resin. Dehydration and sectioning of bladder cells was performed by the Electron Microscopy department at the University of Plymouth (Plymouth, Devon, UK).

To determine the concentration of inoculum required for standardised flask infection, three flasks of HTB-9 cells were grown to confluency, washed twice with PBS and the cells removed using Trypsin-EDTA (0.25%). The average

number of HTB-9 cells per flask was calculated using a haemocytometer. Infected flasks were not centrifuged and the incubation procedure was similar to that performed in section 5.2.4.1.

Confluent flask monolayers were infected with test bacteria in 10ml of MEM (2% FBS v/v) at an adjusted MOI of 100 to increase the potential invasion frequency and chances of observing invaded bacteria. Flasks were incubated for one hour at 37°C in CO_2 then washed six times with PBS and the infected HTB-9 cells disassociated from the flask internal surface using 0.5ml of Trypsin-EDTA. The cells were removed from the flask and centrifuged for five minutes at 600 x*g*, and the lose pellet washed twice in PBS. The pellet was fixed in 1ml of distilled water containing 2.5% glutaraldehyde and incubated overnight at room temperature.

The glutaraldehyde was removed and the sample pellets resuspended in 0.1M sodium cacodylate buffer (pH 7.2) (Agar scientific, Stansted, UK) for 15 minutes. The buffer was removed and the specimen secondary fixed with 1% Osmium tetroxide (w/v) (pH 7.2) for one hour then rinsed twice with sodium cacodylate buffer. Dehydration of the sample pellets was performed by the addition of 30% (v/v) ethanol in distilled water for 15 minutes. The 30% ethanol was removed and the process repeated with 50%, 70%, 90% and 100% (v/v) ethanol to distilled water. The 100% ethanol step was repeated a second and third time. On each occasion ‘dry’ ethanol was used obtained by the addition of molecular sieves (Agar scientific, Stansted, UK) in 100% ethanol.

Once specimen dehydration was complete, the 100% ethanol was removed and a 30:70 ratio of Agar low viscosity resin (Agar scientific, Stansted, UK) and

absolute ethanol, respectively, was added to each specimen. The cells were incubated at room temperature for 12 hours to allow the infiltration of resin. The process was repeated with a 50:50 and 70:30 ratio of resin:ethanol. Finally, 100% resin was applied three times to the specimen with a 12 hour incubation period between each. On the third resin application, the specimen was transferred to a Tabb embedding oven (Tabb, Aldermasten, UK) and the resin polymerised at 60°C for 12 hours.

The hardened resin was then cut into 80nm sections by use of a Leica Ultracut E ultramicrotome with a diatome diamond knife (Agar scientific, Stansted, UK). Sections were supported on a 200 mesh Copper grid and stained first with a saturated solution of uranyl acetate for 15 minutes, gently rinsed in distilled H_2O followed by staining with Reynold's lead citrate (50ml distilled H_2O, 0.1g NaOH 0.25g lead citrate) (Reynolds, 1963) in a petri dish for 15 minutes, surrounded by pellets of sodium hydroxide (Agar scientific, Stansted, UK) to eliminate atmospheric CO_2, which can cause the development of crystalline lead structures that can impair specimen visualisation. Specimens were washed in distilled H_2O and imaged with JEOL 1400 TEM and Gatan computer software.

5.2.5.2 Scanning Electron Microscopy of infected HTB-9 cell monolayers

Only a proportion of the bacterial isolates were selected for Scanning Electron Microscopy (SEM) based on results from the gentamicin invasion assay, along with negative and positive controls. Specimens tested included four ST127 isolates (39, 124L, 124S and 263AS), one ST73 (263TR), K12 C600 and the positive control EC958.

To visualise bacteria bound and possibly invading HTB-9 culture cells, monolayers were grown on sterile cell-culture treated circular coverslips (ThermoFisher, Loughbourgh, UK) that were aseptically positioned on the bottom of 24-well culture plates. All of the specimens were inoculated using a three day static culture at an MOI of ~10 and incubated for one hour at 37°C in CO_2. Specimens 124L and 124S were specifically observed by SEM over a range of incubation times; 10, 20, 30 and 60 minutes. After incubation, specimens were washed three times with PBS, fixed in 2.5% glutaraldehyde and incubated at room temperature for at least one hour. Coverslips were washed twice in sodium cacodylate buffer for 10 minutes and dehydrated with increasing concentrations of ethanol, as described in section 5.2.5.1.

The dehydrated monolayer coverslips were placed in porous specimen pots (Agar Scientific, Stansted, UK) whilst submerged in 100% dry ethanol. Specimens were then subjected to Critical Point Drying (CPD) with CO_2 using an Emitech K850 CPD (Quorum Technologies, Laughton, UK) to remove and replace any remaining water whilst preventing the collapse or deformation of surface detail. The coverslips were placed on aluminium stubs and fixed in position using a carbon infiltrated minitab (Agar Scientific, Stansted, UK). Specimens were sputter coated with gold using an Emitech K550 gold sputter unit (Quorum Technologies, Laughton, UK) and visualised using a JEOL 6610LV SEM (JEOL Ltd, Hertford, UK) with Gatan computer software.

5.3 Results

5.3.1 Motility of test organisms

5.3.1.1 Motility assay

The majority of the UPEC ST127 isolates were recorded as non-motile. Two of the ST127 UPEC isolates (316 and 468), produced atypical growth/motility after several hours incubation under specific test conditions. Both ST73 isolates were highly motile at each temperature range in both 0.3 and 0.4% agar. Table 25 displays a semi-quantification scale of motility observed for each test specimen under two agar concentrations and varying incubation temperatures (Figure 30).

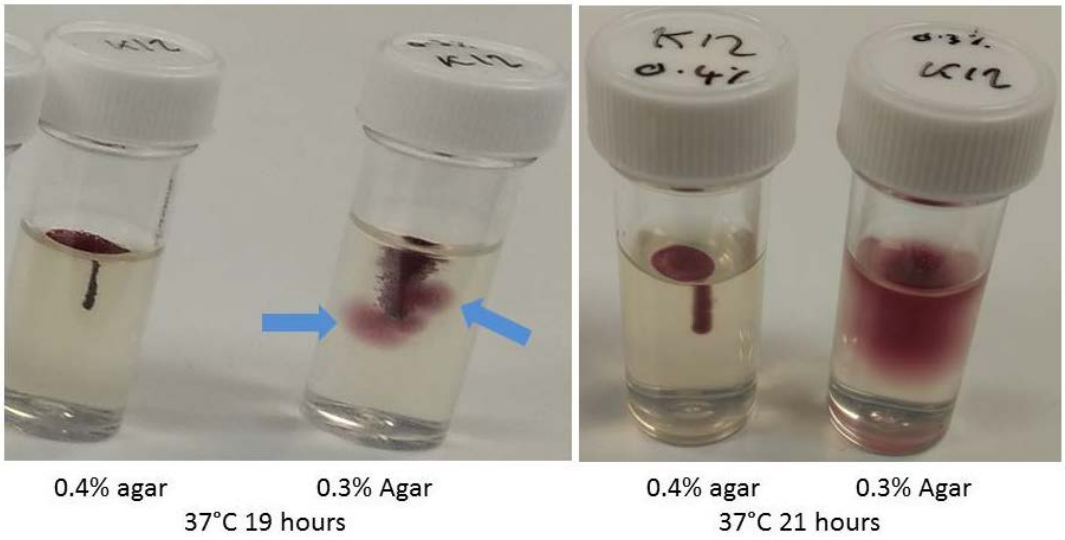

Figure 29. Laboratory strain *E. coli* K12 inoculated into motility media after 3 days static (LB) growth conditions. Motility media was grown at 37°C in both 0.3 and 0.4% agar. The K12 isolate that was grown in an agar concentration of 0.4% displayed no motility. *E. coli* K12 motility was observed at specific points (blue arrows) of the inoculum in 0.3% agar (19 hours). Motility testing for *E. coli* K12 was discontinued at 24 hours.

Both bacterial variants from specimen 124 were the only ST127 isolates to display comparable motility with the highly motile positive control EC958. Both ST73 isolates (263AR and 263TR) also produced comparable motility to the positive control. The difference of only 0.1% variation in agar concentration had

dramatic effects on the results. This was best demonstrated by use of the laboratory strain *E. coli* K12 that produced at least two highly motile sub-populations emanating from specific points of the inoculum after ~16 hours growth and only in the 0.3% agar medium (Figure 29 - 19 hours incubation

Table 24. Semi-quantitative motility results for bacterial isolates incubated at 25, 30 and 37°C, for 16, 24 and 42 hours using 0.3 and 0.4% agar concentration

Agar Conc.	Hours Incub.	Temp	Bacterial Isolate														
			9 ST127	39 ST127	124S ST127	124L ST127	263AS ST127	263AR ST73	263TR ST73	264 ST127	298 ST127	316 ST127	354 ST127	468 ST127	EC958 ST131	*K. pneu*	*S. typhi*
		25°C	-	-	++	++	-	+	+	-	-	-	-	-	++	-	+++
0.3%	16	30°C	-	-	++	++	-	++	+/++	-	-	-/+	-	-	++	-	+++
		37°C	-	-	++	++	-	++	++	-	-	-/+	-	-	+++	-	+++
		25°C	-	-	++	++/+++	-	++	++	-	-	-	-	-	++	-	+++
0.3%	24	30°C	-	-	+++	+++	-	++/+++	+++	-	-	+	-	+	+++	-	+++
		37°C	-	-	+++	+++	-	+++	+++	-	-	+	-	-	+++	-	+++
		25°C	-	-	+++	+++	-	+++	+++	-	-	-	-	-	+++	-	+++
0.3%	42	30°C	-	-	+++	+++	-	+++	+++	-	-	+	-	+++	+++	-	+++
		37°C	-	-	+++	+++	-	+++	+++	-	-	+	-	-	+++	-	+++
		25°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
0.4%	16	30°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
		37°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
		25°C	-	-	+	+	-	+	+	-	-	-	-	-	+	-	++
0.4%	24	30°C	-	-	+	+	-	+	+	-	-	-	-	-	+	-	++
		37°C	-	-	+	+	-	+	+	-	-	-	-	-	+	-	++
		25°C	-	-	+	+	-	+	+	-	-	-	-	-	+	-	++
0.4%	42	30°C	-	-	++	++	-	++	+/++	-	-	-	-	-	++	-	+++
		37°C	-	-	++	++	-	++	++	-	-	-	-	-	++	-	+++

Symbols representation: -, negative motility; +, some significant migration from inoculation point; ++, migration to the inner vertical wall of the bijou; +++, migration to the base of the bijou.

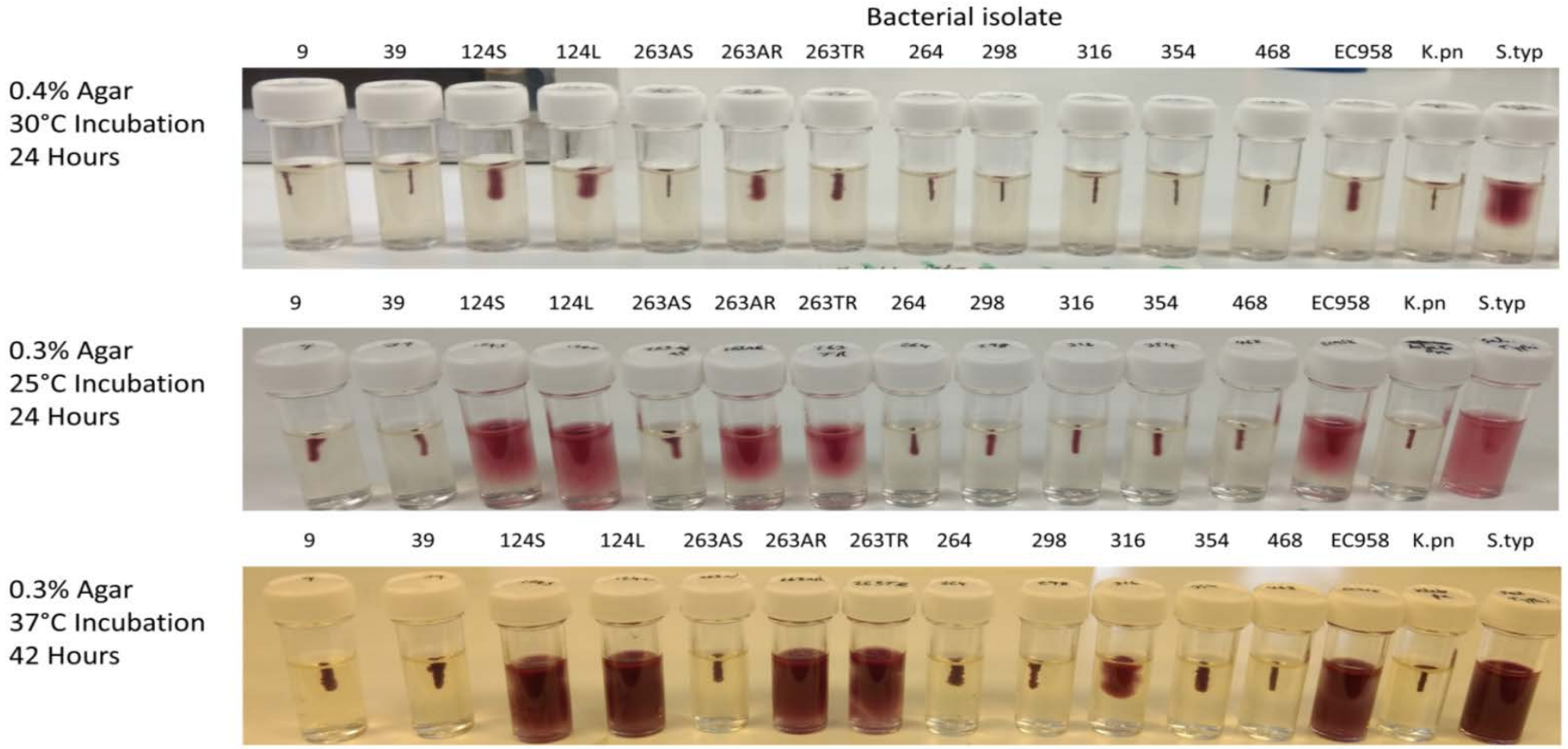

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Figure 30. Examples of motility assay using TTC (red pigment). Bacterial isolates were inoculated by straight wire loop 1cm below the surface. Motile bacteria can be easily observed by the red pigment produced from TTC by cellular respiration. In comparison to the positive control EC958, only 2 two UPEC ST127 Isolates (124S and 124L) and both ST73 isolates displayed clear motility irrespective of incubation temperature or agar concentration. Motility testing including all agar concentrations and incubation temperatures were performed at least twice on each bacterial isolate

Another two ST127 isolates displayed atypical growth/motility in the 0.3% TTC medium. Isolate 316 displayed a distinct migration in comparison to the non-motile ST127 isolates but the migration was greatly reduced in comparison with the positive 124 isolates. Additionally, this atypical growth/motility phenotype was only clearly observable after a full 24 hours growth and only in 0.3% agar concentration (Figure 31 and Figure 30 -bottom row).

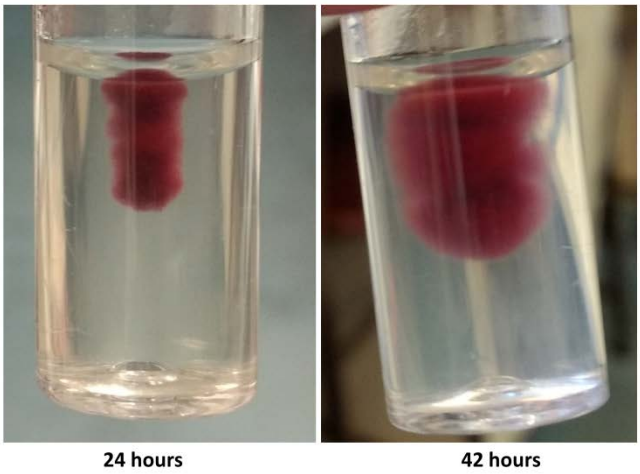

Figure 31. Atypical growth/motility of UPEC ST127 strain 316 grown in 0.3% agar at 37°C. Images display growth after 24 and 42 hours incubation.

In general, ST127 UPEC specimen 468 displayed no motility at all temperatures in all agar concentrations up to ~16 hours. The same sub-population motility observed with *E. coli* K12 laboratory strain (Figure 29) was also displayed with specimen 468 at 18 hours incubation (37°C, 0.3% agar), although the effect was less abundant. The ST127 UPEC 468 isolate produced only one motile sub-population emanating from a single point two thirds of the way down the stab inoculum (Figure 32). Once the sub-population had initiated motility, the speed at which the organism migrated was similar to that observed by the

highly motile UPEC isolates. The same motility test was repeated twice for specimen 468 but upon repeat, no motile sub-population was observed.

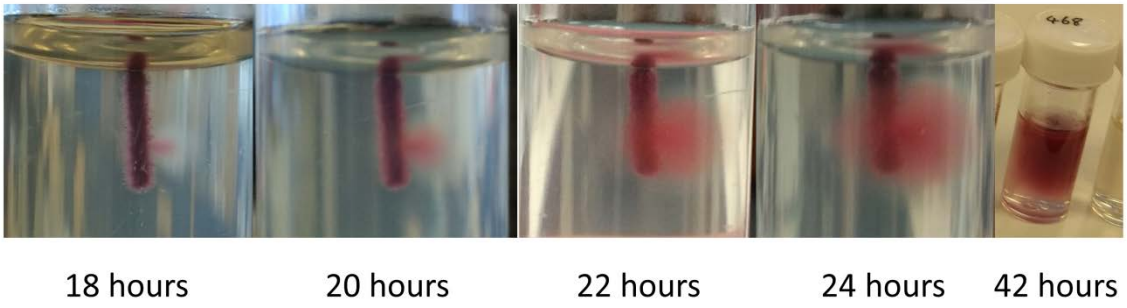

Figure 32. Motile sub-population of ST127 strain 468 grown at 30°C in a concentration of 0.3% agar. The sub-population emerged at ~16 hours and migrated at a speed equivalent to the motility exhibited by ST127 specimen 124.

5.3.1.2 Genomic analysis of chemotaxis and motility genes

A large region of the ST127 genome totalling 72,785bp, containing motility and chemotaxis genes, was analysed for missing/additional genes or mutations. No extra or additional gene sequences were observed in any ST127 UPEC isolate but a total of 19 collective SNP mutations were identified from the ST127 UPEC consensus sequence. Five of these SNPs caused synonymous amino acid mutations and 12 caused non-synonymous amino acid mutations (listed in Table 26). Two point mutations were detected in a gap sequence between the *araG* and *araF* genes (strain 354); and between *fliE* and *fliF* genes (9), their effect, if any, was unknown but as the mutation was not part of any defined motility/chemotaxis genes, it was postulated that these mutations had no effect on motility, as such, no further examination was carried out on these mutations.

Specimen 263AS, 264 and 354 possessed identical sequences to the ST127 UPEC consensus sequence across all motility/chemotaxis genes. Specimen

124L contained the highest number (4) of non-synonymous mutations within this area of the genome, with only one SNP mutation varying from the 124S specimen; an aspartic acid to glycine amino acid in the *uvrY* gene.

The most prominent mutation was the frameshift mutation in the *flhB* gene displayed in all ST127 isolates except specimens 124S, 124L and 316, which possessed a sequence identical to the *flhB of E. coli* K12. The *flhB* gene was 1149bp in length with the point deletion at 336bp possibly affecting over two thirds of the gene translation. Analysis of 246 ST127 scaffolds using SeqFindR (See Appendix IV) identified the *flhB* frameshift mutation in exactly two thirds of these scaffolds (n=164).

Table 25. List of motility and chemotaxis genes possessing SNP mutations in comparison to consensus ST127 sequence and standardised protein sequences from other *E. coli* isolates acquired from the Swiss-Prot database. Brackets denote position of mutation on DNA and protein sequence relative to reference sequences.

Isolate	Gene											
	flhA	***flhB***	***cheA***	***motA***	***uvrY***	***sidA***	***fliF***	***fliI***	***fliK***	***fliP***	***flgI***	***flgJ***
9 DNA Amino acid		G deletion (336) Frameshift (113)				A >T (607) Isoleucine > Phenylalanine (203)			A > T (968) Glutamic acid > Valine (323)			
39 DNA Amino acid		G deletion (336) Frameshift (113)										A > G (896) Aspartic acid > Glycine (299)
124S DNA Amino acid		Homologous To K12 *flhB*					C > A (163) Leucine > Isoleucine (55)	C > T (874) Proline > Serine (292)		A > C (164) Glutamine > Proline (55)		
124L DNA Amino acid		Homologous To K12 *flhB*			A > G (146) Aspartic acid > Glycine (49)		C > A (163) Leucine > Isoleucine (55)	C > T (874) Proline > Serine (292)		A > C (164) Glutamine > Proline(55)		
263 DNA Amino acid		G deletion (336) Frameshift (113)										
264 DNA Amino acid		G deletion (336) Frameshift (113)										
298 DNA Amino acid		G deletion (336) Frameshift (113)									C > T (806) Threonine > Isoleucine (269)	
316 DNA Amino acid		Homologous To K12 *flhB*	G > A (794) Arginine > Histidine (265)	C > T (311) Proline > Leucine (104)						A > C (164) Glutamine > Proline (55)		
354 DNA Amino acid		G deletion (336) Frameshift (113)										
468 DNA Amino acid	A > T (1658) Aspartic acid> Valine (553)	G deletion (336) Frameshift (113)										

Genes names with SNP mutations (Uniprot): *flhA*, Flagella biosynthesis protein; *flhB*, Flagella biosynthetic protein; *cheA* Chemotaxis protein; *motA*, Flagella motor protein; *uvrY*, DNA binding response regulator; *sidA*, Cell division inhibitor; *fliF*, Flagella M-ring protein; *fliF*, Flagellum specific ATP synthase; *fliK*, Flagella hook length control protein; *flip*, Flagellar biosynthetic protein precursor; *flgI*, Flagella P-ring protein precursor; *flgJ*, Peptidoglycan hydrolase

5.3.2 ST127 binding and invasion of HTB-1197 and HTB-9 bladder cells

5.3.2.1 Specimen preparation and optimisation of cell culture assay

The average 1.9cm^2 tissue culture plate well contained approximately half a million HTB-9 cells, thus to achieve an MOI of ~10, approximately five million bacterial cells were inoculated per well. Although an MOI of 10 was the target, it was not always achieved and ranges varied between 4-18 MOI were recorded. However, the vast majority of inocula were 7-13 MOI; each individual result was adjusted to compensate for this variance with the exact inoculation concentration used in the final calculation. Specimen 124L consistently yielded reduced bacterial concentrations from three day static LB broth culture, inoculum levels were adjusted to compensate.

Concerns regarding the lysis solution (0.1/0.4% Triton X100 and 0.25% (v/v) Trypsin-EDTA) affecting the viability of organisms, were dispelled with comparable recoveries to the control in each concentration of Triton X100 and Trypsin-EDTA (data not shown).

Plate dilution assays were performed to determine the level of gentamicin resistance prior to the binding/invasion assay. All isolates were tested and EC958 produced the highest minimum inhibitory concentration (MIC) of 6.25µg/ml, well below the 100/200µg/ml used in the final assay.

5.3.2.2 Yeast agglutination from static culture

Agglutination of the yeast culture *Saccharomyces cerevisiae* was performed on all isolates and visualised under low power light microscopy. All ST127 isolates agglutinated the baker's yeast cells after 24 hours static growth. The *fimH*

negative mutant N2 produced no agglutination at any time in the experiment. The positive invasive control EC958 and the two ST73 isolates (263AR and 263TR) produced agglutination only after three days static growth (Table 26).

Table 26. Results from the agglutination of baker's yeast by *E. coli* isolates grown in static LB broth culture for three days.

isolate	Hours		
	24	48	72
9	+	+	+
39	+	+	+
124S	+	+	+
124L	+	+	+
263AS	+	+	+
264	+	+	+
298	+	+	+
316	+	+	+
354	+	+	+
468	+	+	+
C600	+	+	+
EC958	-	-	+
N2	-	-	-
EC18	+	+	+
SA191	+	+	+
263AR	-	-	+
263TR	-	-	+

5.3.2.3 Invasion and binding assay incorporating a centrifugation stage, 100ug/ml of gentamicin, and 0.4% Triton X100 with 0.25% Trypsin-EDTA as the lysis agent.

The results for the binding and invasion assays were often erratic, even under the same experimental conditions. Several initial attempts were made to achieve consistent results and numerous steps were taken in an attempt to standardise the procedure including a standardised inoculation solution (per isolate) used for each of the six inoculated wells (3 binding and 3 invasion), the use of a multichannel pipette for washing stages and increased dilution and inoculation volumes (100µl) for plate counts. It was believed that one of the major factors concerning extremely high and erratic recovery rates was the two

hour incubation period for the initial infection and gentamicin stages (as proposed by Elsinghorst 1994), presumably due to bacterial growth during the experiment (Elsinghorst, 1994). Accordingly, the results demonstrated in this section were achieved from numerous attempts (data not shown) to optimise the assay and from using only one-hour incubation for both infection and gentamicin treatment.

Figure 33 and 34 display the individual and combined results for the binding and invasion assays, which incorporated a centrifugation stage (five minutes at 600 x*g*) immediately after inoculation. Due to the reduction in the initial incubation time to one hour, it was believed a centrifugation stage was necessary to expedite the binding of test bacteria.

Each graph represents the results of two 24-well culture plates. The positive invasion control was used on each plate, hence two results per graph for EC958. The N2 and C600 negative controls were each inoculated on only one of the two plates used.

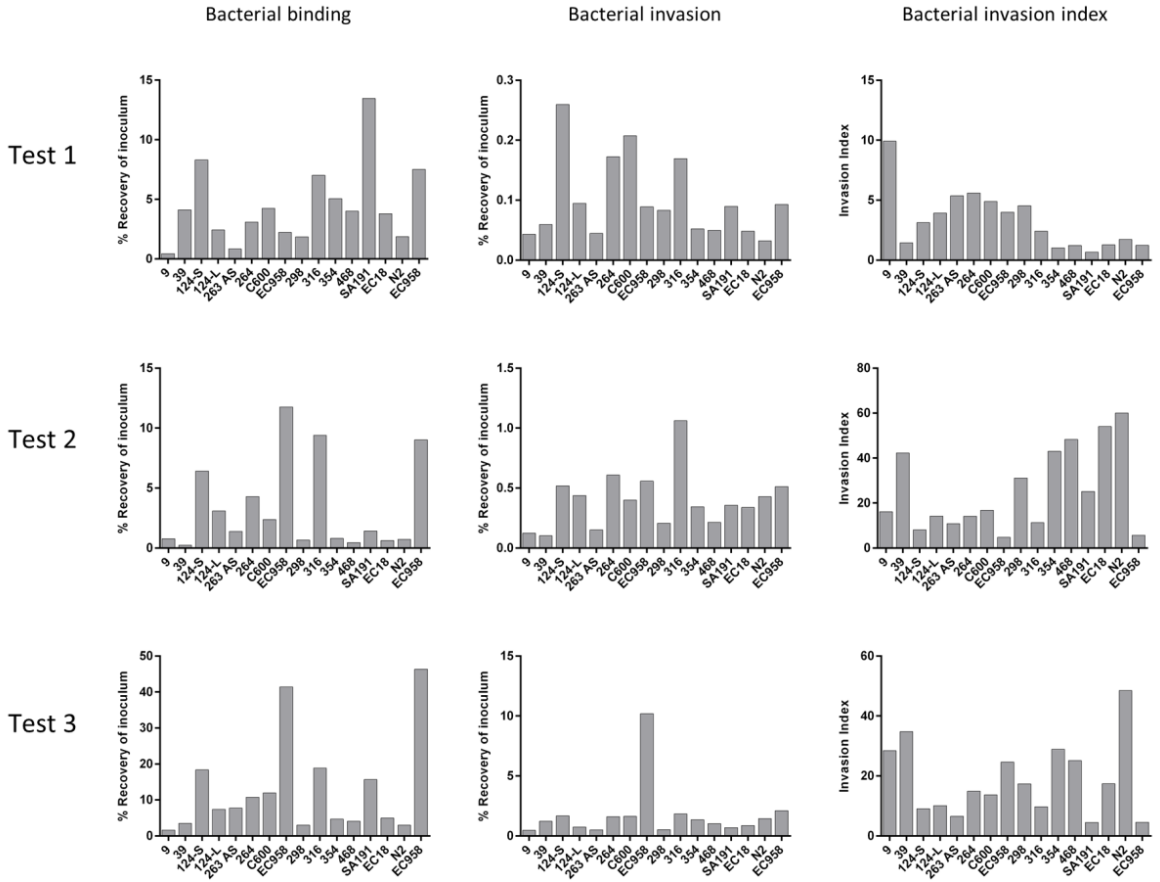

Figure 33. Three independent binding and invasion assays each incorporating triplicate infected monolayers. Bacterial binding (left column) was determined by the number of recovered organisms expressed as a percentage of the original inoculation concentration. The same calculation was used for the bacterial invasion (centre column), post gentamicin incubation. The invasion index (right column) was determined by the recovery expressed as a percentage of the recovered bound bacteria.

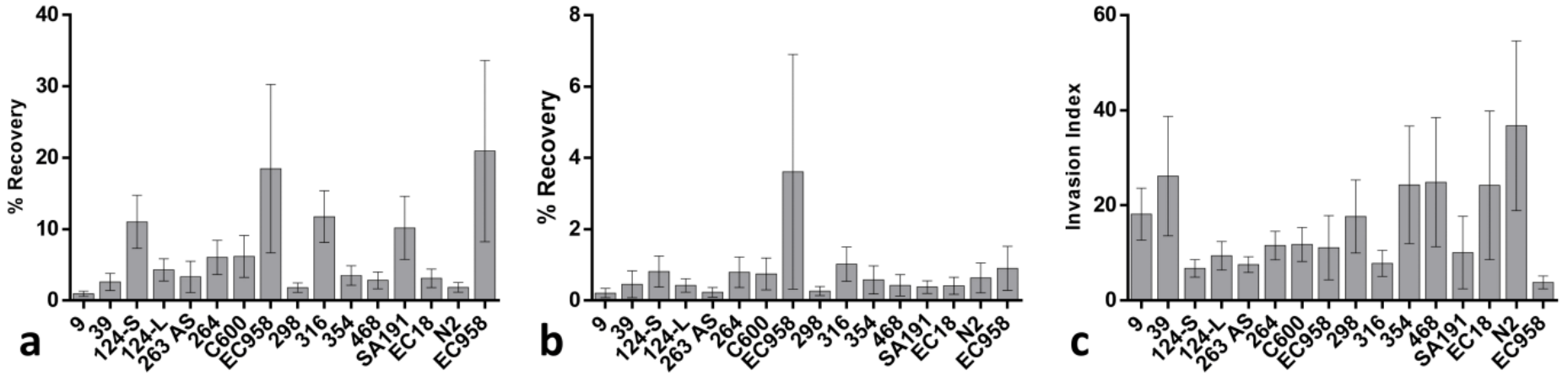

Figure 34. Collective results for the binding and invasion assays. Assays incorporated a 5 minute centrifugation stage and 0.4% Triton X100 with 0.25% Trypsin EDTA lysis stage. (a) Bacterial recovery. (b) Bacterial binding. (c) Invasion index. Values depict the means of three independent experiments performed in triplicate and error bars the standard error of the mean (SDEM).

Results of the invasion/binding assay incorporating a centrifugal stage were inconsistent. Efforts made to standardise the protocol were only partially successful and the inconsistencies with results is illustrated by the SDEM range. The positive control (EC958) displayed increased binding in two of the three assays performed; the invasion as a percentage of the inoculum was also consistently above the average in two of the three assays. The positive binding/negative invasion control C600 produced a binding and invasion capacity relative to many of the ST127 isolates. As expected, the negative control (N2) displayed an extremely poor binding capacity and a low invasion frequency. From the invasion and binding recovery results taken as a percentage of the inoculum, most ST127 isolates displayed no obvious invasive characteristics.

Isolate 9 consistently displayed poor binding ability, even with the added centrifugal stage incorporated into the protocol. The binding results for specimen 9 were consistently below the levels obtained for the negative control.

The invasion index calculation assumes an invasive organism will have a high binding phenotype. If an organism displays poor binding capabilities and the basal recovery of organisms is high, results using this calculation will be misleading, producing an increased invasive index for organisms with poor binding capabilities. As specimen 9 and the negative binding control (N2) display a low binding phenotype, the invasive index for these organisms was disproportionately high and results were disregarded. As binding capabilities were also variable with most ST127 isolates, results from the invasion index calculation were of limited use.

5.3.2.4 Effect of centrifugation and gentamicin concentration on recovery of bacteria.

The concentration of gentamicin appeared to have a negligible effect on recovery rates, especially when no centrifugation was used (Figure 35).

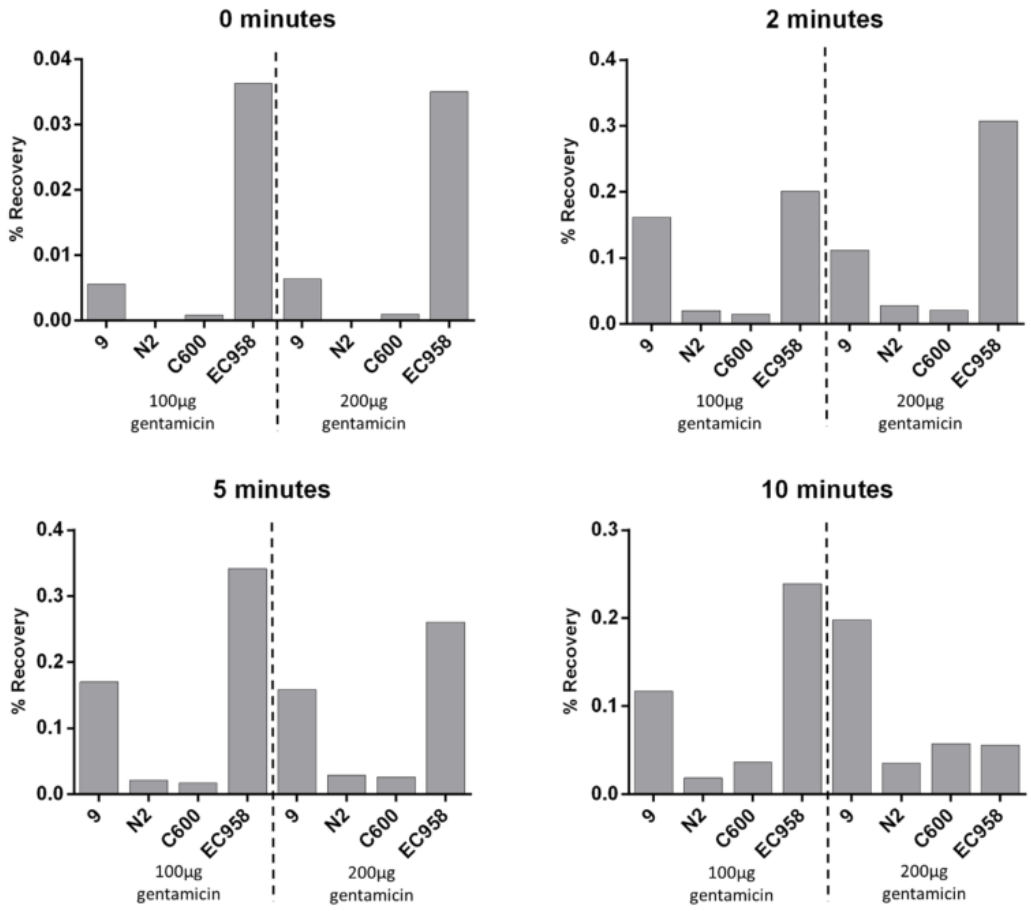

Figure 35. Bacterial recovery with the HTB-9 invasion assay using two concentrations of gentamicin and various centrifugation times.

A large variation in recovery was recorded at 10 minutes centrifugation with both specimen 9 and EC958, however as recoveries were larger with a decreased concentration of gentamicin (a phenomenon not observed to any large extent at other time points) this result was considered an anomaly and accepted as part of the varied results that seem commonplace whilst using this assay.

Centrifugation had a considerable effect on the basal level recoveries. Although basal levels of non-invasive bacteria were consistently low, the effect of centrifugation, at times, caused recoveries to be comparable to the positive control; N2 and C600 at 10 minutes-200µg and the isolate 9 at 2 minutes-100µg.

More significantly, the recovery of the *fimH* negative non-binding isolate N2 was recorded at zero CFU/ml for the non-centrifuged assay, effectively removing the baseline levels of bacteria. As expected, recovery of the positive control for the non-centrifuged assay were also greatly reduced but, with a zero baseline recovery for non-invasive bacteria, the disparity between invasive and non-invasive isolates was much clearer.

5.3.2.5 Invasion and binding assay with no centrifugation stage, 200ug/ml gentamicin concentration and 0.4% Triton X100 as the lysis agent.

Assay results without the centrifugal step were more consistent with an overall reduction in the basal bacterial recovery and reduced levels of recovery inconsistency (Figure 36 and 37). The positive control (EC958) displayed consistently high levels of binding and invasion and recovery of the negative control (N2) was negligible. The binding but non-invasive control (C600) showed some variance with only moderate levels of binding with the first assay, but recorded the highest level of binding on the second. Most ST127 isolates displayed a moderate level of binding for the first assay and low level of binding for the second, the exception being specimen 124S and 264, which both displayed increased binding to HTB-9 cells.

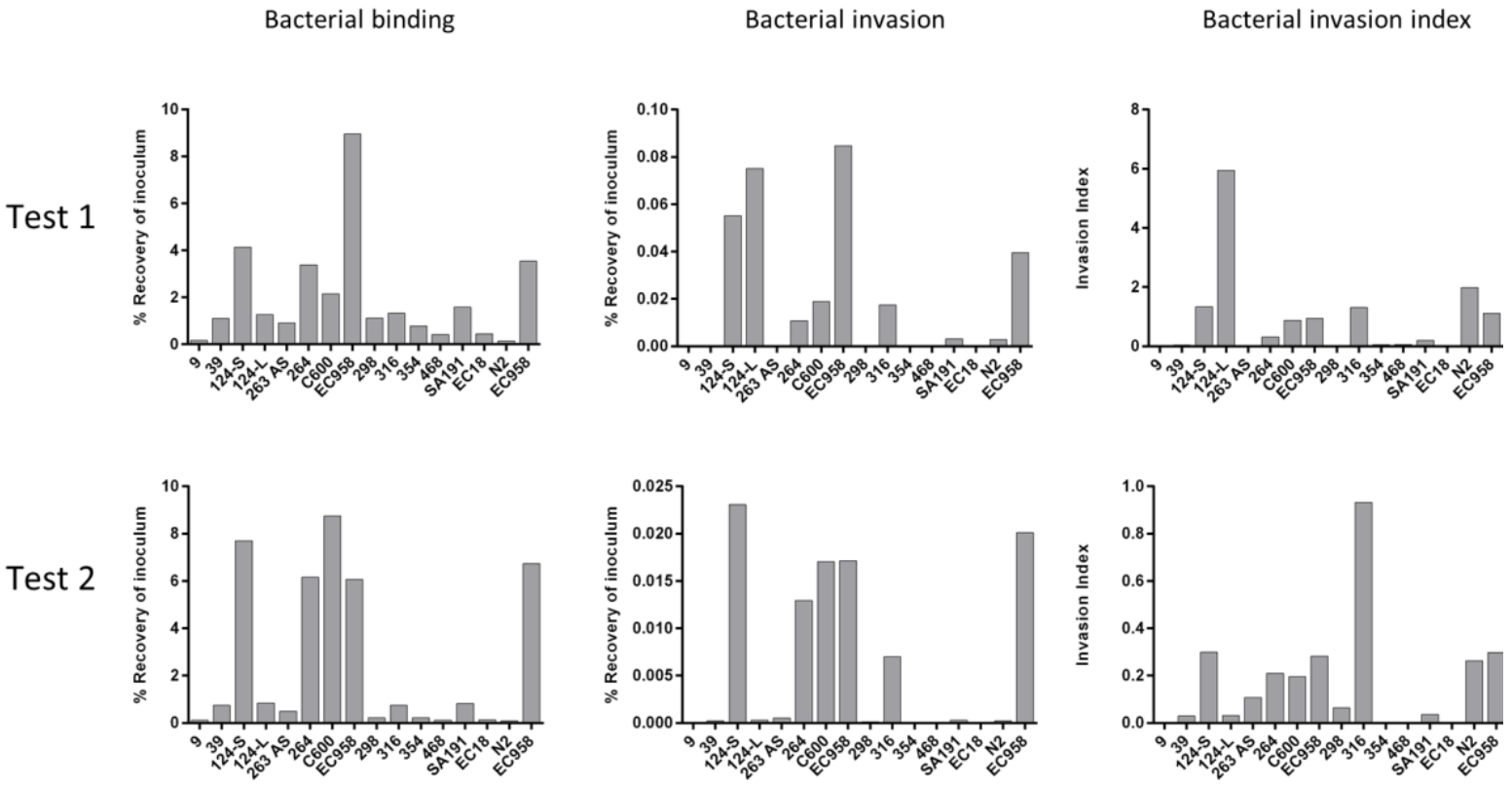

Figure 36. Two independent binding and invasion assays each incorporating triplicate infected monolayers. Calculations were performed as stated in Figure 32. All assays had no centrifugation stage and cell lysis was performed using 0.4 Triton X100 only.

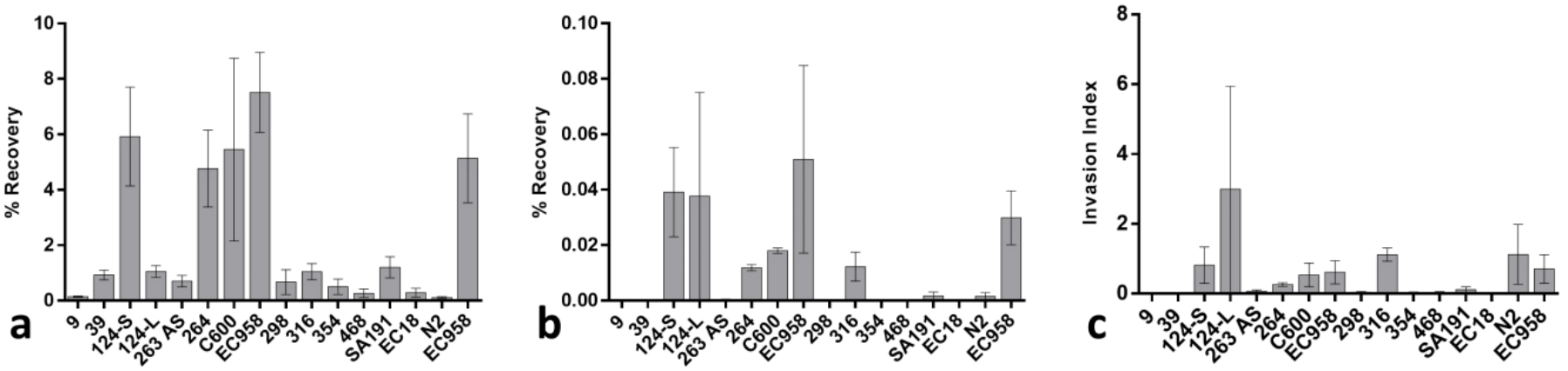

Figure 37. Collective results for the binding and invasion assays with no centrifugation stage and 0.4% Triton used as the lysis agent. (a) Bacterial binding. (b) Bacterial invasion. (c) Invasion index. Values depict the means of two independent experiments performed in triplicate and error bars represent the SDEM.

Specimen 124S was also consistently found to have increased invasion recoveries in the non-centrifuged assay. Its counterpart, 124L, displayed increased levels of invasion on the first assay but not the second. Specimen 316 also displayed a moderate level of invasion but produced levels of cell binding comparable to other, deemed non-invasive ST127.

The low binding phenotype of specimen 9 was also confirmed with this assay producing negligible recoveries for both binding assays.

5.3.2.6 Lysis of HTB-9 monolayer

Concerns were raised over the lysis procedure during the binding and invasion assays. Initial testing with 0.1% Triton as performed by Elsinghorst and Martinez (Elsinghorst, 1994; Martinez & Hultgren, 2002) displayed, what was considered by visual inspection, inadequate lysis of the bladder cell sheet. The lysis procedure was adjusted to include 0.4% Triton X100 as performed by Eto and colleagues (Eto *et al.*, 2007). However, the increased concentration of Triton X100 also seemed to have little or no effect on cell lysis (Figure 38b) and bacterial cells could still be observed embedded in the cell sheet (Figure 38c) once the lysis solution was removed. The effect of Triton X100 caused a cellular reaction more like a cytopathic effect (CPE), rather than the expected complete cell lysis. It was decided to add Trypsin-EDTA at a concentration of 0.25% to the 0.4% Triton X100 solution to aid cell lysis, as performed by Madelung and colleagues (Madelung *et al.*, 2017). Upon initial visualisation of the cell monolayer after incubation with the 0.4% Triton X100 0.25% Trypsin lysis

solution, the cell sheet appeared completely decimated with few cells remaining on the bottom of the tissue culture well.

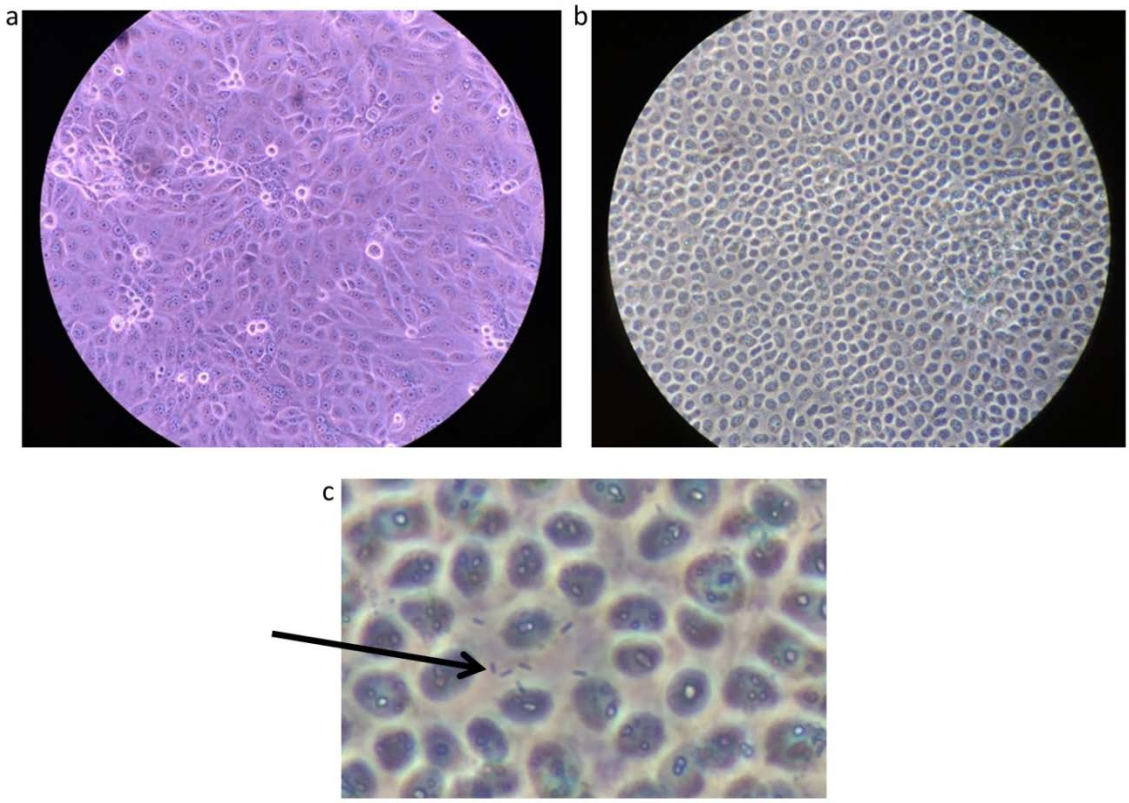

Figure 38. Light microscopy of HTB-9 monolayer. Image a, healthy confluent HTB-9 monolayer in MEM. Image b, HTB-9 124S infected monolayer after 30 minutes incubation with 0.4% Triton X100. Image taken after solution removed. Monolayer does not seem to be lysed but instead produces a reaction indicative of a CPE. Image c, a close-up image of b after removal of Triton X100. Bacteria remain embedded or attached to cell sheet monolayer (black arrow).

Analysis performed to determine if UPEC recoveries were consistent in all lysis solution concentrations ensured that the lysis solution itself had no effect on recovery.

Several erroneous plate dilution results were experienced whilst using lysis solution that contained 0.25% Trypsin-EDTA, in respect to vastly increased bacterial counts from a higher dilution factors. Initially this was attributed to methodological and/or technical error. To circumvent the dilution count problem,

increased volumes of inocula (100µl per dilution) and a single plate per dilution factor was used for the plate dilution series. An additional concern was also raised regarding the consistency of the lysis solution after removal from the HTB-9 monolayer. Pipette tips would either not take up the lysis solution or become blocked when attempting to expel the liquid due to a thick 'gloopy' consistency within parts of the lysis solution. Further investigation identified high bacterial counts in the viscous areas of solution and extra mixing of the solution was performed to result in complete homogenisation. However, the problem persisted even after adequate pipette mixing and vortexing of bacterial lysis solution. Further inspection of the monolayer well and lysis solution revealed large pieces of intact cell sheet removed from the surface of the culture plate and floating in lysis solution, when 0.25% Trypsin was used in the lysis solution.

Although 0.4% Triton X100 seemed inadequate to completely lyse the HTB-9 bladder monolayer, an increased concentration of bacteria at higher dilution factors was not experienced, therefore for the final binding/invasion assay 0.4% triton alone was used as the lysis solution.

5.3.3 Electron Microscopy

5.3.3.1 Transmission Electron Microscopy of UPEC

The positive invasive control (EC958), the two non-motile isolates (9 and 39), the atypical growth/motility isolate 316 and the two highly motile ST127 isolates (124S and 124L) were examined by TEM. Visualisation of individual bacterial cells under TEM revealed the presence of fimbriae on all tested isolates (Figures 39-43).

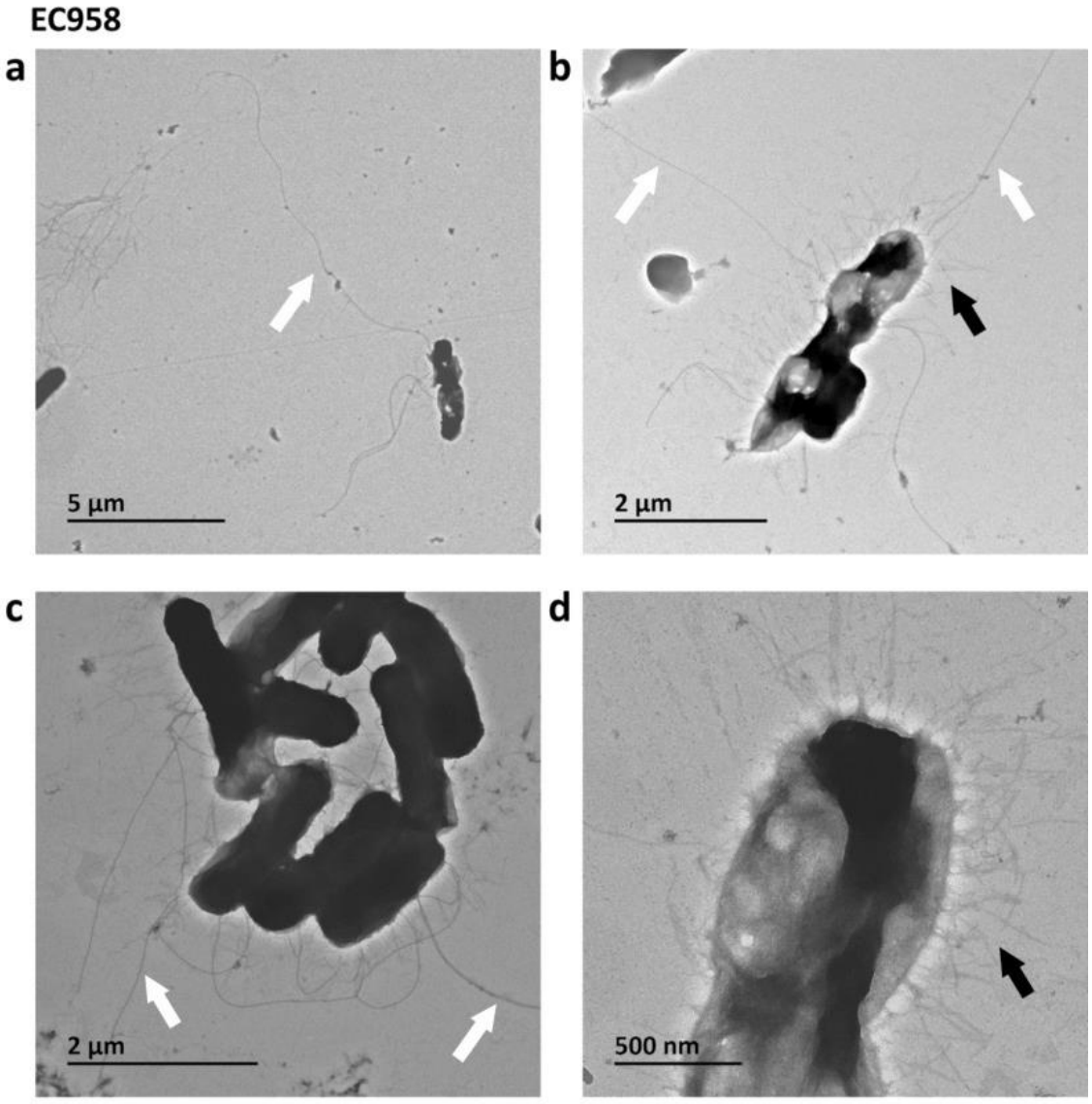

Figure 39. Transmission electron micrograph of invasive positive control ST131 isolate EC958. Images taken after three days static growth. White arrows indicate long flagella structures (image a-c) and black arrows indicate peritrichous fimbrial structures (image b and d) protruding from the outer cell membrane ~1µm in length.

As suspected following results of the motility assay, specimen 9 and 39 (Figure 40) possessed fimbriae but no flagella structures, whereas EC958 (Figure 39) and the ST127 UPEC isolates 124S, 124L and 316 (Figures 41-43) displayed fimbriae as well as extremely long flagella structures. The fimbriae possessed by specimen 9 seemed to be much denser than any of the other tested isolates (Figure 40-42).

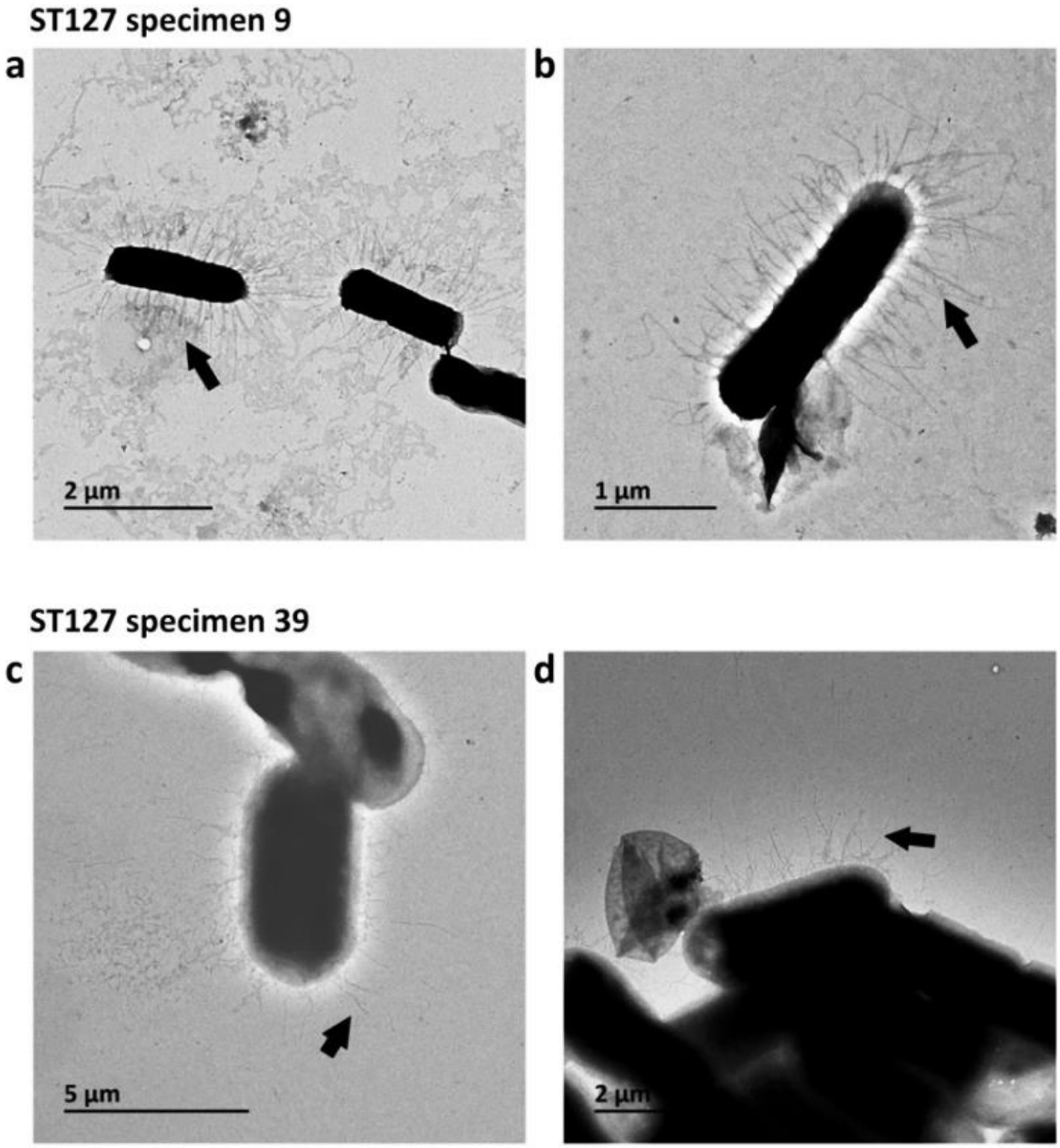

Figure 40. Transmission electron micrograph of two non-motile ST127 isolates. Images taken after three days static growth. Specimen 9 (image a and b) displayed long dense fimbriae (black arrows) whereas specimen 39 (image c and d) possessed more sparse distribution of fimbriae. Neither organism produced any flagella structures.

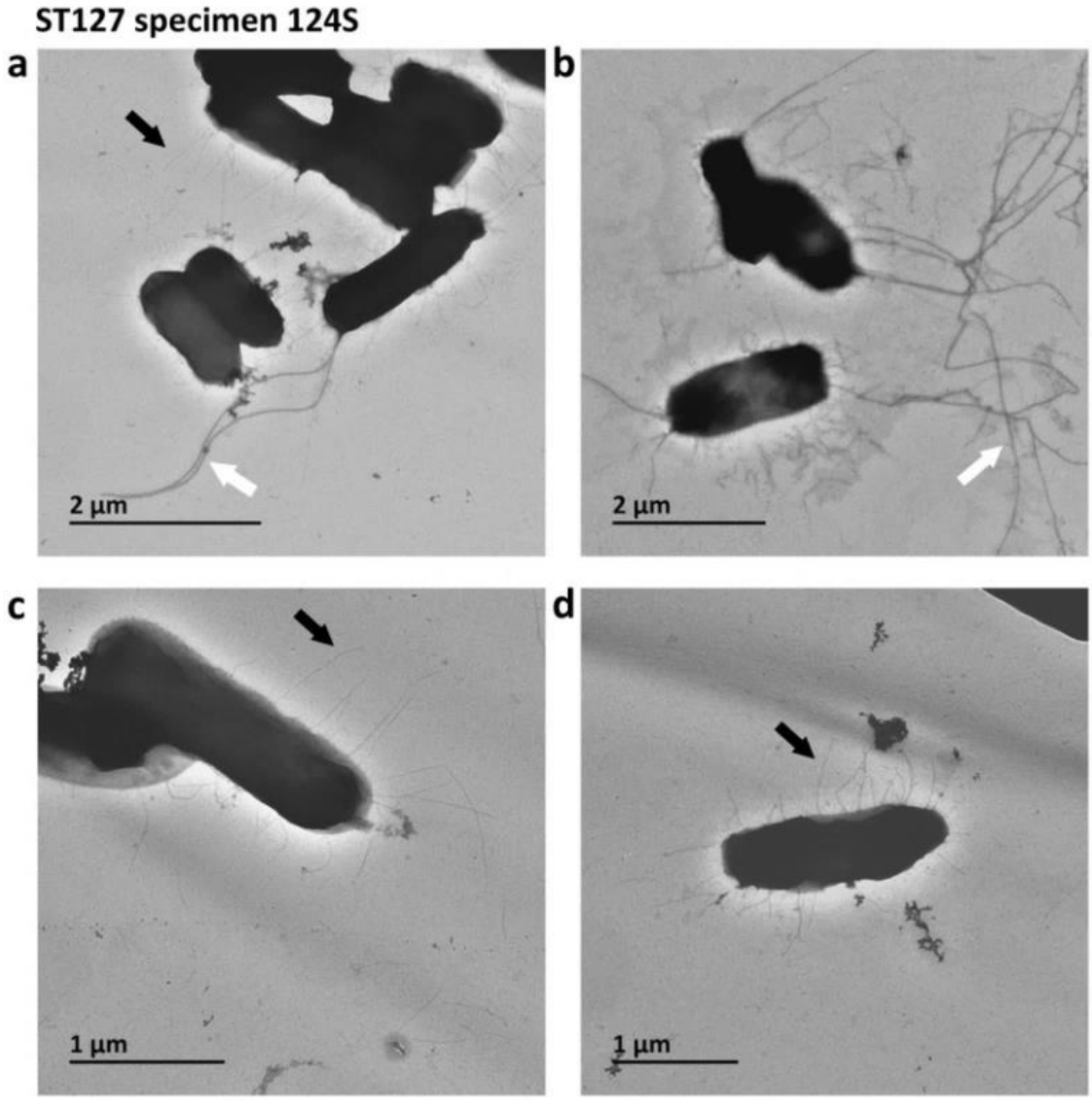

Figure 41. Transmission electron micrograph of ST127 specimen 124S. Images taken after three days static growth. Image a and b displays multiple flagella (white arrows) structures appearing mainly from the polar regions. Image c and d display peritrichous fimbriae (black arrows).

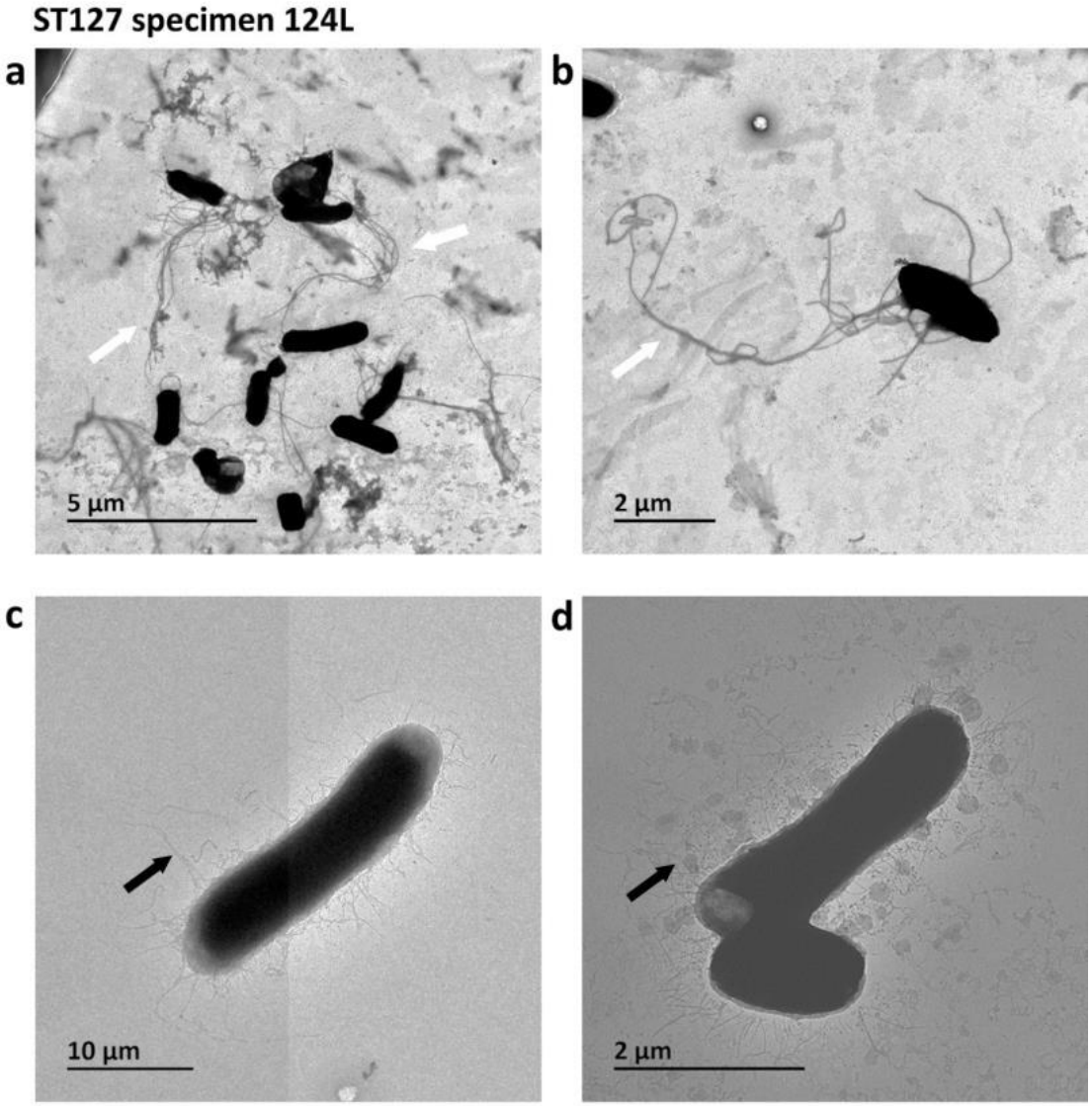

Figure 42. Transmission electron micrograph of ST127 specimen 124L. Images taken after three days static growth. Image a and b displays multiple flagella structures (white arrows) emanating from multiple cell membrane positions. Image c and d displays peritrichous fimbriae (black arrows).

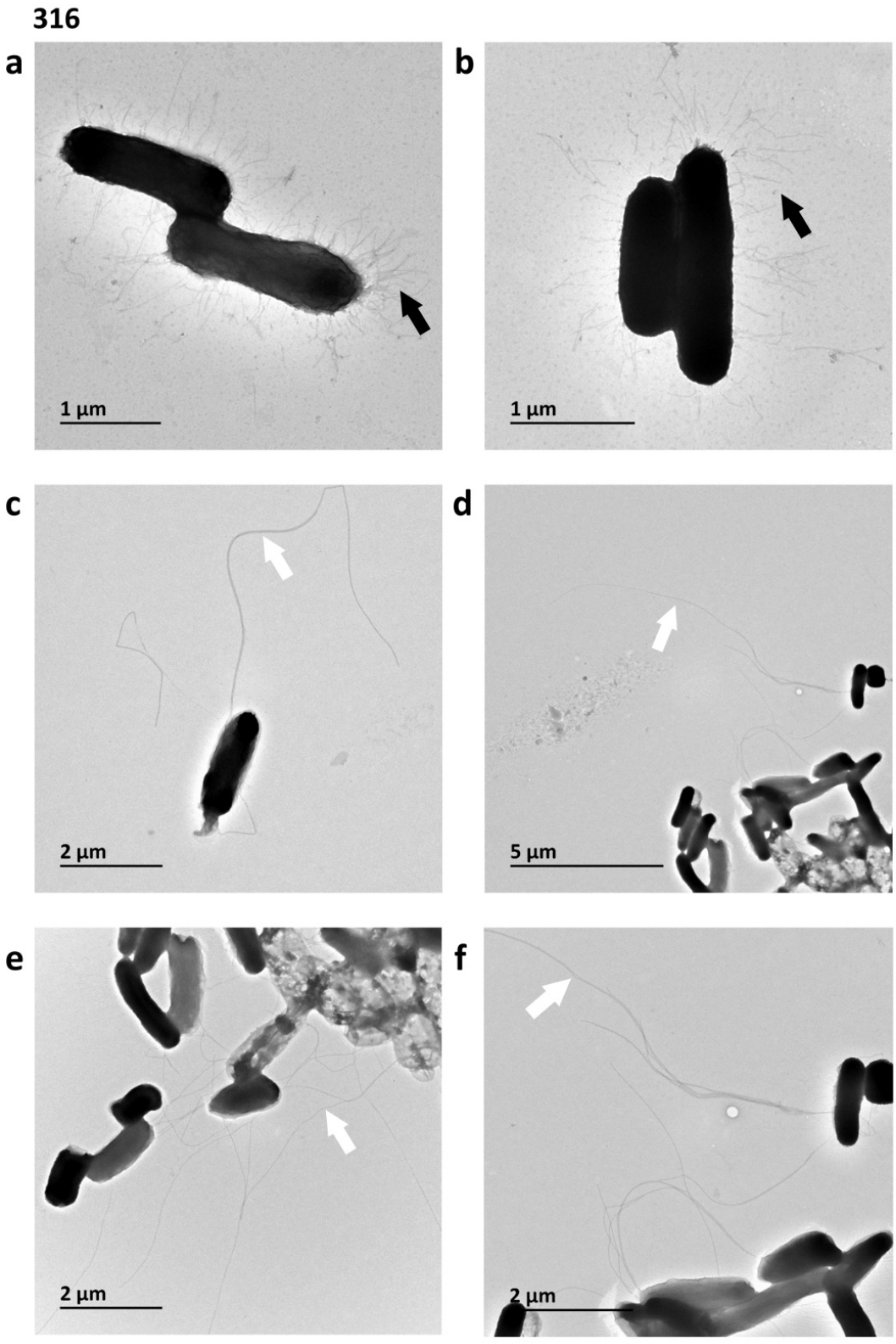

Figure 43. Transmission electron micrograph of ST127 specimen 316. Images taken after three days static growth. Image a and b displays peritrichious fimbriae (black arrows). Image c to f displays multiple flagella structures (white arrows) emanating from multiple cell membrane positions (white arrows).

5.3.3.2 Scanning Electron Microscopy of infected HTB-9 bladder cell monolayers

Two sets of specimens were inoculated and visualised using scanning electron microscopy. In the first session, HTB-9 monolayers were inoculated with the positive invasive control (EC958) and three ST127 isolates (263AS, 124S and 124L) and each incubated for one hour. All specimen inoculations and SEM preparations were performed at the same time and the results are displayed in Figure 44 and 45. The EC958 isolate was imaged with projections bound to the membrane, presumably instigating the formation of a phagocytic cup (Figure 44a and b).

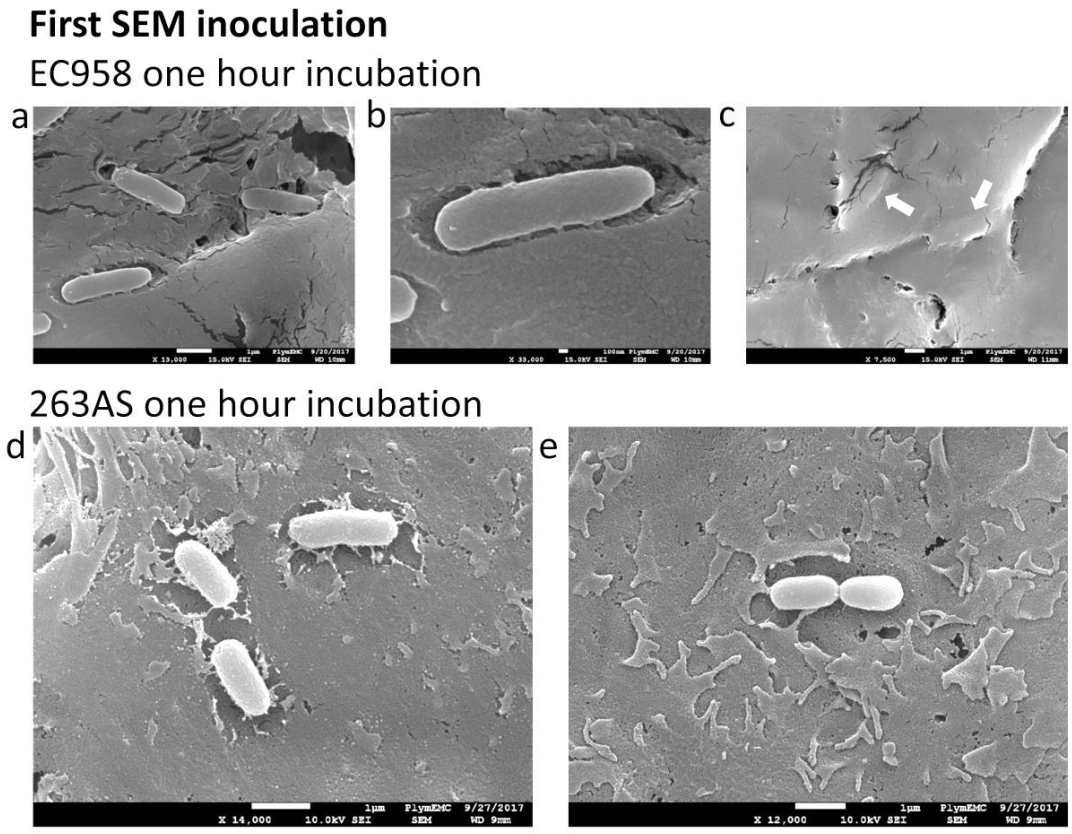

Figure 44. Scanning electron micrograph images of positive invasion control EC958 (a-c) and non-motile ST127 isolate 263AS (d and e). Image a, three EC958 bacteria bound to HTB-9 cell monolayer. Bottom left bacterium clearly initiating the production of a phagocytic cup in response to contact with HTB-9 cell membrane surface; b, zoom image of phagocytic cup formation from image a; c, bacteria embedded in HTB-9 cell membrane (white arrows); d and e, bound 263AS isolates causing a HTB-9 cell membrane reaction with a darkening of the cell membrane around the bound bacteria, but without invasion.

The EC958 isolate was also imaged embedded in the cell sheet (Figure 44c). The ST127 isolate 263 did not show any evidence to suggest invasion but did putative fimbrial binding to the HTB-9 cells that seemed to cause a reaction around the bacteria causing a change in the surface composition displayed by a darkening of the membrane around the bacteria.

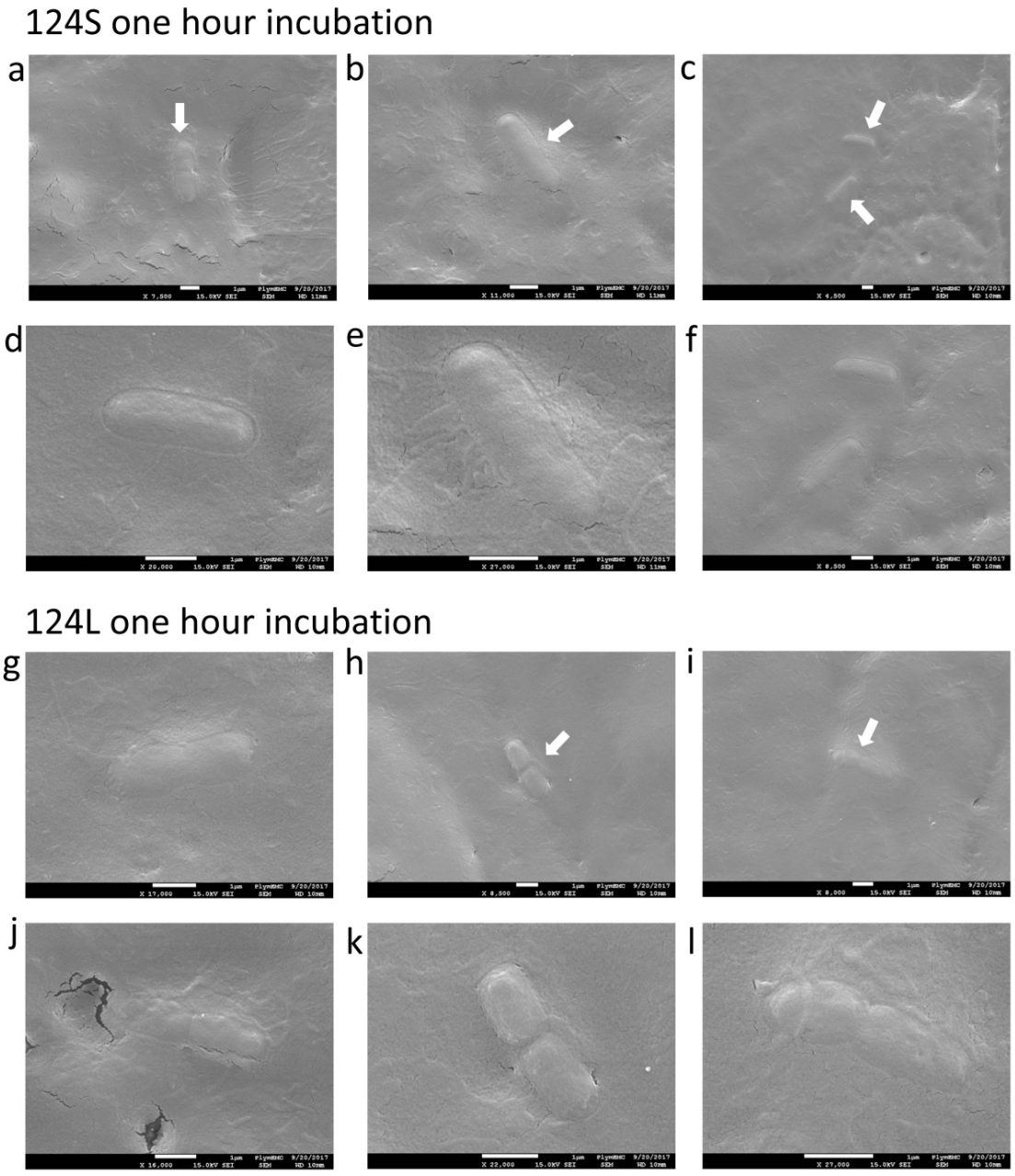

Figure 45. Scanning electron micrograph images of ST127 specimens 124S (a-f) and 124L (g-l). Bacteria embedded in the HTB-9 cell surface (white arrows). Images below b, c, h and l (e, f, k and l) are enhanced focused images of embedded bacteria.

The ST127 124S and 124L isolates showed organisms buried in the HTB-9 cell membrane. The observation was interesting and indicative of invasion, but as the topography of the HTB-9 cell sheet in the images were very smooth and not typical of the images acquired for specimens EC958 and 263AS, it was suspected that the preparations for SEM of the isolates may have been the reason for this observation.

However, as specimen preparations for the first session were performed in parallel and the same effect was observed with specimen EC958 (Figure 44c), it was necessary to confirm the observation by further testing.

The second set of SEM images involved inoculation of isolates EC958, four ST127 isolates (39, 263AS, 124S and 124L), an ST73 isolate (263TR) and the negative invasion/positive binding control C600.

EC958 seemed to display two different mechanisms of invasion. The first and well-documented method, the formation of a phagocytic cup, was identified in both the first SEM session and the second (Figure 46e). The second method of invasion seemed similar to the trigger mechanism, describing the involvement of micro villi and membrane ruffles ‘growing’ around the bacterium. Image 46a and b show examples of this phenomenon, with an organised migration of cell membrane protrusions surrounding the entire bacterium, with no evidence, in these two instances, of phagocytic cup formation. The production of micro villi/membrane ruffles was also widely observed following inoculation of this organism.

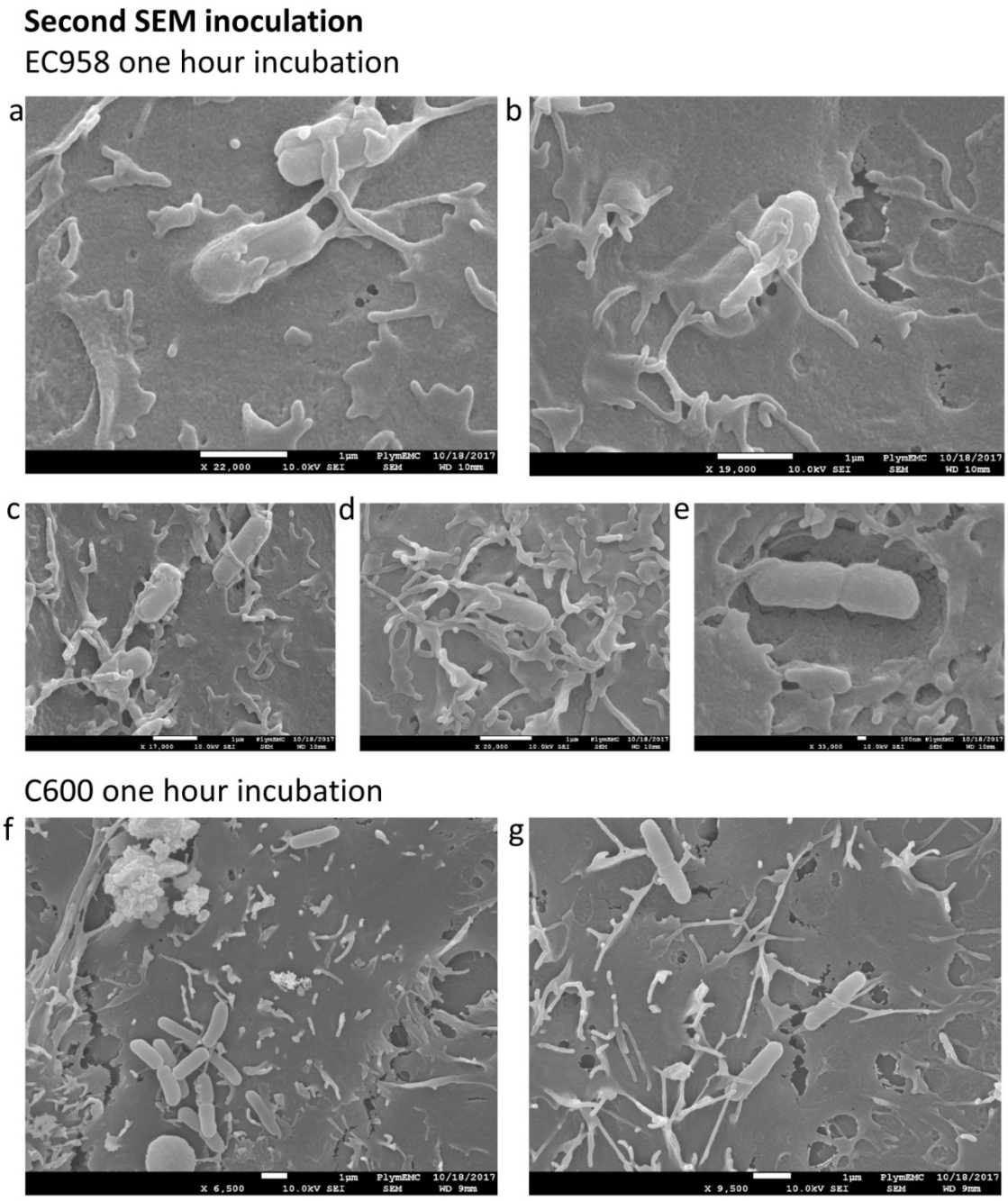

Figure 46. Scanning electron micrographs of positive invasion control EC958 (a-e) and the negative invasion/positive adherence control C600 (f and g). Images a-d, EC958 invading HTB-9 monolayer; e, formation of a phagocytic cup surrounding two EC958 bacteria; f and g, isolates of C600 bound to monolayer. The presence both isolates initiates the formation of membrane ruffles, more so with the EC958 isolate.

The non-invasive control C600 (Figure 46f and g) displayed abundant binding to the cell monolayer and long micro villi was produced by the HTB-9 cell membrane. Although there was some slight evidence from the gentamicin

assay results that this organism was capable of invasion, no evidence was observed using SEM.

The two ST127 specimens 39 and 263AS produced no evidence of invasion (Figure 47a-f), but did produce abundant binding to the cell membrane. However, both isolates seemed to initiate the formation of micro villi on the tissue surface indicating a positive HTB-9 cell reaction to bacterial binding.

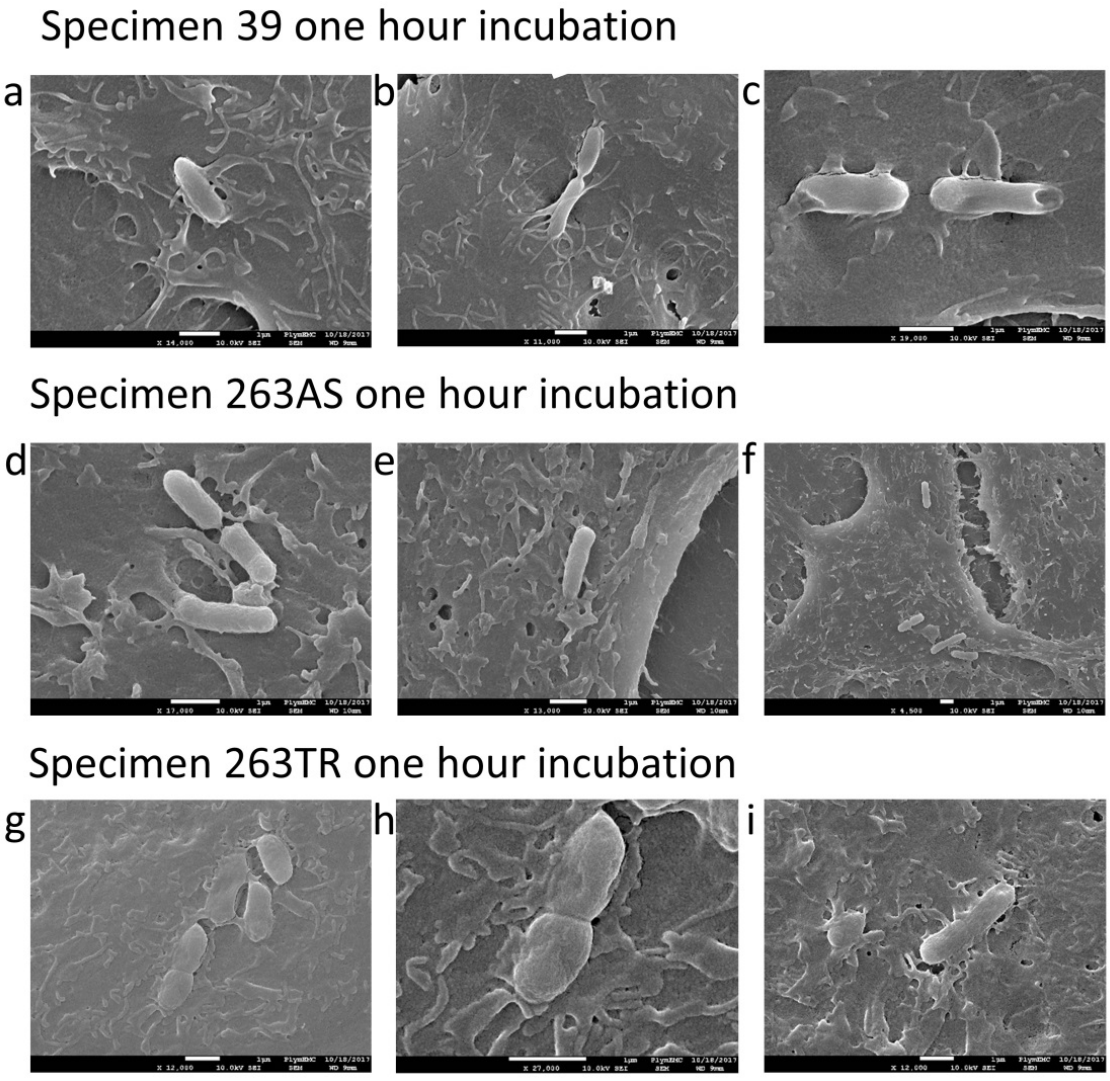

Figure 47. Scanning electron micrograph images of two ST127 isolates (39 and 263AS- images a-c and d-f, respectively) and one ST73 isolate (263TR – images g-i). Specimen 39 and 263AS both exhibited the ability to bind to HTB-9 monolayers, with no evidence of invasion. Specimen 263TR also bound to cell membrane with observations suggesting the possibly HTB-9 of invasion with the bacteria embedded in the membrane possible at the start of an invasion process.

Although specimen 263TR (ST73) was only used for some initial test stages of the gentamicin assay (data not shown), the organism was inoculated and processed for SEM for the purpose of comparison (Figure 47g-i). The 263TR isolate displayed a shorter and fatter bacterial morphology than the other UPEC isolates and binding initiated the production of micro villi. No evidence of invasion was observed but the organism was abundant on the HTB-9 cell surface and did display a proportion of the bacteria embedded in the cell membrane.

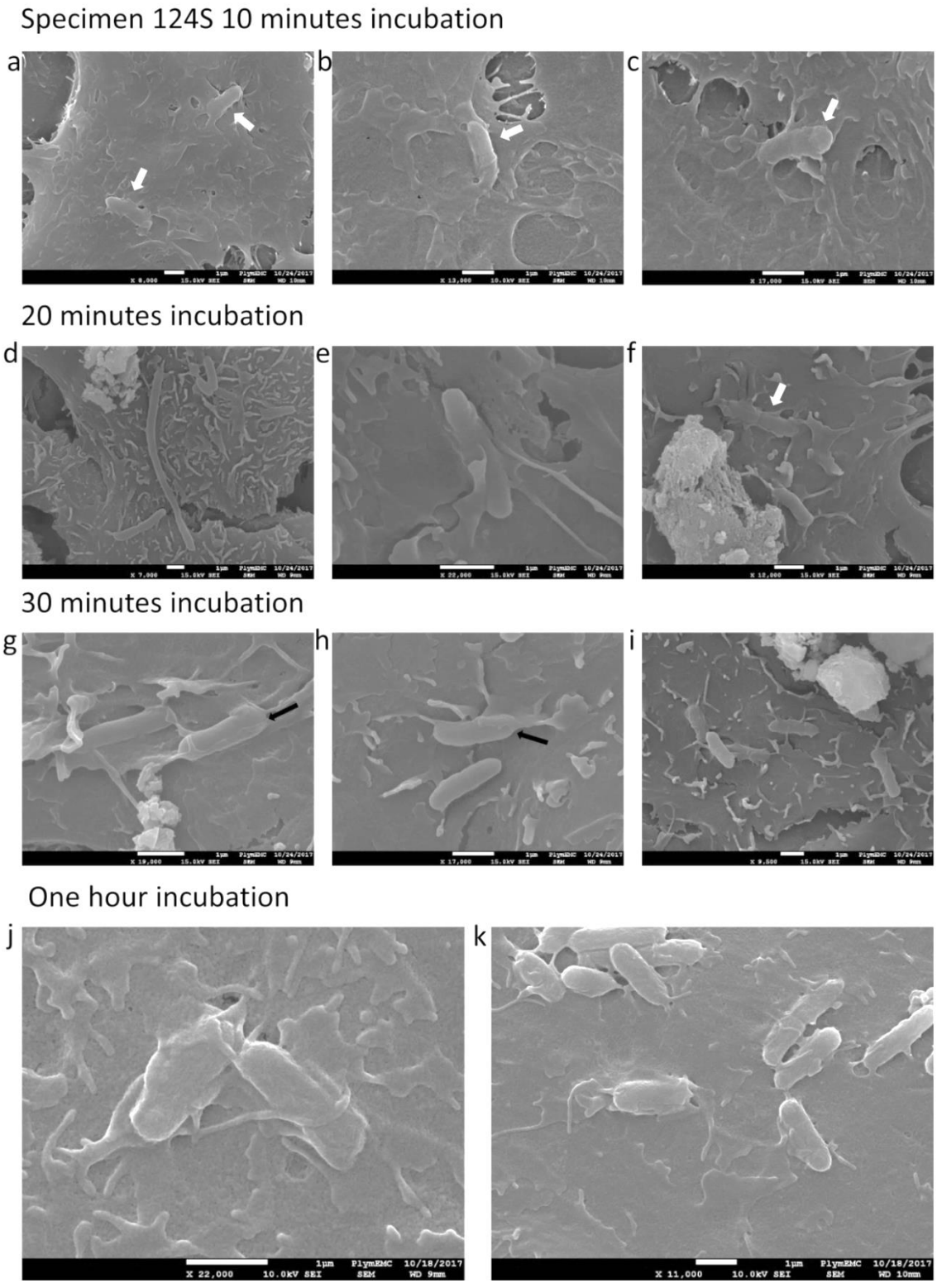

Figure 48. Scanning electron micrograph of ST127 specimen 124S after 10 minutes incubation (a-c), 20 minutes incubation (d-f), 30 minutes incubation (g-i) and one hour incubation (j and k). Evidence of bacterial invasion (white arrows) was observed as early as ten minutes incubation. Membrane ruffles were observed (black arrows) to be in the process of manoeuvring over the bacterial cell in a process characteristic of the trigger-like mechanism of invasion. Image d displays a filamentous form of the 124S bacterium after just 20 minutes incubation.

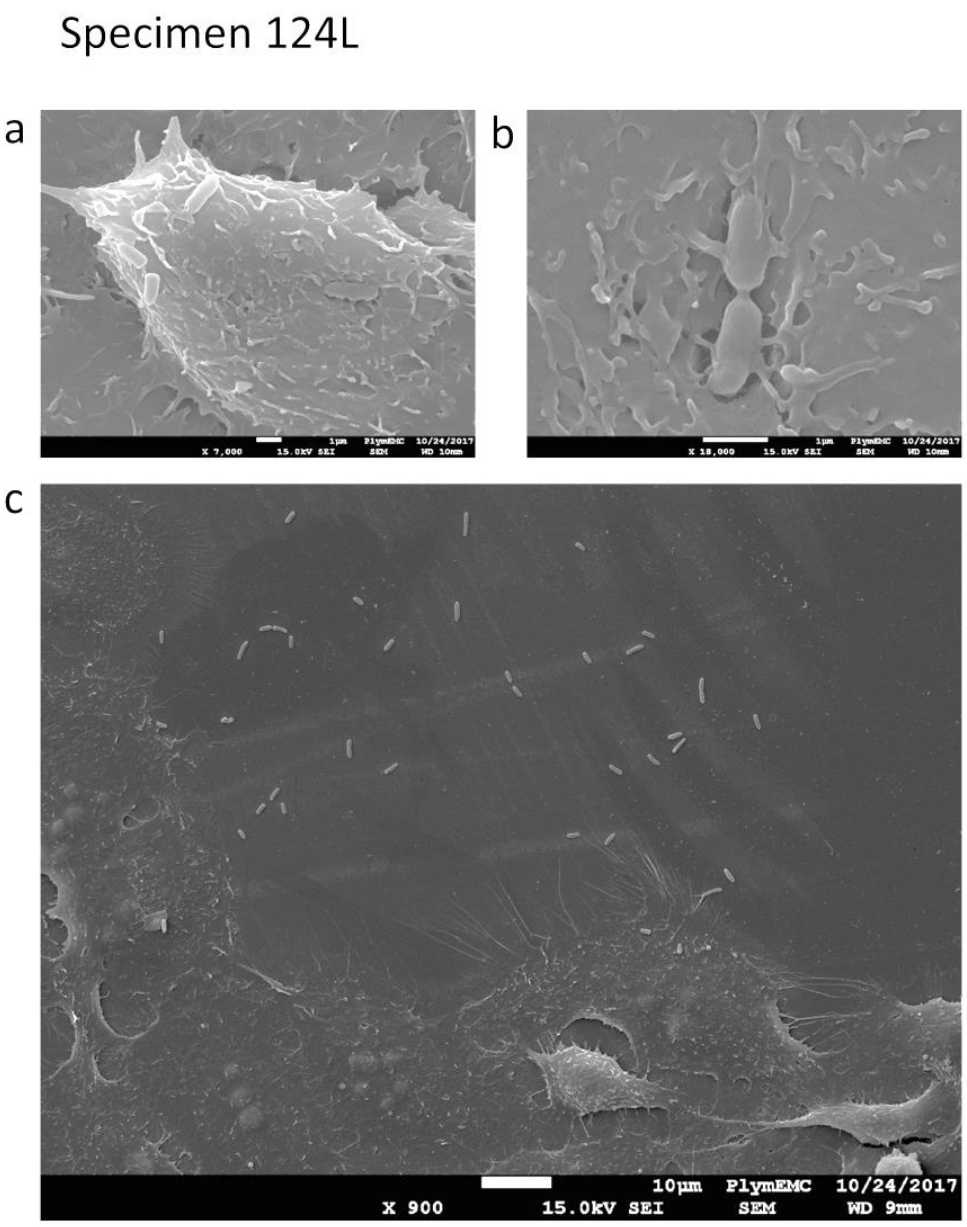

Figure 49. Scanning electron micrograph of ST127 specimen 124L after 30 minutes (a), 60 minutes (b) and 20 minutes (c). Images a and b show the beginning of invasion with the suspected formation of a phagocytic cup. Image c displays the poor binding of isolate 124L; the image was taken in an area where no bladder cells had grown and on this occasion isolate 124L had a higher affinity for the tissue culture well surface than the HTB-9 bladder cells.

The repeat inoculation of specimen 124L showed a very low level of binding for the second SEM experiment (Figure 49). Some instances were recorded with what seemed to be the isolate in the process of invasion (Figure 49a and b) but bacterial binding, in general, was extremely low. Figure 49c displays the low

binding affinity for the uroepithelial surface with 124L binding much more readily to an area of the tissue culture plate in which HTB-9 cell growth was absent. The reason for this result was unknown; however, the 124S specimen (Figure 48) displayed abundant binding and initiation of membrane ruffling. The isolate also displayed clear signs of invasion (white arrows) with as little as ten minutes incubation. The formation of a phagocytic cup was not observed but a phenomenon similar to that of specimen EC958 was observed with a migration of cell membrane protrusions surrounding and encasing the bacterium similar to observations described with the trigger mechanism of entry. Interestingly, this organism also produced a filamentous form of itself whilst bound to the HTB-9 cell membrane (Figure 48d).

5.3.3.3 Transmission Electron Microscopy of infected HTB-9 monolayers

For clear and irrefutable evidence of invasion, TEM was performed on infected HTB-9 monolayers. Isolates EC958, C600, 124L and 124S were inoculated for TEM along with the 263TR isolate for comparative purposes.

The positive control EC958 showed clear evidence of invasion (Figure 50) with bacteria distinct within the cytoplasm of HTB-9 cells (white arrows). Evidence of micro villi protruding (black arrows) from the HTB-9 membrane to surround the bacteria was also observed.

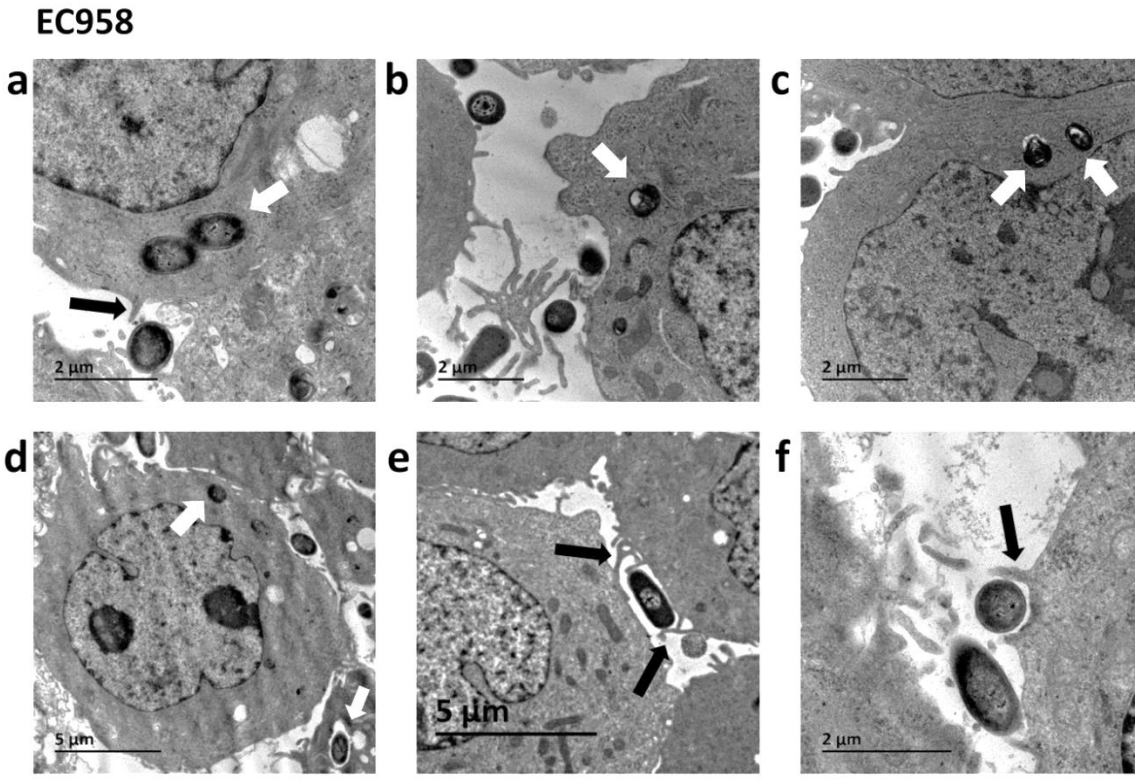

Figure 50. Transmission electron micrograph of infected HTB-9 cells with positive invasive control EC958. Images a-d display invaded bacteria (white arrows) present in the HTB-9 cytoplasm. Images e and f display the formation of micro villi (black arrows) formation around the bacterial cells.

In comparison to the EC958 positive control, isolate 124S produced many more examples of bacterial invasion (Figure 51). The images also provided evidence to support the suggestion of intracellular proliferation of this isolate (51b and e). In contrast to the EC958 isolate, the invaded 124S were consistently detected within distinct membrane-bound vacuoles.

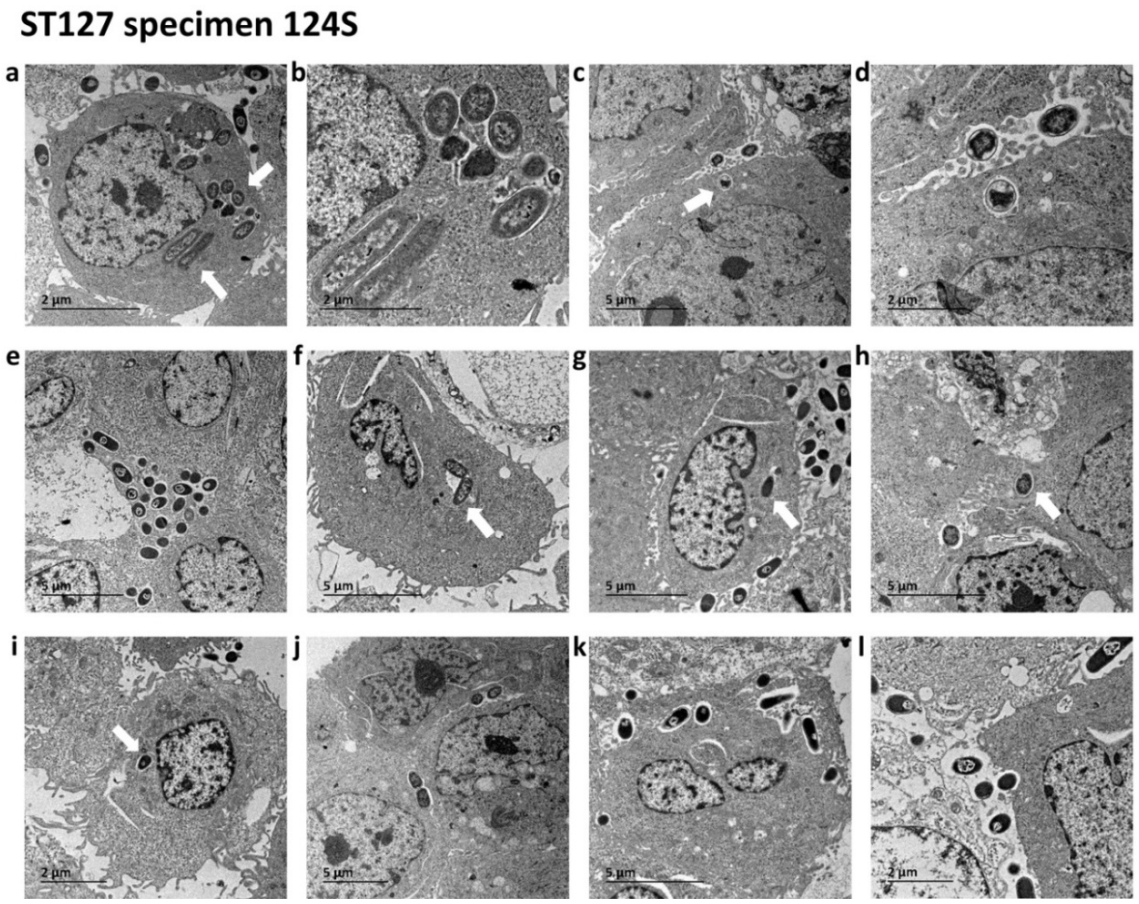

Figure 51. Transmission electron micrograph of infected HTB-9 cells with ST127 isolate 124S. Images a-i display invaded bacteria (white arrows) present in the HTB-9 cytoplasm. Image b and d are zoomed images of a and c, respectively. Images a, b, and e display evidence of intracellular proliferation after just one hour incubation. Images j-l display what may be invaded (j and k) or bacteria in the process of invasion with possible evidence of the formation of a phagocytic cup (l).

The ST127 isolate 124L also provided evidence of bladder cell invasion (Figure 52). Instances were much less abundant compared to its counterpart 124S with only one example of clear invasion observed. Defined membrane-bound vacuoles were also observed.

The ST73 isolate, 263TR (Figure 53), also displayed clear evidence of invasion and internalisation and protruding micro villi. However, only one instance of invasion by 263TR was observed.

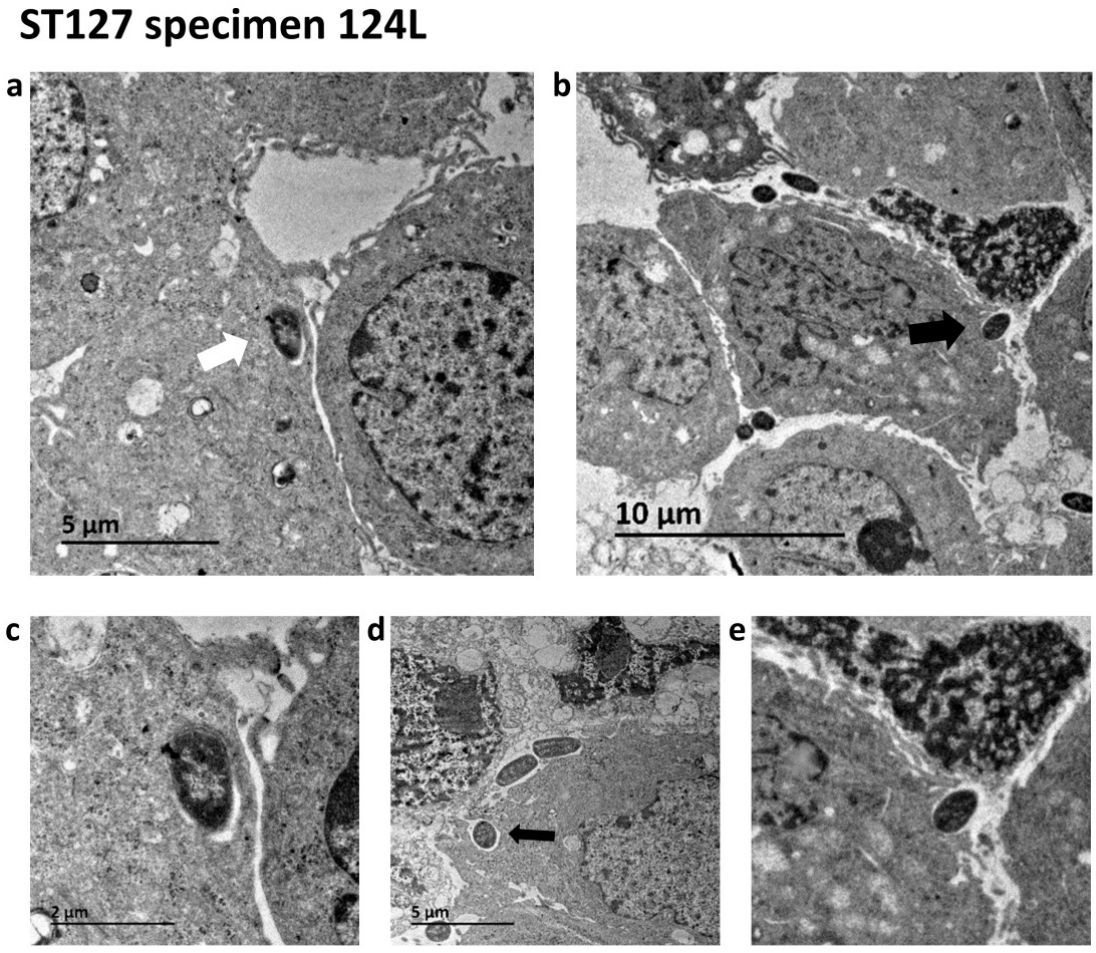

Figure 52. Transmission electron micrograph of HTB-9 cells infected with ST127 isolate 124L. Image a, c and d display invaded bacteria (white arrow), image c is a close-up image of image a. Image d displays what seems to be the final stage of bacterial invasion (black arrow). Image b and e (e enhanced image of b) displays a bacterium in the process of invasion (black arrow).

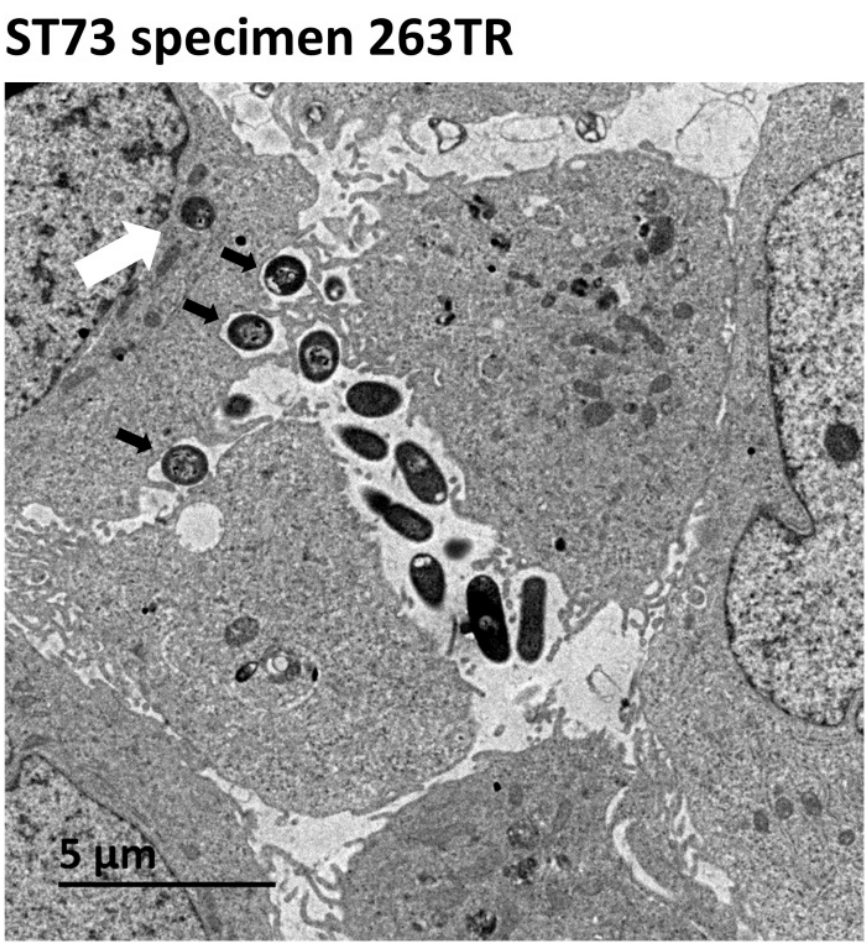

Figure 53. Scanning electron micrograph of HTB-9 cells infected with ST73 isolate 263TR that displays one bacterium within the cytoplasm (white arrow) and three bacteria in the process of invading the HTB-9 cell (black arrows) with micro villi surrounding the bacteria.

Interestingly, the C600 negative invasion/positive adherence control also provided evidence of internalisation (Figure 54). As with EC958, no distinct membrane-bound vacuole was observed surrounding the internalised bacteria, however, one instance of a C600 isolate was observed (54b and c) showing the bacteria in the process of invading an HTB-9 cell.

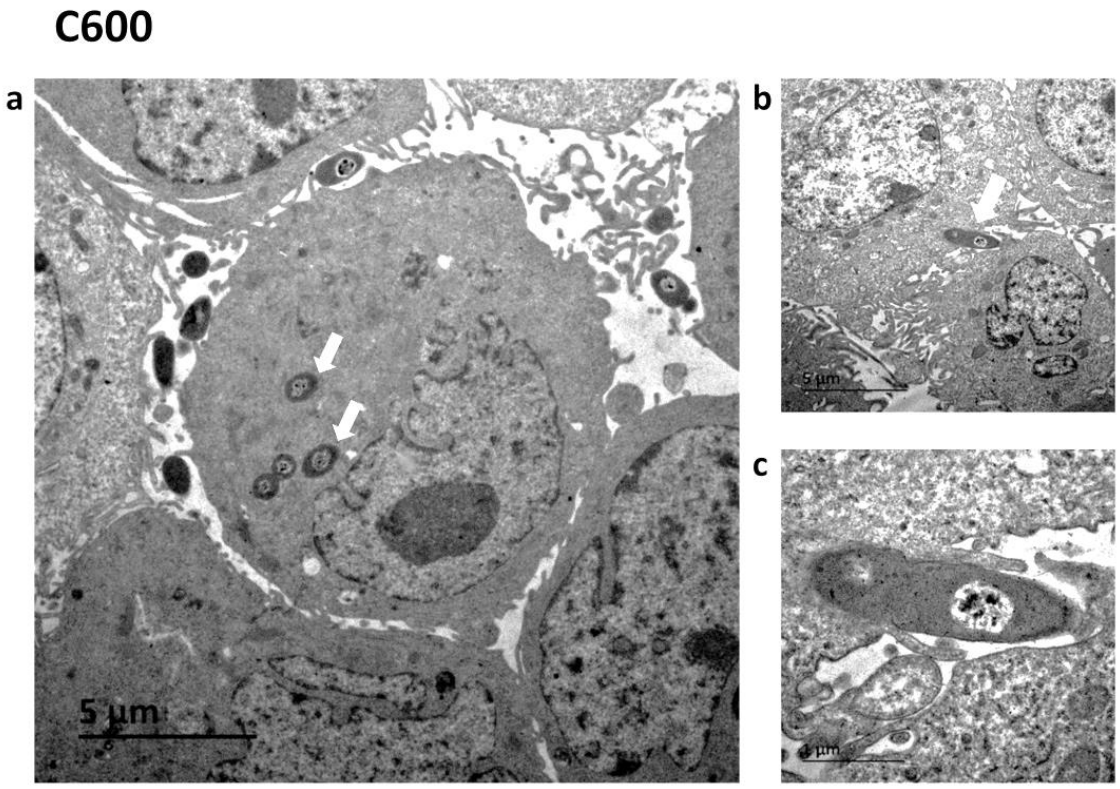

Figure 54. Scanning electron micrograph of HTB-9 cells infected with the positive binding/negative invading control organism C600. Image a displays the clear presence of four bacteria (white arrows) within the HTB-9 cytoplasm. Image b and c (c- enhanced image of b) displays the C600 bacterium in the process of invasion with distinct protrusions of micro villi on either side of the bacteria.

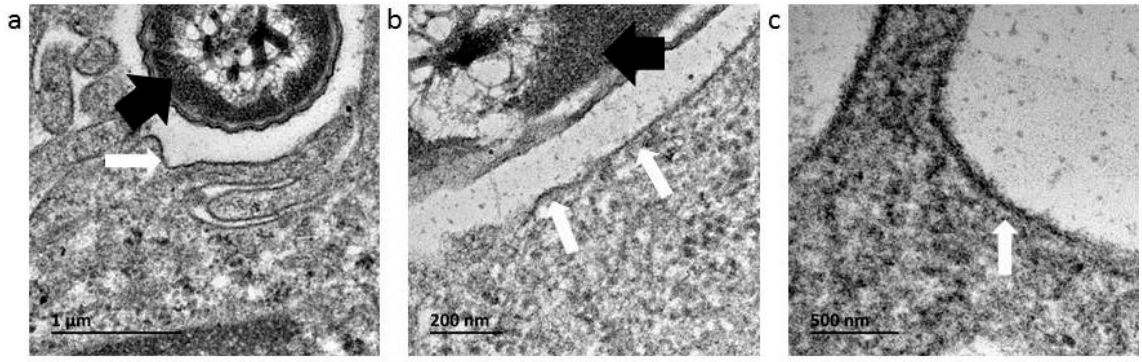

Figure 55. Examples of thickened HTB-9 outer cell membrane (white arrows) when in close contact with bacterial cells (Black arrows, images a and b) and upon the production of microvilli (c).

5.4 Discussion

5.4.1 Motility of UPEC ST127

Considering the modus operandi by which most UPEC are believed to cause uncomplicated UTI (ascending infection), it is not unreasonable to expect the majority of UPEC to be, at least moderately, motile. UPEC ST131, retains a highly motile conserved phenotype (Kakkanat *et al.*, 2015), a characteristic that may well be an essential attribute towards its success as one of the leading UPEC linages (Ben Zakour *et al.*, 2016; Kallonen *et al.*, 2017; Lau *et al.*, 2008b; Totsika *et al.*, 2011). Surprisingly, the majority of the ST127 isolates in this study were non-motile under these experimental conditions; a characteristic that may explain its reduced prevalence in most published studies. The ST127 specimens that displayed some form of motility were isolates 124S and 124L (highly motile phenotype comparable to the ST131 positive control), a sub population of 468 (non-repeatable) and an atypical growth/motility displayed by isolate 316. Appropriately, the 124 isolates were collected from a male patient; the highly motile phenotype likely to be one of the most essential virulence attributes for pathogenesis in the male gender considering the extra urethral distance a pathogen must travel to cause disease (Lane *et al.*, 2007). The 316 specimen was isolated from a 34 year old female and was considered as ‘not significant’ (low bacterial count, low WBC, mixed isolation) and the 468 isolate considered as significant, isolated from a 91 year old patient (high bacterial count, high WBC, pure isolation).

Several different types of motility have been described and categorised. Twitching motility is a flagella independent form of locomotion caused by the extension and retraction of type IV pili (Mattick, 2002) and, gliding motility is

movement along the linear axis thought to be caused by ligand binding and cytoskeleton contraction (Mignot, 2007). The most common and effective forms of bacterial motility are the swimming and swarming phenotypes, both of which phenotypes are flagella mediated and have been reported to be differentiated by a concentration of agar >0.3% to exclude swimming motility (Kearns, 2010). Various culture methods are available to determine an organism's motility (Atlas, 1996; Kakkanat *et al.*, 2015; Lane *et al.*, 2005). Also, the correct incubation temperature can often be essential for motility, previously shown to be one of the most important parameters for strains within the genera of *Campylobacter*, *Yersinia*, *Listeria* and *Escherichia* (Lam, Wheeler & Tang, 2014). The UPEC that displayed a highly motile phenotype in this study were motile at all temperatures tested, though the degree of motility did vary at different temperatures.

As this study has shown, small adjustments in the concentration of agar can have a significant impact on results. Isolates 316, 468 and the laboratory K12 strain displayed no motility with methods using concentrations of 0.4% agar (Atlas, 1996). The motile sub-population of 468 and the atypical growth/motility of isolate 316 (0.3% agar) were considered novel motility phenotypes, a result that may have possibly been overlooked in media without the addition of TTC allowing easy assessment of bacterial locomotion.

In an attempt to understand the presence or absence of motility in the ST127 isolates, genomic analysis of genes known to be involved with the structural features of flagella and their chemotaxis regulation were compared and analysed. It must be appreciated that results from this analysis may be, at times, somewhat speculative, with respect to the large number of structural and

regulatory genes involved in bacterial motility and with the presence of several mutations within the same genome; further experimentation and analysis would be necessary to confirm the impact of any mutation. That being said, the most prominent mutation, in terms of the effect on the entire gene and in relation to the motility results, was the frameshift mutation observed in the *flhB* gene. Isolates 124S, 124L and 316 all possessed a *flhB* gene homologous to the motile *E. coli* K12.

The *flhB* gene is one of six integral membrane components reported to be responsible for the export of the structural flagella components (T3SS) from the cytoplasm through the continuous cell membrane flagellum channel (McMurry *et al.*, 2004; Minamino, Imada & Namba, 2008). The FlhB protein is specifically thought to mediate the switch from hook to flagellin proteins during the growth of flagella. Null mutations in this gene (a likely outcome of an early transcribing frameshift mutation) have previously been shown to cause a severe non-motile phenotype, with reports of flagella assembly failing to proceed beyond the MS ring formation (Kubori *et al.*, 1992; Minamino, Imada & Namba, 2008; Williams *et al.*, 1996); the lack of flagella structure effectively rendering the bacterium immobile.

The hypothesis that a frameshift mutation in the *flhB* gene in ST127 UPEC isolates is the basis for the absence of flagella, is supported by the presence of flagella on specimen 316, which did not possess the frameshift mutation, but did not produce a hyper motile phenotype. The atypical growth/motility displayed by this isolate can be explained by two non-synonymous mutations in the *motA* and *cheA* genes. These genes are reported to be co-transcribed and encode the flagellar motor protein (Blair & Berg, 1990) and a chemotaxis protein kinase

(Parkinson, 1976), respectively. Isolate 316 was the only ST127 UPEC to possess these mutations and several reports have described mutations in either, or both, producing paralysed flagella (Lane *et al.*, 2005; Silverman, Matsumura & Simon, 1976). As isolate 316 produced flagella, it is hypothesised that the two non-synonymous SNP mutations in the *motA* and *cheA* either paralysed the flagella completely, seriously debilitated the torque or may have disrupted the transmission of sensory signals from the chemoreceptors to the flagellar motor (Komatsu *et al.*, 2016; Stewart, Roth & Dahlquist, 1990). It is hypothesized that the presence of flagella was the reason for the atypical growth pattern observed in this isolate, the mere presence of flagella pushing the bacteria apart in the low density agar media, culminating with more diffuse growth pattern in the 0.3% agar motility media, but displaying no diffuse growth pattern in the higher density 0.4% agar media.

At first glance this hypothesis may be easily refuted by the novel motility displayed by 468 as this strain exhibited the frameshift mutation. The motility demonstrated by specimen 468 remains a mystery. As this sub-population motile phenotype was not replicated, it is possible that the motile organism was a contaminant. However, the nature of the observed motility (from a single point and only after 16 hours incubation) suggests this organism was not a contaminant. It may be the case that the *flhB* gene SNP deletion may have been restored to one *E. coli* sub population of isolate 468 during growth within the motility media. Interestingly, isolate 468 was the only ST127 isolate to possess a non-synonymous mutation in the *flhA* gene, another member of the export apparatus in the T3SS. Both *flhA* and *flhB* are postulated to be located within the central pore of the MS ring and are the two major proteins for an

export gate/docking platform for chaperone substrate complexes (Minamino, Imada & Namba, 2008; Xing *et al.*, 2018). Although speculative, the mutation in the *flhA* gene may somehow counteract the effects of the *flhB* mutation and allow the transfer of flagella proteins under certain unknown conditions. To the best of the authors knowledge, this particular mutation has not been reported in the literature, so comparisons cannot be made with previous studies.

Using 246 ST127 genome scaffolds obtained from enterobase website, it was found that two thirds of all the genomes contained the *flhB* frameshift mutation. The genomes originated from isolates collected from across the world and, if considered as a marker for a non-motile phenotype, would suggest that most ST127 UPEC are non-motile, a possible explanation for the contrasting high virulence potential and low prevalence, taken with the understanding that the VF PCR assay does not target motility associated genes. Additionally, an NCBI blast search using the *flhB* gene with the frameshift mutation (Dec 2018) produced only one hit with an exact sequence homology. The organism in question was *E. coli* strain ECONIH2 (accession number PRJNA279612) and was identified as ST127 using the CGE MLST 2.0 programme, suggesting that this particular mutation may be specific to the ST127 lineage.

Several more non-synonymous mutations were identified in the 124 ST127 isolates including changes in the flagella M protein ring (*fliF*), ATPase (*fliI*) and another minor member of the T3SS membrane component, *fliP*. The effect of each of these mutations is unknown without further testing, however, one interesting mutation of note was observed between the two 124 isolates, an aspartic acid to glycine residue in the cognate response regulator (*uvrY* gene) of isolate 124L (Pernestig, Melefors & Georgellis, 2001).

Several studies have examined the functionality of the *E. coli uvrY* gene and its orthologues in other genera (Binnenkade, Lassak & Thormann, 2011; Goodier & Ahmer, 2001; Herren *et al.*, 2006; Mitra *et al.*, 2013; Palaniyandi *et al.*, 2012; Pernestig *et al.*, 2003; Tomenius *et al.*, 2006; Zere *et al.*, 2015). In each genus, the *uvrY* orthologue exists as a two component system (TCS); in *E. coli*, BarA-UvrY; in *Salmonella*, BarA-SirA; in *Vibrio*, BarA-VarA; and GacS-GacA in *Pseudomonas*. The TCS is involved with the phosphorylation and dephosphorylation of intracellular proteins in response to extracellular stimuli, the stimulation of the membrane associated kinase (BarA in *E. coli*) activating the cognate response regulator (uvrY) by transphosphorylation and in combination primarily functioning as a transcription regulator (Pernestig, Melefors & Georgellis, 2001).

In each of the studies mentioned, the TCS transduction system has been shown to universally regulate multiple factors inherent to the organisms' survival and pathogenicity, affecting processes including metabolism, motility, biofilm formation, virulence, persistence, invasion, survival and quorum sensing. It has been estimated that the BarA-UvrY TCS directly or indirectly regulate the transcription levels of more than 200 genes (Binnenkade, Lassak & Thormann, 2011), a process achieved by activating the transcription of genes for regulatory sRNAs, CsrB and CsrC (Zere *et al.*, 2015). The effect of mutations on this TCS in UPEC CFT073 was shown to significantly decrease the virulence of CFT073 in the mouse UTI model, cell culture assays and chicken embryo lethality assays (Palaniyandi *et al.*, 2012). The TCS mutant also reduced the production of specific virulence factors such as haemolysin and LPS.

The evidence for mutations in the TCS attenuating numerous functionalities in the bacterial genome is very convincing and goes far to explaining several features concerning the 124L isolate in comparison to its 124S counterpart. The attenuated growth in LB prior to its inoculation on to HTB-9 cells, may be due to its ineffectiveness to adapt to different carbon sources for metabolism whilst cultured in LB media. Pernestig and colleagues found that mutations in either of the TCS genes drastically affected *E. coli* survival in long term competition cultures proposed to be due to the TCS controlling the carbon storage regulatory (Csr) system, known to be the master switch between glycolysis and gluconeogenesis (Pernestig *et al.*, 2003). As LB media only has gluconeogenesis carbon sources (peptides and casein peptones), and no glycolytic substrates, such as glucose, the growth of the *uvrY* mutant 124L isolate may be hindered due to and inefficiency to utilize the carbon sources in LB media. Also the poor/reduced binding phenotype in comparison to its counterpart 124S is possibly due to a reduction in the number of specific types of adhesive fimbriae. Although this mutation did not affect motility as reported in other genera (Goodier & Ahmer, 2001), on the whole, it is reasonable to suggest that this particular pathoadaptive mutation is one that seems to be selected as strains progress towards commensalism rather than pathogenicity.

5.4.2 The uroepithelial cell binding and gentamicin protection invasion assay

Several repeat attempts at the binding and invasion assay (preliminary data not shown) often produced very erratic and unreliable data. However, a limited number of results were relatively consistent, often leading to a confident

suspicion rather than firm evidence. The positive control strain EC958 often displayed high levels of recovery, although the invasion index results for this isolate were not indicative of a highly invasive organism. The K12 *fimH* mutant negative control (N2) consistently produced poor binding and invasion recoveries. The C600 control regularly displayed strong binding to the HTB-9 cells but also, at times, produced results indicative of an invasive organism. Specimen 9 consistently displayed a poor level of binding, suggesting this isolate did not possess or express the adequate adhesive organelles required for uroepithelial colonisation, under assay conditions. Isolates 124S and 124L often displayed results indicative of an invasive pathogen, especially when no centrifugation was used.

The exceptionally low binding phenotype displayed by isolate 9 correlated with the absence of the S-pilus operon, as confirmed by genome sequence analysis. The S-pilus is known to mediate interactions with sialic acid residues (Morschhauser *et al.*, 1990), glycolipids and plasminogen (Parkkinen, Hacker & Korhonen, 1991; Prasadarao *et al.*, 1993). Sialic acid residues have also been identified as a component of the UPIII transmembrane glycoprotein present on the luminal surface as part of the uroepithelial plaque (Malagolini *et al.*, 2000). The complete absence of the S-pilus in isolate 9, whilst still retaining the FimH binding adhesin, suggests the S-pilus may have an important role for this isolate in uroepithelial colonisation. An important point of note is that isolate EC18 also lacked the sfa operon, encoding the S-pilus. Its binding capability was also consistently low, especially without centrifugation, as was the case for many ST127 isolates, but not to the degree displayed by isolate 9. Further work on this poor binding phenotype will be necessary to confirm any hypotheses,

however the role for the S-pilus in UTI may be underestimated. There is evidence to suggest that breast-fed infants can be 2-3 times less likely to develop a UTI than infants fed with formula milk (Ladomenou *et al.*, 2010; Levy *et al.*, 2009; Mansour & Mansour, 1993; Marild *et al.*, 2004; Pisacane *et al.*, 1992). One of the many protective compounds in mother's milk has been identified as human milk oligosaccharides (HMOs), a compound absent in formula milk. Amongst the many varied HMOs produced in breast milk, a proportion contain sialylated oligosaccharides. Martin-Sosa and colleagues described an increased binding of the sialylated fraction of HMOs to UPEC adhesins preventing UPEC from binding to uroepithelia (Martin-Sosa, Martin & Hueso, 2002). In the report, the authors implied that the sialylated HMOs bound to the P-fimbriae and Prs-adhesins, however, it seems more likely that S-fimbriae would be a more culpable UPEC adhesin in this scenario, suggesting a more significant role for the S-fimbriae in UTI. This hypothesis would be supported by the presence of the sfa operon in the two uropathogenic isolates used in this study (FVL 3 and FVL 25), however, sequence data was not available for these isolates.

Isolate 124S displayed clear signs of invasion. Its counterpart 124L, displayed signs of invasion on the first non-centrifugation assay, but not on the second, once again highlighting the variability of the binding and invasion gentamicin assay using bladder epithelia.

The binding and invasion assay is a procedure frequently used by researchers to measure the tissue tropism and eukaryotic cell penetration of bacterial species. The method is commonly used due to its reported simplicity, in comparison to other more specialised and expensive methods such as direct

observation by EM. As previously mentioned, recovery results for the HTB-9 assay were often erratic and non-reproducible, a situation which has probably been experienced by other users and seems to have culminated with a variety of adaptations on the protocol in an effort to obtain consistent results. Observations, during this study, of partial or fully intact cell sheets after the lysis stage coupled with an increased viscosity in parts of the removed lysis solution, led to the conclusion that the lysis solution was inadequate for this tissue type. Figure 38 (page 235) shows a direct observation of a HTB-9 infected monolayer after 30 minutes treated with 0.4% Triton X100 and the lysis solution removed (theoretically containing the invaded bacteria). As can be seen, bacteria remain embedded in the cell layer after an extensive lysis period. Although the HTB-9 cells exhibit a reaction to the Triton X100 (rounding morphology) they still remain intact. The failure of Triton X100 to adequately lyse the HTB-9 cells is likely to be explained by the composition of the cell membrane/cytoplasmic constituents. The uroepithelial bladder surface is very specialised and impermeable to many compounds, including detergents. Whilst attempting to isolate individual structures of the AUM, Laing and colleagues determined that both the plaques and the plaque hinge regions of the AUM were ‘remarkably’ insoluble to detergents (Liang *et al.*, 1999; Liang *et al.*, 2001; Wu *et al.*, 1990), indicating that the use of the detergent, Triton X100, at 0.4% would be an inadequate lysis solution.

It could be argued that laboratory cultured bladder cell monolayers do not develop a rigid structured AUM with interlocking plaques on their luminal surface as do the *in vivo* mammalian bladder cells (Sun, 2006; Wang, Liang & Kong, 2008). However, as *in vivo* uroepithelia also contain fusiform/discoid

vesicles in their cytoplasm, which contain sections of uroplankin particles used for compensatory endo/exocytosis (Duncan *et al.*, 2004; Khandelwal, Ruiz & Apodaca, 2010), it is reasonable to suspect that human bladder tissue culture cells will also have a high proportion of uroplankins present in their cytoplasm and on the membrane surface, although they may not be structured in such a uniform way. Admittedly, AUM was not observed in cultured uroepithelia using TEM, although a thickened HTB-9 cell membrane was observed in places where bacteria were in close proximity. Distinctive membranes were also observed around internalised bacteria, particularly isolate 124S. However, if this is not the case and bladder tissue culture cells do not have a high number of uroplankins as part of their structure, as seen with *in vivo* bladder cells, then a researcher must question the suitability of human bladder monolayers as a model for bladder cell invasion as many of the fimbrial adhesive binding sites are present on uroplankins.

It is strongly suspected that the sensitivity and specificity results of the binding and invasion assay in its current form (using uroepithelia and Triton X100), would be poor. The binding and gentamicin invasion assay has proved to be a useful technique with non-uroepithelial cells, however it is the author's belief that major adjustments to the lysis reagent are required to ensure the complete lysis of bladder epithelium and consistent results.

Direct observation of invading bacteria by Electron Microscopy

TEM of individual bacterial cells confirmed the presence of flagella in both the highly motile 124 isolates, isolate 316 and in the positive control EC958. The

lack of flagella was also confirmed in a small selection of the non-motile ST127 strains (9 and 39). Each strain observed by TEM possessed fimbriae and each ST127 strain was positive for the yeast agglutination test after just one day of static culture.

TEM examination of isolate 9 confirmed the presence of dense fimbriae after 3 days static growth, which further complicates the reasoning behind the poor binding phenotype. The isolate produced fimbriae that agglutinated yeast cells (as did all ST127 isolates) after only 24 hours static growth, in what is believed to be accomplished in a mannose dependant manner due to the production of the type 1 fimbriae FimH adhesin (Jann *et al.*, 1981; Martinez *et al.*, 2000). Isolate 9 also contained the same monomannose FimH type as the 124 isolates, but, unlike the other ST127, consistently displayed poor binding to the HTB-9 cells.

It may be the case that the yeast agglutination test is not discriminatory enough to determine the production of the specific type-1 FimH fimbriae, or lack thereof. It may be hypothesised that the S-pilus or other fimbriae also agglutinates yeast cells in a non-mannose dependant manner. The exact nature of the yeast agglutination would need to be further elucidated, as would what particular fimbriae were being expressed. As UPEC can possess up to 12 CU pathway fimbriae (Wurpel *et al.*, 2013) and a host of other adhesins, it may be the case that an entirely different fimbriae is being expressed or another ligand type completely is causing the agglutination.

The positive binding but non-invasive control, K12 C600, was reported by the original authors of the gentamicin Invasion assay (Elsinghorst, 1994), as non-

invasive, but was, in fact, invasive. However, *E. coli* K12 may not have been the best choice for control as some reports suggest this organism can be invasive, but not multiply intracellularly (Mulvey, Schilling & Hultgren, 2001).

The EM images presented in this study confirm the first reported incidence of an invasive ST127 UPEC isolate. ST127 has been previously reported to be a non-invasive lineage (Alkeskas *et al.*, 2015), however both variations of the hyper motile isolate 124 were invasive (particularly 124S), whereas none of the non-motile strains tested displayed any credible evidence of invasion. The invasiveness of the 124 isolates seemed to correlate with positive motility, a trait that has previously been reported for other genera to be essential for invasion (Allen-Vercoe, Sayers & Woodward, 1999; Dibb-Fuller *et al.*, 1999; Liu *et al.*, 1988; Merino *et al.*, 1997; Qin *et al.*, 2014).

Although intensely studied in other genera and other diseases, the importance of bacterial motility on the aetiology of UTIs has been largely ignored with only a few authors alluding to its importance in UTI pathogenesis (Kakkanat *et al.*, 2015; Lane *et al.*, 2007; Lane *et al.*, 2005; Pichon *et al.*, 2009). With the pathogenesis of UTI taken as a whole, it is suspected that most clinicians would accept the role of motility as a significant virulence trait of UPEC. However, the virulence factor PCR assay by Johnson and Snell (Johnson & Stell, 2000), which is probably the most widely used and most relevant UPEC virulence factor assay, does not mention or include motility in its assessment. Isolate 124S has proven to possess the highest phenotypic virulence potential of any ST127 in this study. Although still fully sensitive to all empiric antibiotics, this isolate possessed a hyper motile phenotype, was strongly adherent to bladder epithelia, invasive, showed signs of intracellular growth and was isolated from a

male; but in contrast the organism presented with the lowest virulence score of all the ST127 isolates using the virulence factor PCR assay.

It is the author's opinion that the evidence linking motility to virulence is extremely convincing. The association has been proved time and time again with many genera for many different types of infection. Evidence pertaining to the role of motility with cell invasion in *E. coli* is much more heavily weighted towards enteric disease rather than urinary disease (Giron *et al.*, 2002; Luck *et al.*, 2006; Pichon *et al.*, 2009), but with the aetiology of UTI, it is confusing why this area of research has remained only partially explored.

As with most virulence factors associated with UTI, no single one can be specifically identified as essential for pathogenesis. However, evidence from this and other studies suggests the role of flagella mediated motility should be included as one of the most important. Flagella may act in at least one of three ways, firstly to mediate UPEC proximity to bladder epithelia, secondly to aid bacterial binding to epithelia and thirdly, to act as some form of effector invasin molecule (Pichon *et al.*, 2009).

It has been shown that flagella attachment and subsequent invasion can be antigen specific depending on the H type (Kakkanat *et al.*, 2015). Additionally, active flagella may be just as important (Pichon *et al.*, 2009). There are 56 different H type variants in *E. coli*, with ST127 expressing the H31 serotype (possibly in less than one third of the isolates). An interesting future study would be to determine any correlation between flagellin serotype and uroepithelial invasion, once an adequate lysis solution has been validated for the gentamicin invasion assay.

An interesting hypothesis may be that broken flagella exposing their inner conserved region, may act as a mechanism of adherence and/or invasion. Its well known that flagella are delicate crystalline structures that are easily and often broken (Evans *et al.*, 2013). The incredible speeds that flagella rotate could possibly ensure some breakage upon contact with host cells. Paradis and colleagues observed that broken flagella had a tendency to stick to the coverslip and the bacterial cell body when performing flagella regrowth experiments (Paradis *et al.*, 2017). Roy and colleagues demonstrated the role of putative adhesins belonging to the two-partner secretion (TPS) family, reporting that the exoprotein, EtpA, which specifically binds to the surface of intestinal cells also bound to the, usually inaccessible, conserved region of the flagellin structure. The resulting flagella/EtpA complex formed a binding bridge between the flagella and the epithelial surface. The author believes that further study on UPEC in respect to their motility, H antigen type and their direct or indirect role in uropathogenesis, is justified.

As well as possessing a highly motile phenotype and the ability to invade HTB-9 cells, the ST127 124S isolate was observed under SEM to also have the ability to produce a filamentous form. This was an interesting observation, originally reported by Mulvey in 2001 (Mulvey, Schilling & Hultgren, 2001) and further examined by Justice and colleagues (Justice *et al.*, 2004; Justice *et al.*, 2006) whilst observing stages of IBC development with UPEC strains UTI89 and NU14. Both authors observed filamentous forms of UPEC upon eruption of an IBC, the earliest observation recorded at 6 hours post inoculation (Mulvey, Schilling & Hultgren, 2001). A filamentous form of ST127 isolate 124S was observed after just 20 minutes post inoculation. Whether this unusual

morphology occurred extremely quickly, in response to contact with the uroepithelia or during static LB growth, is unknown. Although no filamentous forms of bacteria were observed with the 124S isolate whilst observing individual bacterial using TEM, it may still be considered more likely that the filamentous form occurred during static growth. Irrespective of this, the ability to form an elongated morphology may be considered as another virulence trait of this isolate. Using time-lapse fluorescence video microscopy, Justice and colleagues demonstrated that filamentation may confer an increased resistance to polymorphonuclear leukocytes (PMN) (Justice *et al.*, 2004). PMNs are the first line of innate immune response to bacteria invading the bladder, recruited from the bloodstream and quickly migrating to the site of UPEC attachment, phagocytosing bacteria and destroying them with digestive enzymes (Justice *et al.*, 2006). PMNs were observed phagocytosing regular bacillus shaped UPEC but moving over filamentous forms of UPEC (Justice *et al.*, 2004). Unfortunately, how elongated UPEC are able to circumvent the PMN attack is not completely understood and further study is required. Additionally, filamentous forms of UPEC may also aid bacterial attachment to the uroepithelia simply by an increased number of adhesins bound to the bladder preventing displacement upon voiding.

5.4.2 Concluding remarks

Here we demonstrate that a highly motile strain of ST127 was able to invade uroepithelial bladder cells. The strain in question displayed evidence of intracellular growth and was suspected to be highly virulent. Based on these results, we hypothesized that bacterial motility may be one of, if not the most,

important virulence factor associated with UPEC. Analysis of the motility and chemotaxis genes suggested that the majority of ST127 isolates may be non-motile due to a frameshift deletion in the *flhB* gene, and consequently be the cause for the low prevalence but high virulence potential reported with this sequence type.

Chapter 6

6.1 Final conclusions

From the patient's point of view, the majority of UTIs are not often a cause of medical emergency, but the presence and recurrence can severely affect the quality of life. From the financial point of view, the burden is enormous, consultation and treatment costing billions each year. However, viewing the problem from one of the most important aspects, antimicrobial resistance, UTIs are amongst the most common infections treated with antibiotics and, therefore, serve as a major driver towards the selection of antibiotic resistant bacteria. In 2016 the most common cause of BSI was *E. coli*, in which 41% of all isolates were resistant to co-amoxiclav (Wiffen, 2018). In 2017, the chief medical officer for England reported that

"the world may be facing a postantibiotic apocalypse that could lead to the end of modern medicine as we know it".

Most likely, this opinion will be considered as alarmist by short sighted individuals, or disregarded by pharmaceutical companies as the global market for antibiotic production is worth billions of dollars per year. However, it is the responsibility of medical researchers to take heed of the growing number of resistant pathogens and respond accordingly.

The constant drive in research to better understand bacterial pathogens must be guided by the development of new alternative treatments or strategies that must mainly be aimed towards preventative measures.

This study could not conclusively determine if ST127 UPEC was isolated at a higher prevalence in polymicrobial culture, presumably due to the low numbers of antibiotic susceptible ST127 isolated from clinical collections. However, some

evidence was presented to suggest that a higher prevalence of ST127 UPEC may be present in the community. Although it was not possible during this study to answer the above question definitively, the work has provided a greater insight into the biology and genomic plasticity of members of this lineage, which may inform future studies and help to limit the negative impacts of this clone.

The design and validation of an ST127 UPEC negative screen PCR and a specific PCR, exposed new aspects of polymicrobial UTI, i.e. the presence of multiple STs in a single infection and the pathoadaptive nature of ST127. Genomic analysis of UPEC ST127 further identified the pathoadaptive features revealing the role that individual SNP mutations and IS can have on the pathogenicity of an organism in this niche. The final part of this study focused on the ability of ST127 to invade uroepithelial tissue and supplied evidence to suggest that bacterial motility could be the crucial attribute necessary for uroepithelial cell invasion.

The idea that bacterial motility is one of the most important pathogenic characteristics of UPEC has long been suspected, but never proven outright. The large proportion of all uropathogenic isolates possessing the non-motile phenotype refutes this hypothesis. However, with the hyper motile and low pathogenic potential of the leading global lineage, ST131, the author is inclined to give preference to simplicity and follow the philosophy of William of Ockham (Duignan, 2017); supportive of the theory that motility aids in ascending infection. Furthermore, it seems logical that the outermost structure of a bacterium would possess epithelial cell binding ability.

It has been proven that motility plays a crucial role in the distribution of UPEC from erupted pods (containing IBC) and in the development of pyelonephritis. Logic would suggest the role of motility in cystitis may be just as important. To that end, effective alternative treatments for patients that suffer from rUTI my lie in the development of compounds with the ability to inhibit motility (Hidalgo, Chan & Tufenkji, 2011; Ochi *et al.*, 1991; Shao *et al.*, 2013; Varga *et al.*, 2012) in an effort to reduce antimicrobial use.

During this research, the ability of ST127 to cause UTI has been investigated. It may be the case that ST127 is an extremely virulent clone, exemplified by isolate 124 with its highly motile phenotype, its ability to invade the uroepithelium and its presumptive capacity to proliferate intracellularly. However, the same 124 isolate has also shown the ability to be a highly adaptive organism; genomic alterations leading to what has been observed as a reduction of virulence with a reduced growth rate in LB media and a decreased ability to bind and invade the uroepithelium.

6.2 Future work

The design and validation of the specific ST127 UPEC PCR will allow clinical researchers to quickly detect and assess any variation in the prevalence of this lineage. Additionally, assessment of these isolates for motility may give credence to the hypothesis that the hyper-motile phenotype may correlate with an increased prevalence.

The ability of this lineage to adapt may also be an interesting focus for future research. This study has supplied some evidence of the regulatory effect IS can

have on an organism’s phenotype, highlighted by the loss of the O-antigen in at least two of the ST127 isolates. The loss of the O-antigen is reported to be a common phenomenon with UPEC isolates. It would be interesting to determine whether this loss is reversible (as suggested by some authors) or is specific to the ST127 lineage.

It is the author’s opinion that the gentamicin protection assay using uroepithelial cells, in its current form, is inadequate to reliably determine the true invasive nature of UPEC. The inability for Triton X-100 to adequately lyse the uroepithelial cells is concerning and future studies should identify a more appropriate lysis solution that can completely degrade the uroplankin structure whilst leaving the bacterial isolates unaffected.

Appendices

Appendix I

List of all specimens and information collected from Derriford hospital during the polymicrobial study (Chapter 2). Abbreviations: ID, Anonymous Identifier; MS2/3, Maldi-TOF identification score (≥1.8 acceptable) for organism 2 and 3; G, Gender; Loc, Location; Spe, Specimen type; WBC, White Blood Cell; Epi, Epithelial Cell; Amo, Amoxicillin; Cef, Cefpodoxime; Cep, Cephradine; CoA, Co-Amoxiclav; Lev, Levofloxicin; Nit, Nitrofurtantoin; Tri, Trimethoprim; Sc, Scanty; (numbers), Numbers of colonies; BH, Beta haemolytic; NBH, Non-Beta haemolytic; U, Urine; CSU, Catheter Specimen Urine; Uro, Urostomy Urine; In, Inpatient; Out, Outpatient

ID	E. coli	Organism 2	MS2	Organism 3	MS3	Age	G	Loc	Spe	WBC	Epi	AMO	CPD	CEP	AMC	LEV	NIT	W
1	2++	Ent. faecalis 2++	2.3	Staph epidermidis Sc(5)	2.1	74	F	In	U	2++	2++/3+++							
2	3+++	E. coli 2++		Ent. faecalis 1+	2.3	95	F	In	U	3+++	2++/3+++	S	S	S	S	S	S	S
3	3+++	Ent. faecalis 3+++	2.1	Staph haemolyticus Sc (3)	2.3	62	F	In	U	3+++	2++/3+++							
4	3+++	Kleb. pneumoniae 3+++	2.4			88	M	In	U	3+++	NS	R	S	S	S	S	S	S
5	3+++	G Sc(2)				86	F	In	U	2++	NS	R	S	S	R	R	S	R
6	3+++	Candida tropicalis 2++	2			76	F	In	CSU			R	R	R	R	R	S	S
7	Sc (2)	Kleb. oxytoca Sc(7)	2.3			55	F	In	CSU									

8	3+++					86	F	In	U	3+++	1+/-	S	S	S	S	S	S	S
9	2++	G 2++				69	F	In	U	3+++	2++/3+++	R	S	S	R	S	S	S
10	3+++	Strep agalactiae 1+	2.5			64	F	In	U	3+++	2++/3+++	R	S	S	S	S	S	S
11	2++	Ent. faecalis 3+++	2.3			29	F	In	CSU									
12	3+++	Ent. faecalis 2++ (BH)	2.4	Ent. faecalis 2++ (NBH)	2.3	69	F	In	U	3+++	1+/-	S	S	S	S	S	S	S
13	3+++					52	F	In	CSU			S	S	S	S	S	R	S
14	3+++					8	F	Out	U	3+++	1+/-	S	S	S	S	R	S	R
15	3+++					55	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
16	3+++					78	F	Out	U	3+++	2++/3+++	R	S	S	S	S	S	S
17	2++	Ent. faecalis 3+++	2.3	Staph hominis 1+	2.2	26	F	Out	U	2++	2++/3+++							
18	3+++	Ent. faecalis 3+++	2.2			78	F	Out	U	3+++	2++/3+++							
19	3+++	G Sc(1)				77	F	Out	U	3+++	2++/3+++	R	S	S	S	S	S	R
20	3+++	Kleb. pneumoniae 3+++	2.4			8	F	Out	U	1+/-	1+/-							
21	3+++	Kleb. oxytoca 3+++	2.4			26	F	Out	U	2++	2++/3+++	R	S	S	S	S	S	S
22	2++	Ent. faecalis 3+++	2.4			71	F	In	CSU									
23	2++					65	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
24	3+++	Staph haemolyticus Sc(1)		Staph haemolyticus Sc(2)	2	83	F	In	U	2++	2++/3+++	R	S	S	S	R	S	R
25	3+++					61	F	In	U	2++	1+/-							

26	3+++					82	M	In	U	3+++	1+/-	R	S	S	S	S	S	R
27	3+++					74	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
28	3+++					96	M	In	U	3+++	1+/-	R	S	S	R	S	S	R
29	3+++					8	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
30	3+++					33	F	Out	U	2++	1+/-	S	S	S	S	S	S	R
31	3+++	Ent. faecalis Sc (3) (BH)	2.2	Ent. faecalis Sc (NBH)	2.2	70	F	Out	U	2++	2++/3+++	R	S	S	R	S	S	R
32	3+++	Ent. faecalis Sc(3) (BH)	2.3	Ent. faecium Sc (NBH)	2.6	73	F	Out	U	3+++	1+/-	R	S	S	S	R	S	S
33	3+++					28	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
34	Sc	Strep lutenensis 3+++	2.3			16	F	Out	U	3+++	2++/3+++	S	R	R	S	S	S	R
35	3+++					6	F	Out	U	3+++	1+/-	S	S	S	S	S	S	R
36	Sc (3)					34	F	Out	U	3+++	2++/3+++							
37	3+++					30	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
38	3+++	Ent. faecalis Sc(5)	2.3			48	F	Out	U	3+++	2++/3+++	R	S	S	S	S	S	S
39	3+++	Ent. faecalis Sc(1)	2.3			35	F	Out	U	3+++	2++/3+++	R	S	S	S	S	S	S
40	3+++					64	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
41	3+++					30	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
42	2++	Strep agalactiae 1+	2.3			69	F	Out	U	3+++	1+/-	R	R	R	R	R	S	R
43	Sc (8)					3	F	Out	U	2++	1+/-							
44	2++	Citro freundii Sc(2)	2.3	Staph epidermidis 2++	2.2	68	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S

45	2++	G Sc				85	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
46	3+++					66	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
47	3+++	Ent. faecalis Sc(5)	2.3			59	F	Out	U	3+++	1+/-	R	S	S	R	S	S	R
48	3+++					78	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
49	3+++					74	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
50	3+++	G Sc(1)				68	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
51	3+++	Green Ecoli 3+++				90	F	Out	U	3+++	2++/3+++	S	S	S	S	R	R	R
52	3+++					65	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
53	3+++					91	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
54	2++					25	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
55	3+++					96	F	Out	U	3+++	1+/-	S	S	S	S	R	S	R
56	3+++	Ent. faecalis 3+++	2.3			91	M	Out	U	3+++	1+/-							
57	1+	Ent. faecalis 1+				68	F	Out	CSU									
58	3+++	Kleb. pneumoniae 2++	2.4			74	F	In	U	3+++	1+/-	R	S	S	S	S	S	S
59	3+++					63	M	Out	U	3+++	1+/-	R	S	S	S	S	S	S
60	3+++	G Sc(1)				78	F	Out	U	2++	2++/3+++	R	R	R	R	S	S	S
61	3+++	G 1+				65	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
62	3+++	E. coli 3+++	2.2	Ent. faecalis 3+++	2.4	14	F	Out	U	3+++	2++/3+++							

63	3+++	Strep sciuri Sc(8)	1.8	Staph haemolyticus Sc(6)	2.1	79	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
64	3+++					79	M	Out	U	3+++	1+/-	R	S	S	S	S	S	R
65	3+++					42	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
66	2++	Strep agalactiae Sc(1)	2.4			57	F	Out	U	1+/-	2++/3++	R	S	S	S	S	S	S
67	3+++					68	F	Out	U	3+++	1+/-	S	S	S	S	S	S	R
68	3+++					40	M	Out	U	3+++	1+/-	R	S	S	S	S	S	R
69	3+++					81	F	Out	U	3+++	1+/-	S	S	S	S	S	R	S
71	2++					63	M	Out	U	3+++	2++/3++	R	S	S	S	S	S	S
72	3+++	Ent. faecium 1+	2.4			81	F	In	U	3+++	2++/3++	R	R	R	R	R	S	R
73	3+++					98	F	In	U	3+++	1+/-	R	S	S	S	S	S	R
74	3+++	Bac lichenformis Sc(1)	1.71			91	F	In	U	3+++	1+/-	S	S	S	S	S	S	S
75	Sc(5)					6	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
76	Sc	Ent. faecalis 3+++	2.21			14	F	In	U	2++	2++/3++	S	R	R	S	S	S	R
77	3+++					66	F	In	U	3+++	2++/3++	R	S	S	R	S	S	R
78	3+++					66	F	In	U	3+++	1+/-	R	S	S	S	S	S	S
79	2++	Strep gallolyticus Sc	2			54	F	In	U	2++	1+/-	R	S	S	S	S	S	S
80	Sc(1)	Strep anginosus Sc(7)	2			38	F	In	U	3+++	1+/-							

81	3+++					79	M	Out	U	3+++	1+/-	R	R	R	R	S	S	S
82	3+++					76	F	In	U	3+++	1+/-	R	R	R	R	S	S	S
83	3+++	Kleb. pneumoniae Sc(7)	2.4			65	F	In	U	3+++	1+/-	R	S	S	S	S	S	S
84	3+++					74	F	In	U	3+++	1+/-	R	S	S	S	S	S	S
85	2++					63	M	In	U	3+++	1+/-	S	S	S	S	S	S	S
86	3+++					80	F	In	CSU			R	S	S	R	R	R	R
87	3+++	Ent. faecalis 1+	2.3			72	F	In	U	3+++	2++/3+++	R	S	S	S	S	S	R
88	Sc(7)					61	M	In	CSU			S	S	S	S	S	S	S
89	Sc(1)	Ent. faecalis 2++	2.3			69	F	In	U	3+++	2++/3+++	S	R	R	S	S	S	R
90	3+++					61	M	In	U	2++	1+/-	S	S	S	S	S	S	S
91	3+++					90	F	In	U	1+	1+/-	S	S	S	S	R	S	S
92	3+++					79	M	In	U	3+++	1+/-	R	S	S	S	S	S	R
93	3+++					69	F	In	U	3+++	1+/-	S	S	S	S	S	S	S
94	3+++	Ent. faecalis 1+	2.3			19	F	In	U	2++	2++/3+++	R	S	S	S	S	S	R
95	3+++	Kleb. oxytoca 2++	2.2			55	F	In	Uro	3+++	1+/-	S	S	S	S	S	S	R
96	3+++	G 3+++ (No reliable ID)		Staph simulans 1+	2.1	81	F	In	U	2++	2++/3+++							
97	3+++	Ent. faecalis Sc(2)	2.2			90	F	In	U	3+++	2++/3+++	S	S	S	S	S	S	S
98	2++	E. coli	2.3			28	F	In	CSU			R	S	S	S	R	S	S
99	3+++	Ent. faecalis Sc(1)	2.2			98	F	In	U	3+++	2++/3+++	R	S	S	S	S	S	R

100	3+++	Kleb. pneumoniae 3+++	2.4	Ent. faecalis 3+++	2.3	73	M	Out	CSU									
101	3+++					29	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
102	3+++					41	F	Out	U	2++	1+/-	R	S	S	S	S	S	R
103	3+++	Ent. faecalis Sc(1)	2.1			68	M	Out	CSU			S	S	S	S	S	S	S
104	3+++					89	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
105	3+++					54	F	Out	U	3+++	1+/-	R	R	R	S	S	S	R
106	3+++	Ent. faecalis Sc(1)	2.3			76	F	Out	U	3+++	1+/-	R	S	S	R	S	S	R
107	Sc(3)	Ent. avium Sc(2)	2.2	Staph haemolyticus Sc(1)	2.1	7	F	Out	U	2++	1+/-							
108	3+++	Ent. faecalis Sc(1)	2.1			35	F	Out	U	2++	2++/3++	S	S	S	S	S	S	S
109	3+++					60	F	Out	U	3+++	2++/3++	S	S	S	S	S	S	S
110	3+++	Ent. faecium Sc(2)	2.2			79	F	Out	U	3+++	1+/-	R	S	R	S	S	S	R
111	1+					71	M	Out	U	3+++	1+/-	R	S	S	S	S	S	R
112	3+++	Ent. faecalis Sc(5)	2.2			80	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
113	2++	Serr liquefaciens 3+++	2.4			3	F	Out	U	2++	1+/-							
114	3+					52	M	Out	U	2++	1+/-	R	S	S	S	S	S	S
115	Sc(7)					32	F	Out	U	2++	2++/3++	S	S	S	S	S	S	S
116	3+++	Ent. faecalis 1+	2.1			61	F	Out	U	2++	2++/3++	S	S	S	S	S	S	S

117	3+++	Ent. faecalis Sc(3)	2.1			63	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
118	3+++	Ent. faecalis 2++	2.3			72	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
119	3+++					68	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
120	3+++					5	F	Out	U	1+	1+/-	S	S	S	S	S	S	S
121	3+++					49	F	Out	U	1+	2++/3++	S	S	S	S	S	S	S
122	3+++					71	F	Out	U	3+++	1+/-	S	S	S	S	S	S	R
123	3+++					35	F	Out	U	3+++	2++/3++	S	S	S	S	S	S	S
124	3+++					82	M	Out	U	3+++	1+/-	S	S	S	S	S	S	R
125	3+++					68	F	Out	U	3+++	1+/-	R	S	S	S	R	S	R
126	3+++					36	F	Out	U	2++	2++/3++	R	S	S	S	S	S	S
127	3+++	Ent. faecalis 1+	2.3			81	M	Out	U	2++	1+/-							
128	3+++					84	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
129	3+++					89	F	Out	U	3+++	1+/-	R	R	R	R	S	S	S
130	3+++	Staph epidermidis 1+	2.1			29	F	Out	U	2++	2++/3++	R	S	S	S	S	S	S
131	3+++					89	F	Out	U	3+++	2++/3++	S	S	S	S	S	S	S
132	3+++	G 2++				19	F	Out	U	2++	2++/3++							
133	3+++	G Sc(1)				84	F	Out	U	2++	2++/3++	R	S	S	S	S	S	S
134	3+++	Staph cohnii Sc(1)	1.75			87	M	Out	U	2++	1+/-	R	R	R	S	R	S	R

135	3+++					20	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
136	3+++					35	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
137	3+++					35	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
138	3+++	Gr Sc(2)				46	F	Out	U	1+	1+/-	R	S	S	R	S	S	R
139	3+++	proteus mirabilis 2++	2.4			68	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
140	3+++					61	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
141	2++					52	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
142	3+++	Ent. faecalis Sc(6)	2.3			73	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
143	3+++	Kleb. pneumoniae (3)Sc	2.3			77	F	Out	CSU			R	S	S	S	S	S	S
144	2++	Entero cloacae Sc(2)	2.3			85	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
145	1+	Staph aureus 3+++	2.4			49	F	Out	U	2++	2++/3+++							
146	3+++					85	F	Out	U	2++	1+/-	R	S	S	R	S	S	R
147	3+++					19	F	Out	U	2++	1+/-	R	S	S	S	S	S	R
148	3+++	Ent. faecalis 2++	2.4			85	M	Out	CSU									
149	3+++	Ent. faecalis 2++	1.75			87	F	Out	CSU			R	R	R	S	R	S	R
150	3+++	Pseu aeruginosa 3+++				98	F	Out	U									
151	3+++	Entero cloacae 3+++	2.4	Acin baumannii 3+++	2.3	94	F	Out	U	2++	1+/-							

152	3+++	Ent. faecalis 2++	2.1			78	F	Out	U	1+	2++/3+++	S	S	S	S	S	S	S
153	2++	Ent. faecalis Sc(1)	2.1			40	M	Out	U	2++	1+/-	R	R	R	R	S	S	S
154	3+++					83	F	Out	U	1+/-	1+/-	S	S	S	S	S	S	S
155	2++	Ent. faecalis 3+++	2.3			72	M	Out	U	1+	1+/-	S	R	R	S	S	S	R
156	3+++	Ent. faecalis Sc(6)	2.3	Bac simplex Sc	1.8	34	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	R
157	Sc(1)	Ent. faecalis 1+	2.3			70	F	Out	U	2++	2++/3+++	S	R	R	S	S	S	R
158	2++	Strep agalactiae 1+	2.4			20	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
159	3+++					82	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
160	3+++	G Sc(5)				36	F	Out	U	1+	1+/-	R	S	S	S	S	S	S
161	2++					10	F	In	U	3+++	2++/3+++	S	S	S	S	S	S	S
162	3+++	G 3+++				69	M	In	CSU			S	S	S	S	S	S	S
163	3+++					98	F	In	U	3+++	1+/-	R	S	S	S	S	S	R
164	3+++	Strep urinalis 1+	2.4			45	M	In	U	3+++	NS	S	S	S	S	S	S	S
165	2++					59	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
166	3+++	E. coli ++ (PINK HALO)	2.4			79	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
167	3+++	Glob sulfidifaciens 2++	2			10	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S
168	Sc(4)					68	M	Out	U	2++	1+/-	R	S	S	S	S	S	R
169	3+++	Glob sulfidifaciens	1.7			37	F	Out	U	2++	2++/3+++	R	R	R	R	S	S	S

		1+																
170	2++					79	M	Out	U	3+++	1+/-	R	S	R	S	S	S	S
171	3+++	G 2++				35	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S
172	3+++	Cory minutissimum 2++	1.8			50	F	Out	U	3+++	2++/3+++	R	S	S	R	S	S	S
173	3+++	G 1+				42	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
174	2++					33	M	Out	U	3+++	1+/-	R	S	S	S	S	S	S
175	2++					40	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
176	2++	Bac megaterium Sc(1)	2.3			55	F	Out	U	2++	1+/-	R	S	S	S	S	S	R
177	3+++					87	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
178	1+	Ent. faecalis Sc(8)	2.2			3	F	Out	U	2++	1+/-							
179	3+++					30	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
180	3+++	Ent. faecalis 1+	2.3			71	F	Out	U	2++	1+/-	R	S	S	R	S	S	S
181	3+++					43	M	Out	U	2++	1+/-	S	S	S	S	S	S	S
182	3+++					74	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
183	3+++	Entero asburiae Sc(4)	2.2			28	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
184	2++	Ent. faecalis Sc(2)	2.3			81	F	Out	U	3+++	2++/3+++	R	S	R	S	S	S	S
185	3+++					56	F	Out	U	1+	NS	S	S	S	S	S	S	S
186	1+					54	M	Out	CSU			R	S	S	S	S	S	R
187	3+++					27	F	Out	U	2++	2++/3++	S	S	S	S	S	S	S

											+							
188	1+	G 2++				28	F	Out	U	2++	2++/3++ +							
189	3+++					64	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
190	3+++					26	F	Out	U	2++	2++/3++ +	S	S	S	S	S	S	S
191	2++	E. coli Sc(1) (small col)	2.5	Entero cloacae Sc(1)	3.3	64	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
192	3+++	G 1+				9	F	Out	U	1+	1+/-	S	S	S	S	S	S	S
193	3+++					66	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
194	3+++					40	F	Out	U	3+++	2++/3++ +	S	S	S	S	S	S	S
195	3+++					82	F	Out	U	3+++	1+/-	S	S	S	S	S	S	R
196	3+++					77	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
197	3+++	Ent. faecalis Sc(7)	2.1			84	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
198	3+++	Ent. faecalis 2++	2.4			71	F	Out	U	3+++	2++/3++ +	S	S	S	S	S	S	S
199	3+++	Ent. faecalis 2++	2.3			83	M	Out	CSU			S	S	S	S	R	S	R
200	3+++					91	M	Out	U	3+++	1+/-	R	S	S	S	S	S	R
201	3+++					91	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
202	3+++					74	F	Out	U	3+++	1+/-	R	R	R	R	R	S	R
203	3+++					25	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
204	3+++	E. coli 3+++ (PINK HALO)	2.4			82	F	Out	U	1+	1+/-	R	S	S	S	R	S	R
205	3+++	E. coli 3+++ (PINK HALO)	2.4			82	F	Out	U	2++	1+/-	R	S	S	S	R	S	R

206	3+++	G 1+ (UNABLE)				77	F	In	U	1+	1+/-	R	S	S	S	S	S	S
207	Sc(3)	Staph simulans Sc(7)	2.2			76	F	In	U	2++	1+/-							
208	3+++	Staph epidermidis 3+++	2.1	Strep constellatus	2.1	90	M	In	CSU			R	S	S	S	S	S	R
209	3+++					25	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S
210	1+	Strep agalactiae Sc(1)	2.3			29	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
211	3+++	Strep agalactiae 1+	2.4			23	F	Out	U	1+	1+/-	R	S	S	S	S	S	S
212	3+++					87	F	In	U	3+++	2++/3+++	S	S	S	S	S	S	S
213	3+++	Entero cloacae 2++	2.1			46	F	In	U	2++	2++/3+++	S	S	S	S	S	S	S
214	Sc(2)	Staph epidermidis Sc(1)	2			88	F	In	CSU									
215	1+	Gemella haemolysans 1+	2.2			20	F	Out	U	1+	2++/3+++							
216	3+++					90	F	Out	U	3+++	1+/-	S	S	S	S	R	R	R
218	3+++					78	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
219	3+++	Ent. faecalis 1+	2.2			74	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
220	1+	Staph capitis Sc(1)	2.2			9	F	Out	U	1+	1+/-	R	S	R	S	S	S	S
221	3+++					7	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
222	Sc(1)	Staph epidermidis Sc(1)	2.1			45	F	Out	U	2++	1+/-							

223	3+++	Ent. faecalis Sc(7)	2.2			39	F	Out	U	2++	1+/-	S	S	S	S	S	S	R
224	3+++					75	F	Out	U	2++	1+/-	S	S	S	S	S	S	R
225	3+++	Ent. faecalis 1+	2.3			81	M	Out	U	3+++	1+/-	R	R	R	R	R	S	R
226	3+++					49	F	Out	U	1+	2++/3+++	S	S	S	S	S	S	S
227	3+++	Strep anginosus 1+	2.2			93	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
228	3+++					67	M	Out	U	3+++	1+/-	R	S	S	S	S	S	S
229	3+++	G 1+ (No reliable ID)				80	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
230	3+++	Strep agalactiae 2++	2.2			21	F	Out	U	3+++	2++/3+++	R	S	S	S	S	S	S
231	3+++					29	F	Out	U	1+	2++/3+++	S	S	S	S	S	S	S
232	3+++					63	F	Out	U	3+++	2++/3+++	R	S	S	S	S	S	S
233	3+++	Ent. faecalis Sc(1)	2.1			16	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S
234	3+++	Ent. raffinosus Sc(5)	2.1	Staph haemolyticus Sc(2)	2	97	F	Out	U	2++	2++/3+++	R	S	S	S	S	S	R
235	3+++					9	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
236	3+++	Strep gallolyticus 3+++	2.4			90	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
237	3+++	E. coli2 3+++		Strep agalactiae 2++	2.2	62	F	Out	U	2++	2++/3+++	R	S	S	R	S	S	S
238	3+++					20	F	Out	U	3+++	2++/3+++	R	S	S	S	S	S	R
239	2++					16	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S

244	3+++					55	M	In	U	2++	1+/-	S	S	S	S	S	S	S
245	3+++					63	M	In	U	3+++	1+/-	S	S	S	S	S	S	S
246	3+++					63	M	In	U	3+++	1+/-	S	S	S	S	S	S	S
247	3+++					23	F	Out	U	1+	2++/3+++	S	S	S	S	S	S	S
248	3+++					72	F	Out	U	3+++	1+/-	R	S	S	S	S	R	R
249	Sc(5)					67	M	Out	U	3+++	1+/-	S	S	S	S	S	S	R
250	3+++	Myco maritypicum 3+++	2.2			95	M	Out	U	2++	1+/-	R	R	R	R	R	S	R
251	3+++					52	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
252	3+++	Ent. faecalis 3+++	2.3			65	M	Out	U	3+++	1+/-							
253	3+++					88	M	Out	U	3+++	1+/-	R	S	S	S	S	S	R
254	3+++					6	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
255	3+++					65	F	Out	U	3+++	1+/-	R	R	R	R	S	S	S
256	3+++					15	F	In	U	3+++	2++/3+++	S	S	S	S	S	S	S
257	3+++	Ent. faecalis Sc(5)	2.4			42	F	Out	U	2++	1+/-	R	R	R	S	S	S	R
258	3+++	Ent. faecalis Sc(2)	2.3			67	F	Out	U	2++	1+/-							
259	1+	G Sc (3)				82	F	In	U	2++	1+/-	S	S	S	S	R	R	R
260	3+++	G 3+++				86	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
261	3+++					45	F	Out	U	3+++	2++/3+++	R	S	S	R	S	R	R
262	3+++					81	M	Out	CSU			R	S	S	S	R	R	R

263	2++	Ent. faecalis Sc(9)	2.3			57	F	Out	U	2++	2++/3++							
264	2++	Ent. faecalis Sc(2)	2.3			72	F	Out	U	3+++	2++/3++							
265	3+++	Ent. faecalis 2++	2.1			7	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
266	3+++					64	F	Out	U	2++	1+/-	R	S	R	R	S	S	R
267	3+++	Ent. faecalis Sc(7)	2.4			56	F	Out	U	2++	2++/3++	S	S	S	S	S	S	S
268	3+++	G 2++				84	F	Out	U	2++	1+/-	R	S	S	S	R	S	R
269	2++	Ent. faecium Sc (5)	2.6			93	F	Out	U	2++	1+/-							
270	3+++	Kleb. oxytoca 3+++	2.4			100	F	Out	U	2++/3++	1+							
271	3+++	Ent. faecalis 2++	2.3			75	M	Out	U	2++	1+/-	R	S	S	S	S	S	R
272	3+++					75	F	Out	U	3+++	1+/-	R	R	R	S	R	S	R
273	3+++	G 1+				30	F	Out	U	3+++	2++/3++	S	S	S	S	S	S	R
274	3+++	Staph haemolyticus 3+++	2			83	F	Out	U	3+++	2++/3++							
275	3+++					80	F	In	U	1+	1+/-	S	S	S	S	S	S	R
276	3+++	Ent. faecalis Sc(2)	2.3			74	F	In	U	3+++	1+/-	R	S	S	R	S	S	S
277	3+++	Ent.e feacium 3+++	2.5			79	F	In	CSU									
278	3+++	Ent. avium 2++	2	Staph hominis Sc(4)	2.3	81	F	In	U	2++	1+/-	R	S	S	S	S	S	S
279	3+++					89	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
280	3+++					85	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S

281	3+++	E. coli Sc(8) (Halo)	2.5			58	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S
282	3+++					74	M	Out	U	2++	1+/-	R	S	S	S	S	S	S
283	3+++	Ent. faecalis 1+	2.3			80	F	Out	U	1+	1+/-	R	S	S	S	S	S	R
284	2++					43	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
285	3+++	Strep lutetiensis 3+++	2.2	Pseu aeruginosa 1+	2.5	82	F	Out	U	3+++	1+/-							
286	3+++	G 1+				89	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
287	3+++					71	F	Out	U	3+++	1+/-	S	S	S	S	S	S	R
288	3+++					42	F	Out	U	1+	1+/-	S	S	S	S	S	S	R
289	3+++	Strep vistibularis Sc(5)	2.1			89	F	Out	U	2++	1+/-	S	S	S	S	S	S	R
290	3+++	Ent. faecalis 3+++	2.2			90	M	Out	CSU									
291	3+++					86	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
292	3+++	Glob sulfidifaciens Sc(2)	1.9	Morg morganii Sc(2)	2.4	91	F	Out	U	3+++	2++/3+++	R	S	S	S	S	S	S
293	3+++					55	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
294	Sc(1)					64	F	Out	U	1+	1+/-							
295	2++	Entero asburiae 2++	2.2			1	F	Out	U	1+/-	1+/-							
296	3+++	G Sc(2)				31	F	Out	U	2++	2++/3++	R	S	S	S	S	S	S
297	1+	E. coli 1+	2.3	Pseu aeruginosa Sc(1)	2.4	86	M	Out	U	3+++	2++/3+++							

298	2++					44	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
299	3+++					45	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
300	2++	Ent. faecalis 1+	2.3			20	F	Out	U	1+/-	2++/3+++	S	S	S	S	S	S	S
301	3+++	Staph simulans Sc(2)	2.1			95	F	Out	U	3+++	1+/-	R	S	S	S	R	S	R
302	3+++	Ent. faecalis 2++	2.3			98	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
303	3+++	Proteus vulgaris 3+++	2.4			92	M	In	U	2++	1+/-							
304	3+++	Ent. faecalis Sc(1)	2.3			80	F	Out	U	3+++	1+/-	S	S	S	S	S	S	R
305	Sc(2)	Ent. faecalis Sc(6)	2.2			75	M	Out	CSU									
306	3+++	G Sc(3)				49	F	Out	U	2++	2++/3++	R	S	S	S	S	S	R
307	3+++					85	F	Out	U	2++	1+/-	R	S	R	S	S	R	R
308	Sc(3)					53	F	Out	U	2++	1+/-							
309	2++	Ent. faecalis Sc(8)	2.2			90	F	Out	U	3+++	1+/-	R	S	S	S	R	S	R
310	3+++	Ent. faecalis Sc(1)	2.2	Strep agalactiae Sc(4)	2.4	91	F	In	U	2++	1+/-	R	S	S	S	S	S	R
311	3+++					84	F	In	CSU			R	S	S	S	R	S	R
312	3+++	Ent. faecalis Sc(2)	2.2			75	M	Out	U	3+++	1+/-	R	S	S	R	S	S	S
313	3+++	Ent. faecalis Sc(2)	2.2			42	M	In	U	2++	1+/-	R	S	S	S	S	S	S
314	3+++	Ent. faecalis Sc(1)	2.4			78	F	In	U	3+++	1+/-	S	S	S	S	S	S	S
315	Sc(3)					50	F	Out	U	3+++	1+/-							

316	1+	Ent. faecalis 1+	2.4			34	F	Out	U	1+	2++/3+++							
317	3+++					46	F	Out	U	2++	2++/3+++	R	S	S	S	S	S	S
318	2++	Ent. faecalis Sc(1)	2.1			78	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
319	3+++					73	M	Out	U	3+++	1+/-	R	S	S	R	S	S	R
320	3+++					53	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
321	3+++					37	M	Out	U	2++	1+/-	R	S	S	R	S	S	R
322	2++	Staph epidermis 2++	2.1			74	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	R
323	3+++					84	F	Out	U	3+++	1+/-	R	S	S	S	S	R	S
324	3+++	Ent. faecalis 3+++	2.3			36	F	Out	U	3+++	2++/3+++							
325	3+++	Ent. faecalis 1+	2.3			68	M	Out	U	3+++	1+/-	R	R	R	R	S	S	R
326	Sc(1)	Strep anginosus 3+++				93	F	Out	U	1+	1+/-							
327	3+++					89	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
328	2++					70	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
329	3+++					66	F	Out	U	1+	1+/-	S	S	S	S	S	S	S
330	2++	Staph haemolyticus Sc(2)	2.3			40	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
331	3+++	Ent. faecalis Sc(1)	2.2			75	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
332	3+++					83	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
333	3+++	Ent. faecalis 2++	2.3			65	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S

334	3+++					90	F	Out	U	1+	1+/-	R	S	S	R	S	S	S
335	Sc(7)	Ent. faecalis 1+	2.4			74	F	Out	U	3+++	2++/3+++							
336	Sc(3)	Ent. faecium 1+	2.5			66	F	Out	U	3+++	2++/3+++							
338	3+++	G Sc(3)				87	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
339	Sc(6)	Ent. faecalis 1+	2.4			27	F	Out	U	3+++	2++/3+++							
340	2++	Staph haemolyticus Sc(2)	2.2	Glob sulfidifaciens 1+		56	F	Out	U	2++	1+/-	R	S	S	S	S	S	R
341	3+++					68	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
342	3+++					43	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
343	3+++					23	F	Out	U	1+	1+/-	R	S	S	S	S	S	S
345	3+++	Ent. faecium Sc(2)	2.5			60	F	Out	U	3+++	1+/-	R	S	S	S	R	S	R
346	3+++	G Sc(4)				91	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
347	3+++					27	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S
348	3+++					41	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
349	Sc(1)	Ent. faecalis Sc(2)	2.4	Strep oralis 1+	2.1	56	F	Out	U	2++	1+/-							
344	3+++	Ent. faecalis 3+++	2.3	Pseu aeruginosa 3+++	2.2	30	F	Out	U	2++	2++/3+++							
350	2++					11	F	Out	U	2++	1+/-	R	R	R	S	S	S	S
351	Sc(1)					26	F	Out	U	1+	2++/3+++							
352	3+++	Ent. faecium Sc(2)	2.5	Staph warneri Sc(1)	2	80	F	Out	Clean	2++	2++/3+++	R	S	S	S	S	R	R

									Catch U									
353	3+++	Ent. faecalis 3+++	2.6			0	M	In	U	1+	1+/-							
354	3+++	Ent. faecalis Sc(1)	2.4			86	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
355	3+++					82	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
356	Sc(2)					93	M	In	U	1+	1+/-							
357	3+++					85	M	In	U	3+++	1+/-	S	S	S	S	S	S	S
358	3+++					60	F	In	U	1+	1+/-	S	S	S	S	S	S	S
359	2++					61	F	In	U	3+++	1+/-	S	S	S	S	S	S	S
360	3+++					25	F	In	U	2++	2++/3+++	S	S	S	S	S	S	S
361	3+++	Strep agalactiae 3+++	2.2	Prot mirabilis 2++	2.5	90	F	In	U	3+++	1+/-							
362	3+++	Strep agalactiae 3+++	2.3	Prot mirabilis 2++	2.5	90	F	In	U	3+++	1+/-							
363	3+++					35	F	In	U	2++	2++/3+++	S	S	S	S	S	S	S
366	3+++					41	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
367	3+++					21	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
368	3+++					69	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
369	3+++					9	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
370	3+++					50	F	Out	U	2++	1+/-	R	S	S	S	S	S	R
371	3+++	Ent. faecalis Sc(3)	2.2			68	F	Out	U	2++	1+/-	S	S	S	S	S	S	S

372	3+++	Ent. faecalis Sc(3)	2.1			76	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	R
373	3+++	E. coli 2++	2.5	Strep agalactiae 2++	2.3	63	F	Out	U	3+++	2++/3+++							
374	3+++					84	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
375	3+++					73	M	Out	U	2++	1+/-							
376	1+	Ent. faecalis 2++	2.4	Morg morganii 1+	2.6	58	F	Out	U	1+	1+							
377	3+++	Entero asburiae 3+++	2.3			3	F	Out	U	2++	1+/-							
378	Sc(1)					28	F	Out	U	1+/-	1+/-							
379	3+++	Ent. faecalis Sc(8)	2.2			51	F	In	U	3+++	2++/3+++	S	S	S	S	S	S	S
380	3+++ ? 2 Ecolis	Ent. faecalis 3+++	2.4			70	F	Out	U	3+++	2++/3+++							
381	3+++	G Sc(3)				86	F	Out	U	2++	1+/-	R	S	S	S	S	S	R
382	Sc(3)					29	F	Out	U	3+++	2++/3+++							
383	3+++					78	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
384	3+++					74	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
385	3+++					45	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S
386	3+++					94	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
387	3+++					26	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S
388	3+++					46	M	Out	U	3+++	1+/-	R	S	S	S	S	S	S
389	3+++	Ent. faecalis Sc(8)	2.2			89	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
390	3+++					82	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R

391	3+++					3	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
392	3+++	Strep gallolyticus Sc(1)	2.3			72	F	Out	U	2++	1+/-	R	S	S	S	S	S	S
393	3+++	G Sc(7)				72	F	Out	U	2++	1+/-	R	S	S	S	S	S	R
394	3+++					20	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S
395	3+++					40	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
396	3+++					67	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
397	3+++	Ent. faecalis Sc(1)	2			94	M	Out	U	3+++	1+/-	R	S	S	S	R	S	R
398	3+++					77	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
399	3+++	Ent. faecalis 2++	2.3			0	F	In	U	not seen	1+/-	S	S	S	S	S	S	S
400	3+++	Kleb. pneumoniae 3+++	2.5			0	F	In	U	1+/-	1+/-	R	S	S	S	S	S	S
401	Sc(2)					61	F	In	U	2++	1+/-							
402	3+++					88	F	In	U	2++	1+/-	R	S	S	S	R	S	R
403	2++					71	M	Out	U	3+++	2++/3+++	R	S	S	S	S	S	R
404	3+++	Ent. faecalis 2++	2.4			90	F	In	U	1+	1+/-	S	S	S	S	S	S	S
405	3+++					92	M	In	U	3+++	1+/-	S	S	S	S	R	S	R
406	1+	Candida albicans Sc(3)	2.1			84	M	In	U									
407	3+++	Kleb. pneumoniae 3+++	2.2			50	M	In	U	1+	1+/-							

408	3+++	Ent. faecalis 2++	2.1			3	F	Out	U	2++	2++/3+++	S	S	S	S	S	S	S
409	3+++					78	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
410	3+++					73	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
411	3+++					15	F	Out	U	2++	2++/3+++	R	S	S	S	S	S	R
412	Sc(3)					90	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
413	3+++					34	M	In	U	3+++	1+/-	R	S	S	S	R	R	R
414	3+++					69	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
415	3+++	Ent. faecalis 1+	2.3			76	F	Out	U	2++	1+/-	R	S	S	R	S	S	R
416	3+++	Pseu aeruginosa Sc(1)	2.4			35	F	Out	CSU			S	S	S	S	S	S	S
417	3+++	Ent. faecalis Sc(7)	2.4			70	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
418	3+++					75	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
419	3+++	Ent. faecalis 3+++	2.4			84	M	Out	CSU									
420	2++	Kleb. pneumoniae 3+++				88	F	Out	U	1+	1+/-							
421	3+++	G Sc(1)				73	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
422	1+	Acin towneri Sc(8)	2.3			4	F	Out	U	1+	1+/-							
423	3+++					51	F	Out	U	1+	1+/-	S	S	S	S	S	S	S
424	3+++	Ent. faecalis 3+++	2.4	Del acidovorans 3+++	2.1	34	F	Out	U	2++	2++/3+++							
425	3+++					63	F	Out	U	1+	1+/-	S	S	S	S	S	S	S

426	3+++					77	F	Out	U	3+++	2++/3+++	R	S	S	S	S	S	S
427	3+++					20	F	Out	U	2++	2++/3+++	R	S	S	S	S	S	R
428	3+++					73	F	Out	U	3+++	1+/-	R	R	R	S	S	R	R
429	3+++	Ent. avium 1+	2.2			85	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
430	3+++					60	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
431	1+	Strep gallolyticus Sc(2)	2.4			51	F	Out	U	2++	2++/3+++							
432	3+++					46	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
433	3+++					7	F	Out	U	1+	1+/-	R	S	R	S	S	S	R
434	3+++	Ent. faecalis 2++	2.2	Strep agalactiae 1+	2.4	48	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
435	3+++					81	F	Out	U	2++	1+/-	R	S	S	S	S	S	R
436	3+++					37	F	Out	U	1+	2++/3+++	R	S	S	S	R	S	S
437	3+++					72	F	Out	U	2++	1+/-	R	S	S	S	S	S	R
438	3+++					85	F	Out	U	3+++	1+/-	S	S	S	S	S	R	R
439	3+++					79	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
440	3+++	Ent. faecalis 1+	1.9			84	M	Out	U	3+++	1+/-	R	S	S	R	S	S	S
441	3+++	Ent. faecium 1+	2.5			95	F	Out	U	1+	1+/-	R	S	S	S	S	S	R
442	3+++	Ent. faecalis Sc(1)	2.3			59	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
443	3+++					26	F	Out	U	2++	1+/-	R	R	R	S	S	S	R
444	3+++	Ent. faecalis 1+	2.3			88	M	Out	U	3+++	1+/-	R	S	S	R	S	S	S

445	3+++	Ent. faecalis 3+++	2.3	Staph aureus 3+++	2.3	93	M	Out	U	3+++	1+/-							
446	3+++					72	M	Out	U	3+++	1+/-	R	S	S	R	S	S	R
447	3+++					94	F	Out	U	3+++	1+/-	R	R	R	R	R	S	R
448	3+++					68	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
449	3+++	Staph haemolyticus Sc(1)	2.2			45	F	Out	U	2++	2++/3+++	R	S	S	S	S	S	R
450	3+++	Ent. faecalis Sc(1)	2.4			89	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
451	3+++	Ent. faecalis 3+++	2.2			83	F	Out	U	3+++	1+/-							
452	3+++					70	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
453	3+++	Ent. faecalis Sc(2)	2.3			71	F	Out	U	3+++	1+/-	R	S	S	S	S	S	R
454	3+++	Ent. faecalis 1+	2.3			16	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
455	2++					53	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
456	3+++					74	F	Out	U	3+++	1+/-	R	S	S	S	S	S	S
457	Sc(2)					23	F	Out	U	3+++	2++/3+++							
458	3+++					32	F	Out	U	1+	1+/-	R	S	S	R	S	S	R
459	3+++	Ent. faecalis 3+++	2.3			79	F	Out	U	3+++	1+/-							
460	3+++					48	F	Out	U	1+	1+/-	S	S	S	S	S	S	R
461	3+++	Ent. faecalis 3+++	2.4			82	F	Out	CSU									
463	2++	Kleb. pneumoniae 2++	2.4			99	F	Out	U	2++	1+/-							

464	3+++					77	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
465	2++					51	M	Out	U	2++	1+/-	S	S	S	S	S	S	R
466	Sc(6)					77	F	Out	U	2++	1+/-	S	S	S	S	S	S	S
467	Sc(9)	Strep gallolyticus 3+++	2.4			81	F	In	U	2++	2++/3+++							
468	3+++					91	F	In	U	3+++	1+/-	R	S	S	S	S	S	S
469	3+++					89	F	In	U	3+++	2++/3+++	R	S	S	S	S	S	R
470	3+++	Kleb. pneumoniae Sc(6)	2.6			55	F	In	U	3+++	1+/-	S	S	S	S	S	S	R
471	3+++					47	F	Out	U	3+++	2++/3+++	R	S	S	R	S	S	S
472	3+++	Lacto lactis 3+++	2.4			4	F	Out	U	3+++	1+/-							
473	3+++					71	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
474	3+++					86	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S
475	3+++	Ent. faecalis1 2++	2.3	Ent. faecalis2 2++	2.2	52	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	S
476	2++					46	F	Out	U	3+++	2++/3+++	S	S	S	S	S	S	R
477	3+++					10	F	Out	U	1+	2++/3+++	R	S	S	R	S	S	R
479	Sc(3)	Strep mitis 1+	2.3			20	F	Out	U	2++	2++/3+++							
480	3+++					71	M	Out	U	3+++	1+/-	S	S	S	S	S	S	S
481	3+++	Strep lutenensis 2++	2.3			93	F	Out	U	2++	1+/-							
482	3+++	Strep anginosus	1.8	Prot mirabilis 3+++	2.3	90	F	In	U	3+++	1+/-							

		3+++																
483	3+++	Ent. faecalis Sc(3)	2.4			80	F	Out	U	3+++	1+/-	R	S	S	S	S	R	R
484	3+++					83	F	Out	U	2++	3+++	S	S	S	S	S	S	R
485	Sc(3)	Ent. faecalis 3+++	2.2			82	M	Out	U	3+++	1+/-	S	R	R	S	S	S	R
486	3+++	Ent. faecalis Sc(1)	2.3			69	F	Out	U	3+++	1+/-	S	S	S	S	S	S	S

Appendix II

List of specimens and all information received from the Cardiff study (Chapter 4).

U Number	Purity	UTI POS	ORG 1	Organism 1 colony count
U118004	Pure	YES	Escherichia coli	5.60E+06
U118007	1 Predominant	YES	Escherichia coli	2.80E+05
U118015	Pure	YES	Escherichia coli	2.00E+05
U118030	1 Predominant	YES	Escherichia coli	1.10E+07
U118032	1 Predominant	YES	Escherichia coli	4.20E+04
U118037	Pure	YES	Escherichia coli	1.90E+07
U118041	Pure	YES	Escherichia coli	1.50E+04
U118047	Pure	YES	Escherichia coli	1.20E+05
U118048	Pure	YES	Escherichia coli	6.00E+06
U118057	Pure	YES	Escherichia coli	2.30E+07
U118063	Pure	YES	Escherichia coli	3.20E+06
U118064	1 Predominant	YES	Escherichia coli	1.80E+06
U118065	Pure	YES	Escherichia coli	2.20E+08
U118067	Pure	YES	Escherichia coli	8.00E+04
U118073	Pure	YES	Escherichia coli	2.80E+04
U118076	1 Predominant	YES	Escherichia coli	9.00E+06
U118083	1 Predominant	YES	Escherichia coli	5.30E+07
U118095	1 Predominant	YES	Escherichia coli	6.60E+07
U118096	Pure	YES	Escherichia coli	1.10E+08
U118102	Pure	YES	Escherichia coli	1.40E+06
U118104	1 Predominant	YES	Escherichia coli	1.90E+07
U118111	1 Predominant	YES	Escherichia coli	2.80E+06
U118113	Pure	YES	Escherichia coli	1.50E+07
U118116	Pure	YES	Escherichia coli	8.00E+05
U118126	Pure	YES	Escherichia coli	8.40E+06
U118128	Pure	YES	Escherichia coli	1.00E+05
U118131	Pure	YES	Escherichia coli	1.90E+07
U118132	1 Predominant	YES	Escherichia coli	7.80E+05
U118159	Pure	YES	Escherichia coli	3.20E+07
U118169	Pure	YES	Escherichia coli	7.80E+05
U118171	Pure	YES	Escherichia coli	7.50E+06
U118200	Pure	YES	Escherichia coli	2.80E+05
U118201	1 Predominant	YES	Escherichia coli	1.10E+08
U119023	1 Predominant	YES	Escherichia coli	8.70E+07
U119029	Pure	YES	Escherichia coli	3.90E+07
U119035	1 Predominant	YES	Escherichia coli	1.30E+08
U119037	1 Predominant	YES	Escherichia coli	9.80E+06
U119041	Pure	YES	Escherichia coli	1.20E+06

U119044	1 Predominant	YES	Escherichia coli	1.10E+05
U119069	1 Predominant	YES	Escherichia coli	1.70E+05
U119070	1 Predominant	YES	Escherichia coli	9.00E+07
U119072	Pure	YES	Escherichia coli	9.30E+07
U119082	1 Predominant	YES	Escherichia coli	2.60E+08
U119088	Pure	YES	Escherichia coli	2.20E+08
U119092	Pure	YES	Escherichia coli	7.60E+07
U119093	Pure	YES	Escherichia coli	1.90E+08
U119097	Pure	YES	Escherichia coli	4.10E+07
U119101	1 Predominant	YES	Escherichia coli	2.80E+06
U119107	1 Predominant	YES	Escherichia coli	8.90E+07
U119109	1 Predominant	YES	Escherichia coli	1.10E+08
U119117	Pure	YES	Escherichia coli	5.10E+06
U119122	Pure	YES	Escherichia coli	2.80E+08
U119128	Pure	YES	Escherichia coli	4.00E+06
U119134	1 Predominant	YES	Escherichia coli	1.00E+05
U119135	Pure	YES	Escherichia coli	5.50E+07
U119136	Pure	YES	Escherichia coli	6.40E+06
U119137	1 Predominant	YES	Escherichia coli	4.20E+05
U119138	1 Predominant	YES	Escherichia coli	9.40E+06
U119140	Pure	YES	Escherichia coli	2.00E+07
U119146	Pure	YES	Escherichia coli	2.30E+07
U119154	1 Predominant	YES	Escherichia coli	2.60E+07
U119156	Pure	YES	Escherichia coli	4.20E+05
U119166	Pure	YES	Escherichia coli	9.30E+07
U119167	Pure	YES	Escherichia coli	1.70E+08
U119170	Pure	YES	Escherichia coli	4.10E+07
U119173	Pure	YES	Escherichia coli	1.40E+07
U119180	Pure	YES	Escherichia coli	7.40E+05
U119182	Pure	YES	Escherichia coli	2.00E+05
U119190	Pure	YES	Escherichia coli	2.40E+07
U119440	1 Predominant	YES	Escherichia coli	4.40E+05
U119453	Pure	YES	Escherichia coli	2.00E+06
U119454	Pure	YES	Escherichia coli	1.10E+08
U119461	1 Predominant	YES	Escherichia coli	5.50E+07
U119476	Pure	YES	Escherichia coli	1.20E+05
U119480	1 Predominant	YES	Escherichia coli	1.80E+05
U119505	1 Predominant	YES	Escherichia coli	1.10E+05
U119510	Mixed 2 organisms	YES	Escherichia coli	6.90E+04
U119513	Pure	YES	Escherichia coli	1.10E+04
U119515	1 Predominant	YES	Escherichia coli	2.40E+05
U119536	Pure	YES	Escherichia coli	4.00E+05
U119554	1 Predominant	YES	Escherichia coli	1.40E+05
U119562	Pure	YES	Escherichia coli	1.40E+07
U119574	Pure	YES	Escherichia coli	7.20E+03

U119594	Pure	YES	Escherichia coli	4.80E+05
U119599	Pure	YES	Escherichia coli	1.00E+05
U119613	1 Predominant	YES	Escherichia coli	5.80E+07
U119629	1 Predominant	YES	Escherichia coli	4.50E+07
U119634	Pure	YES	Escherichia coli	1.80E+05
U119635	1 Predominant	YES	Escherichia coli	1.90E+07
U119640	Pure	YES	Escherichia coli	1.40E+08
U119673	1 Predominant	YES	Escherichia coli	7.60E+07
U119684	Pure	YES	Escherichia coli	1.10E+05
U119693	Pure	YES	Escherichia coli	7.20E+04
U119699	Pure	YES	Escherichia coli	1.80E+05
U119700	1 Predominant	YES	Escherichia coli	2.00E+07
U119716	Pure	YES	Escherichia coli	6.50E+07
U119717	Pure	YES	Escherichia coli	1.60E+07
U119718	Pure	YES	Escherichia coli	9.60E+07
U119728	Pure	YES	Escherichia coli	8.50E+04
U119730	Pure	YES	Escherichia coli	1.90E+07
U119738	Pure	YES	Escherichia coli	2.60E+05
U119739	Pure	YES	Escherichia coli	2.80E+07
U119749	Pure	YES	Escherichia coli	1.40E+04
U119759	Pure	YES	Escherichia coli	1.80E+07
U119787	Pure	YES	Escherichia coli	4.70E+06
U119788	1 Predominant	YES	Escherichia coli	1.50E+06
U119798	Pure	YES	Escherichia coli	1.60E+05
U119803	Pure	YES	Escherichia coli	4.00E+07
U119811	Pure	YES	Escherichia coli	3.60E+05
U119820	Pure	YES	Escherichia coli	8.60E+05
U119822	1 Predominant	YES	Escherichia coli	1.20E+04
U119836	Pure	YES	Escherichia coli	5.80E+05
U119838	Pure	YES	Escherichia coli	4.90E+07
U119846	1 Predominant	YES	Escherichia coli	1.80E+05
U119847	1 Predominant	YES	Escherichia coli	6.20E+05
U119850	Pure	YES	Escherichia coli	1.90E+08
U119858	1 Predominant	YES	Escherichia coli	6.00E+05
U119864	1 Predominant	YES	Escherichia coli	6.00E+04
U119865	Pure	YES	Escherichia coli	2.20E+08
U119869	1 Predominant	YES	Escherichia coli	4.90E+06
U119875	Pure	YES	Escherichia coli	3.80E+05
U119876	Pure	YES	Escherichia coli	3.40E+06
U119882	1 Predominant	YES	Escherichia coli	1.80E+08
U119889	Pure	YES	Escherichia coli	3.60E+06
U119892	1 Predominant	YES	Escherichia coli	7.60E+05
U119902	Pure	YES	Escherichia coli	1.50E+05
U119907	Pure	YES	Escherichia coli	6.00E+04
U119908	Pure	YES	Escherichia coli	1.00E+08

U119915	Pure	YES	Escherichia coli	6.00E+05
U119927	1 Predominant	YES	Escherichia coli	6.50E+07
U119941	1 Predominant	YES	Escherichia coli	8.10E+04
U119945	Pure	YES	Escherichia coli	1.80E+07
U119948	Pure	YES	Escherichia coli	9.40E+07
U119950	Pure	YES	Escherichia coli	2.60E+05
U119951	1 Predominant	YES	Escherichia coli	2.00E+07
U119966	Pure	YES	Escherichia coli	8.00E+04
U119970	1 Predominant	YES	Escherichia coli	1.00E+05
U119975	Pure	YES	Escherichia coli	1.50E+07
U119976	Mixed 2 organisms	YES	Escherichia coli	2.30E+06
U119982	Mixed 2 organisms	YES	Escherichia coli	1.70E+06
U119985	Pure	YES	Escherichia coli	1.10E+04
U119997	1 Predominant	YES	Escherichia coli	9.60E+05
U119999	Pure	YES	Escherichia coli	8.00E+07
U119194	Pure	YES	Escherichia coli	9.10E+07
U119195	Pure	YES	Escherichia coli	1.70E+06
U119196	1 Predominant	YES	Escherichia coli	1.00E+08
U119200	1 Predominant	YES	Escherichia coli	1.10E+08
U119203	Pure	YES	Escherichia coli	1.80E+05
U119208	1 Predominant	YES	Escherichia coli	1.60E+06
U119213	1 Predominant	YES	Escherichia coli	3.40E+08
U119218	Pure	YES	Escherichia coli	3.40E+07
U119222	Pure	YES	Escherichia coli	6.40E+05
U119223	Pure	YES	Escherichia coli	6.10E+03
U119229	Pure	YES	Escherichia coli	2.60E+05
U119230	1 Predominant	YES	Escherichia coli	2.00E+07
U119232	Pure	YES	Escherichia coli	7.80E+07
U119245	1 Predominant	YES	Escherichia coli	1.60E+08
U119246	Pure	YES	Escherichia coli	2.80E+08
U119256	Pure	YES	Escherichia coli	1.00E+08
U119257	Pure	YES	Escherichia coli	3.80E+04
U119258	Pure	YES	Escherichia coli	3.20E+03
U119259	1 Predominant	YES	Escherichia coli	5.20E+05
U119263	1 Predominant	YES	Escherichia coli	4.00E+06
U119266	1 Predominant	YES	Escherichia coli	5.80E+05
U119275	Pure	YES	Escherichia coli	1.00E+07
U119278	1 Predominant	YES	Escherichia coli	2.90E+07
U119280	Pure	YES	Escherichia coli	2.10E+03
U119285	1 Predominant	YES	Escherichia coli	2.40E+05
U119291	Pure	YES	Escherichia coli	1.90E+06
U119294	Pure	YES	Escherichia coli	4.00E+05
U119299	Pure	YES	Escherichia coli	1.10E+08
U119304	Pure	YES	Escherichia coli	1.20E+06
U119306	1 Predominant	YES	Escherichia coli	1.80E+05

U119307	1 Predominant	YES	Escherichia coli	1.40E+06
U119309	Pure	YES	Escherichia coli	3.50E+07
U119314	Pure	YES	Escherichia coli	4.20E+07
U119315	Pure	YES	Escherichia coli	4.60E+05
U119316	Pure	YES	Escherichia coli	6.00E+04
U119323	Pure	YES	Escherichia coli	1.10E+08
U119324	1 Predominant	YES	Escherichia coli	8.70E+07
U119327	Pure	YES	Escherichia coli	3.00E+05
U119330	Pure	YES	Escherichia coli	4.70E+04
U119332	1 Predominant	YES	Escherichia coli	3.60E+07
U119336	Pure	YES	Escherichia coli	6.50E+07
U119342	Pure	YES	Escherichia coli	1.10E+04
U119345	Pure	YES	Escherichia coli	2.20E+05
U119352	Pure	YES	Escherichia coli	7.00E+07
U119355	Pure	YES	Escherichia coli	3.10E+06
U119357	1 Predominant	YES	Escherichia coli	1.30E+08
U119359	Pure	YES	Escherichia coli	2.00E+04
U119360	Pure	YES	Escherichia coli	1.10E+08
U119361	Pure	YES	Escherichia coli	4.40E+04
U119362	Pure	YES	Escherichia coli	3.00E+05
U119366	Pure	YES	Escherichia coli	1.70E+08
U119368	Pure	YES	Escherichia coli	4.00E+04
U119371	1 Predominant	YES	Escherichia coli	3.40E+05
U119373	Pure	YES	Escherichia coli	3.00E+06
U119382	Pure	YES	Escherichia coli	1.90E+06
U119383	Pure	YES	Escherichia coli	4.20E+05
U119384	Pure	YES	Escherichia coli	1.30E+07
U119389	Pure	YES	Escherichia coli	1.40E+08
U119390	Pure	YES	Escherichia coli	4.50E+04
U119391	Pure	YES	Escherichia coli	3.00E+05
U119394	Pure	YES	Escherichia coli	1.80E+07
U119398	Pure	YES	Escherichia coli	8.80E+05
U119410	Pure	YES	Escherichia coli	1.80E+06
U119412	Pure	YES	Escherichia coli	8.00E+04
U119420	Pure	YES	Escherichia coli	4.00E+04
U119422	1 Predominant	YES	Escherichia coli	6.70E+06
U119424	Pure	YES	Escherichia coli	6.70E+07
U119425	1 Predominant	YES	Escherichia coli	3.10E+07
U119430	1 Predominant	YES	Escherichia coli	2.00E+08
U119442	Pure	YES	Escherichia coli	2.80E+05
U119446	1 Predominant	YES	Escherichia coli	1.80E+06
U119452	Pure	YES	Escherichia coli	3.60E+05
U119487	Pure	YES	Escherichia coli	3.60E+06
U119514	Pure	YES	Escherichia coli	1.10E+08
U119526	1 Predominant	YES	Escherichia coli	3.90E+07

Appendix III

List of all ST127 genomes downloaded from the enterobase website. Files were prefixed with continuous numbers for ease of use.

1_ESC_AA8616AA_AS.scaffold.fasta
2_ESC_AA8875AA_AS.scaffold.fasta
3_ESC_AA9405AA_AS.scaffold.fasta
4_ESC_AA9512AA_AS.scaffold.fasta
5_ESC_BA0085AA_AS.scaffold.fasta
6_ESC_BA0548AA_AS.scaffold.fasta
7_ESC_BA0846AA_AS.scaffold.fasta
8_ESC_BA1129AA_AS.scaffold.fasta
9_ESC_BA1250AA_AS.scaffold.fasta
10_ESC_BA1345AA_AS.scaffold.fasta
11_ESC_BA2289AA_AS.scaffold.fasta
12_ESC_BA2314AA_AS.scaffold.fasta
13_ESC_BA2674AA_AS.scaffold.fasta
14_ESC_BA3402AA_AS.scaffold.fasta
15_ESC_BA3662AA_AS.scaffold.fasta
16_ESC_BA3720AA_AS.scaffold.fasta
17_ESC_BA3727AA_AS.scaffold.fasta
18_ESC_BA4265AA_AS.scaffold.fasta
19_ESC_BA4503AA_AS.scaffold.fasta
20_ESC_BA4632AA_AS.scaffold.fasta
21_ESC_BA4666AA_AS.scaffold.fasta
22_ESC_BA4789AA_AS.scaffold.fasta
23_ESC_BA5464AA_AS.scaffold.fasta
24_ESC_BA5763AA_AS.scaffold.fasta
25_ESC_BA6358AA_AS.scaffold.fasta
26_ESC_BA6584AA_AS.scaffold.fasta
27_ESC_BA6647AA_AS.scaffold.fasta
28_ESC_BA6709AA_AS.scaffold.fasta
29_ESC_BA7040AA_AS.scaffold.fasta
30_ESC_BA7063AA_AS.scaffold.fasta
31_ESC_BA7070AA_AS.scaffold.fasta
32_ESC_BA7152AA_AS.scaffold.fasta
33_ESC_BA7450AA_AS.scaffold.fasta
34_ESC_BA7554AA_AS.scaffold.fasta
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36_ESC_BA7845AA_AS.scaffold.fasta
37_ESC_BA7985AA_AS.scaffold.fasta
38_ESC_BA8367AA_AS.scaffold.fasta
39_ESC_BA8995AA_AS.scaffold.fasta
40_ESC_BA9187AA_AS.scaffold.fasta
41_ESC_BA9412AA_AS.scaffold.fasta
42_ESC_BA9468AA_AS.scaffold.fasta
43_ESC_BA9568AA_AS.scaffold.fasta
44_ESC_BA9575AA_AS.scaffold.fasta
45_ESC_BA9725AA_AS.scaffold.fasta
46_ESC_BA9819AA_AS.scaffold.fasta
47_ESC_CA0419AA_AS.scaffold.fasta
48_ESC_CA0989AA_AS.scaffold.fasta
49_ESC_CA1120AA_AS.scaffold.fasta
50_ESC_CA1289AA_AS.scaffold.fasta
51_ESC_CA1934AA_AS.scaffold.fasta
52_ESC_CA2017AA_AS.scaffold.fasta
53_ESC_CA2069AA_AS.scaffold.fasta
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58_ESC_CA2543AA_AS.scaffold.fasta
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61_ESC_CA2830AA_AS.scaffold.fasta
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91_ESC_GA0031AA_AS.scaffold.fasta
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98_ESC_GA4823AA_AS_genomic.fna
99_ESC_GA5315AA_AS.scaffold.fasta
100_ESC_GA6736AA_AS.scaffold.fasta
101_ESC_GA6892AA_AS.scaffold.fasta
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226_ESC_SA3766AA_AS.result.fasta
227_ESC_SA5314AA_AS.result.fasta
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229_ESC_SA7351AA_AS.result.fasta
230_ESC_SA7950AA_AS.result.fasta
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232_ESC_BA9222AA_AS.scaffold.fasta
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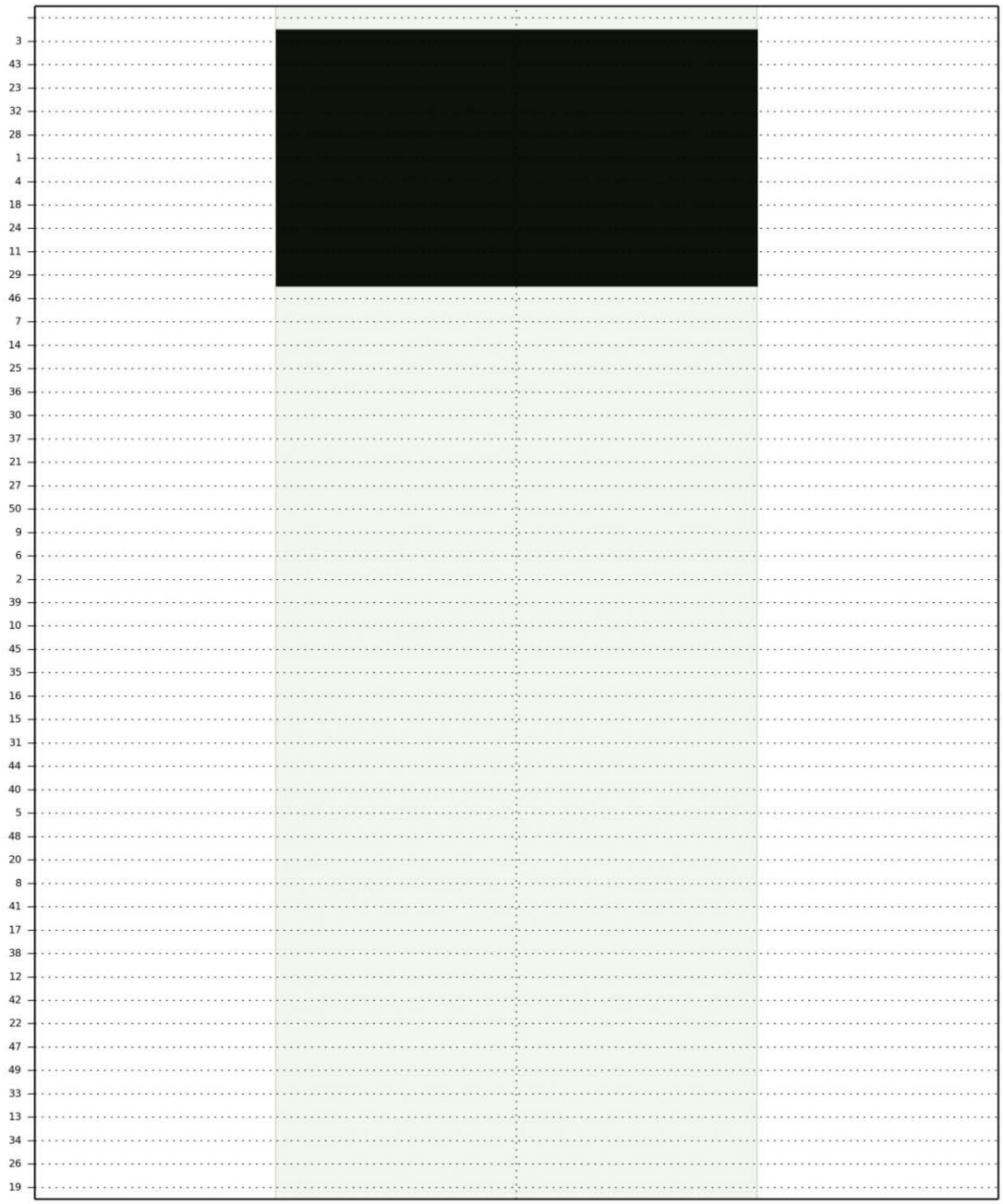
240_ESC_HA7580AA_AS.scaffold.fasta
241_ESC_HA7581AA_AS.scaffold.fasta
242_ESC_HA7672AA_AS.scaffold.fasta
243_ESC_RA5369AA_AS.result.fasta
244_ESC_GA6739AA_AS.scaffold.fasta
245_ESC_HA5574AA_AS.scaffold.fasta
246_ESC_LA5913AA_AS.scaffold.fasta

Appendix IV

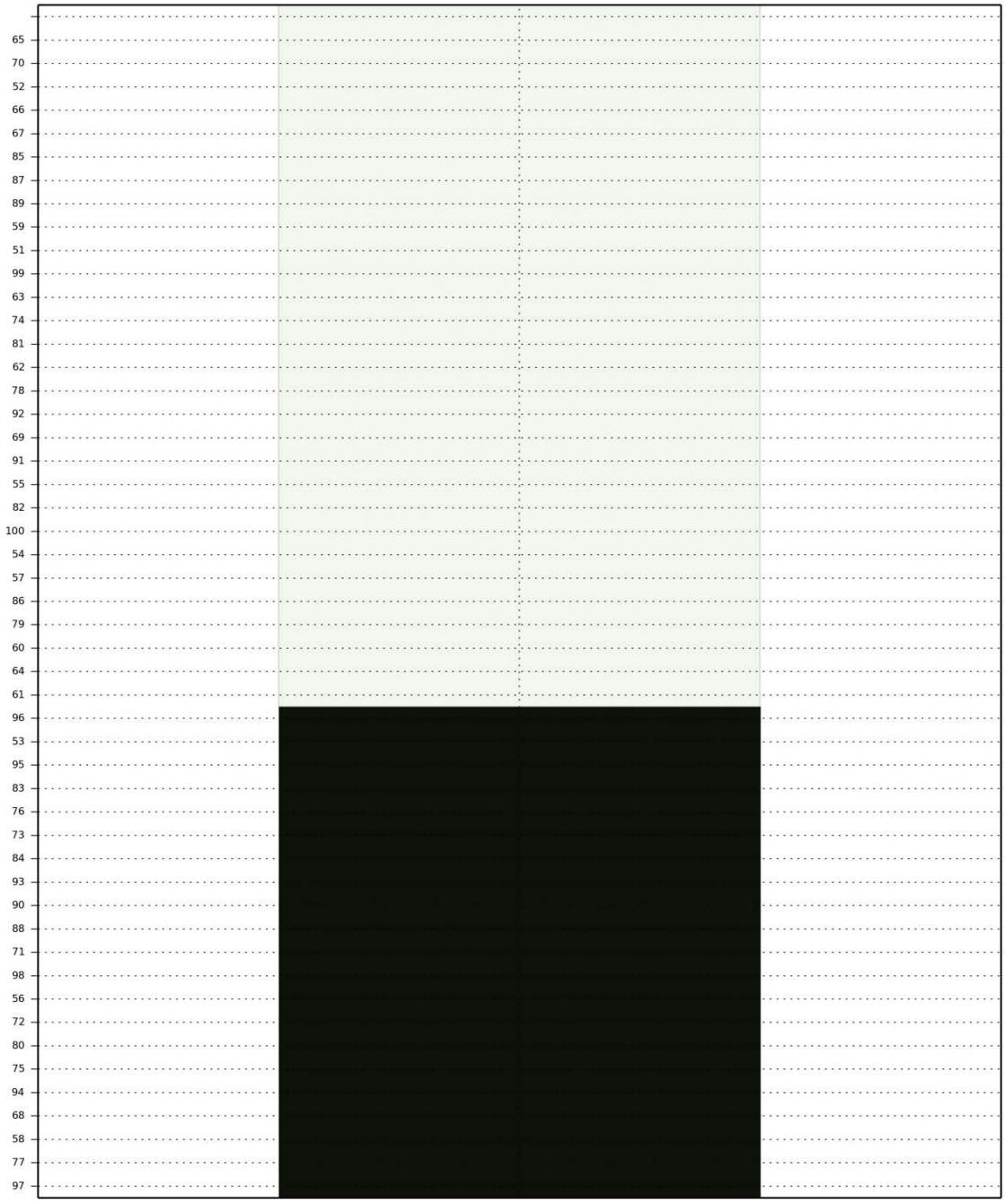
Results from SeqFindR programme for the detection of an intact *flhB* gene without the identified frameshift mutation. Black boxes indicate an intact gene. Genomes were processed in runs of 50 for the easy interpretation of results. Specimen designation on left hand side.

Run 1



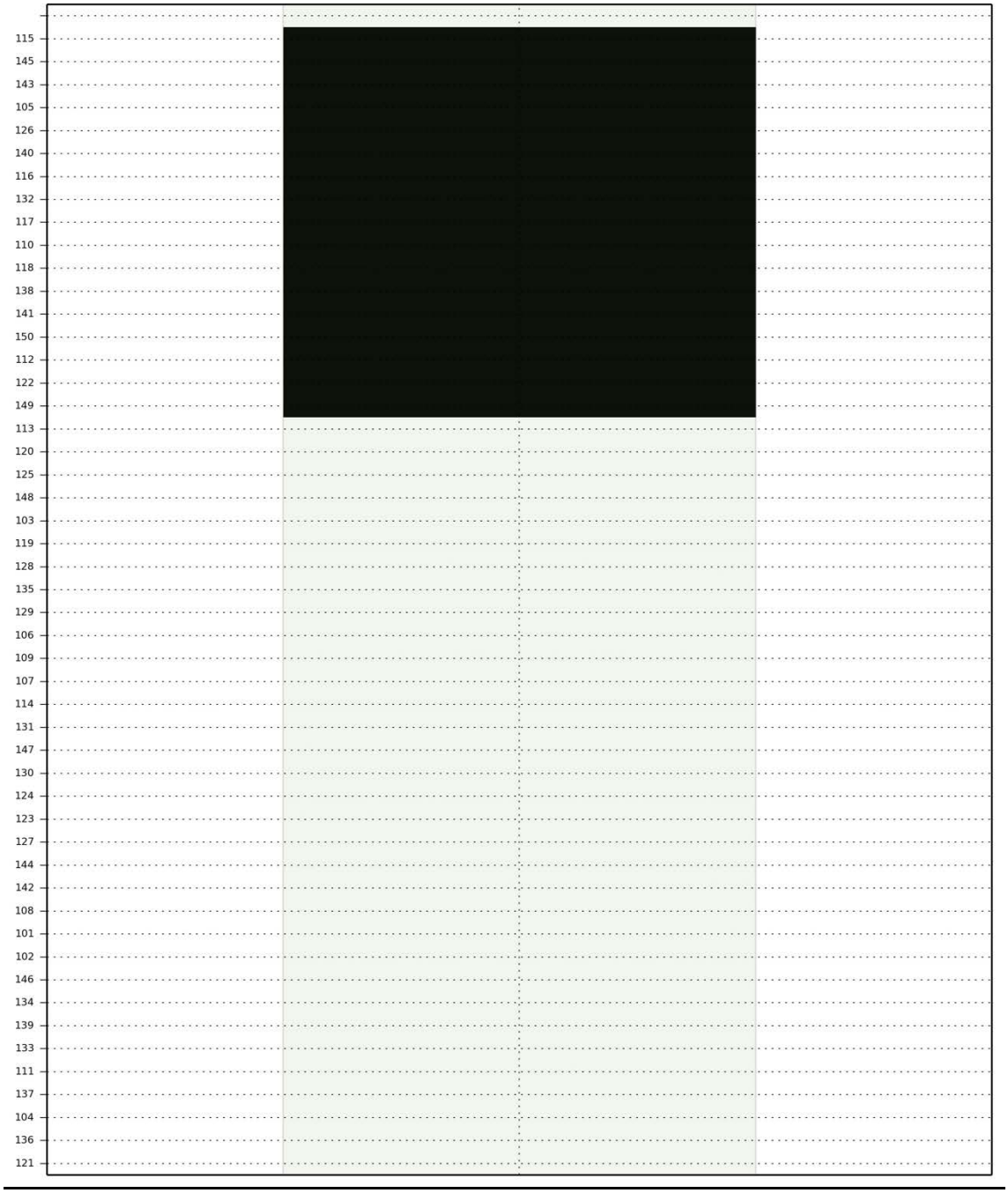
SeqFindR results continued

Run 2



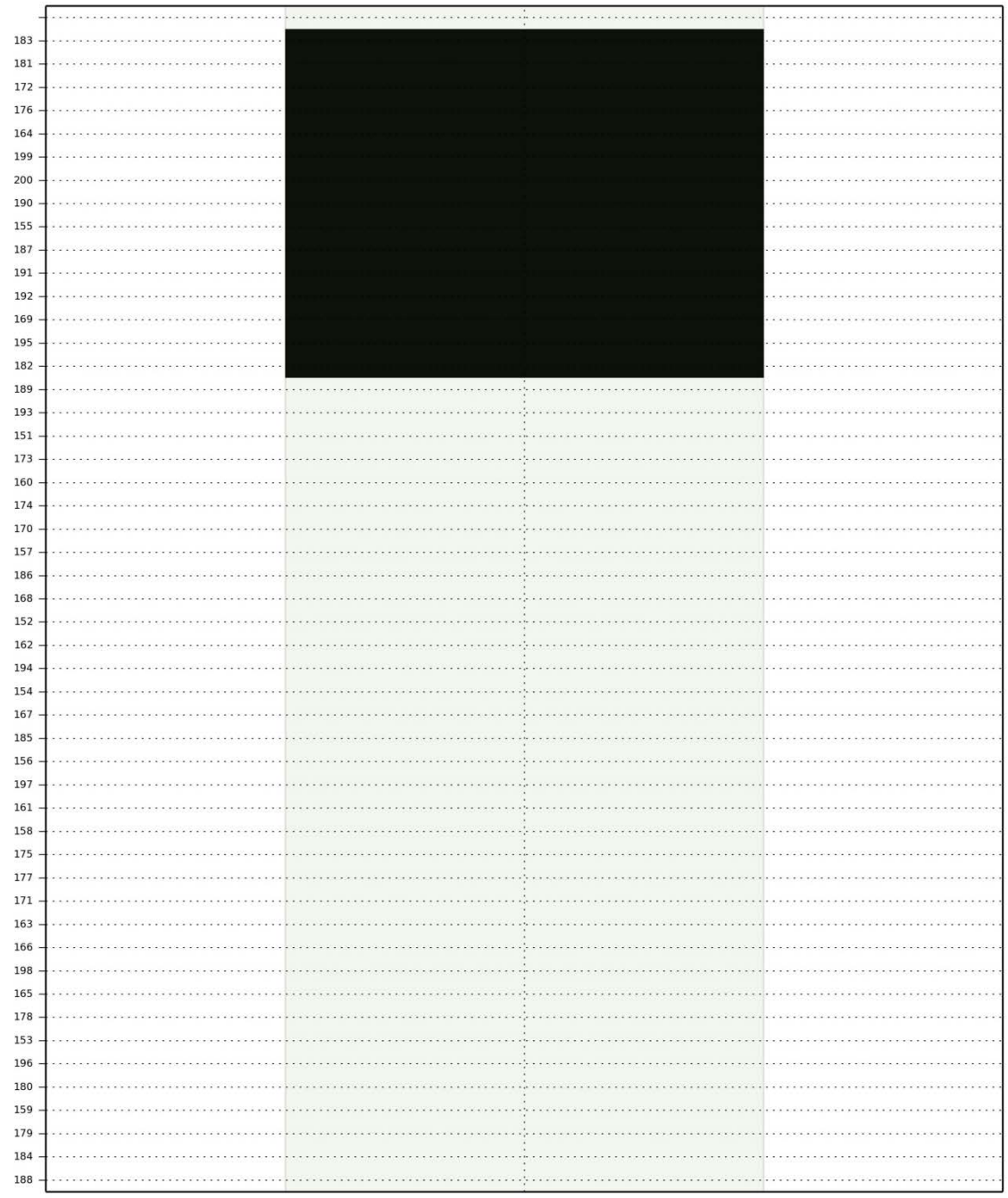
SeqFindR results continued

Run 3



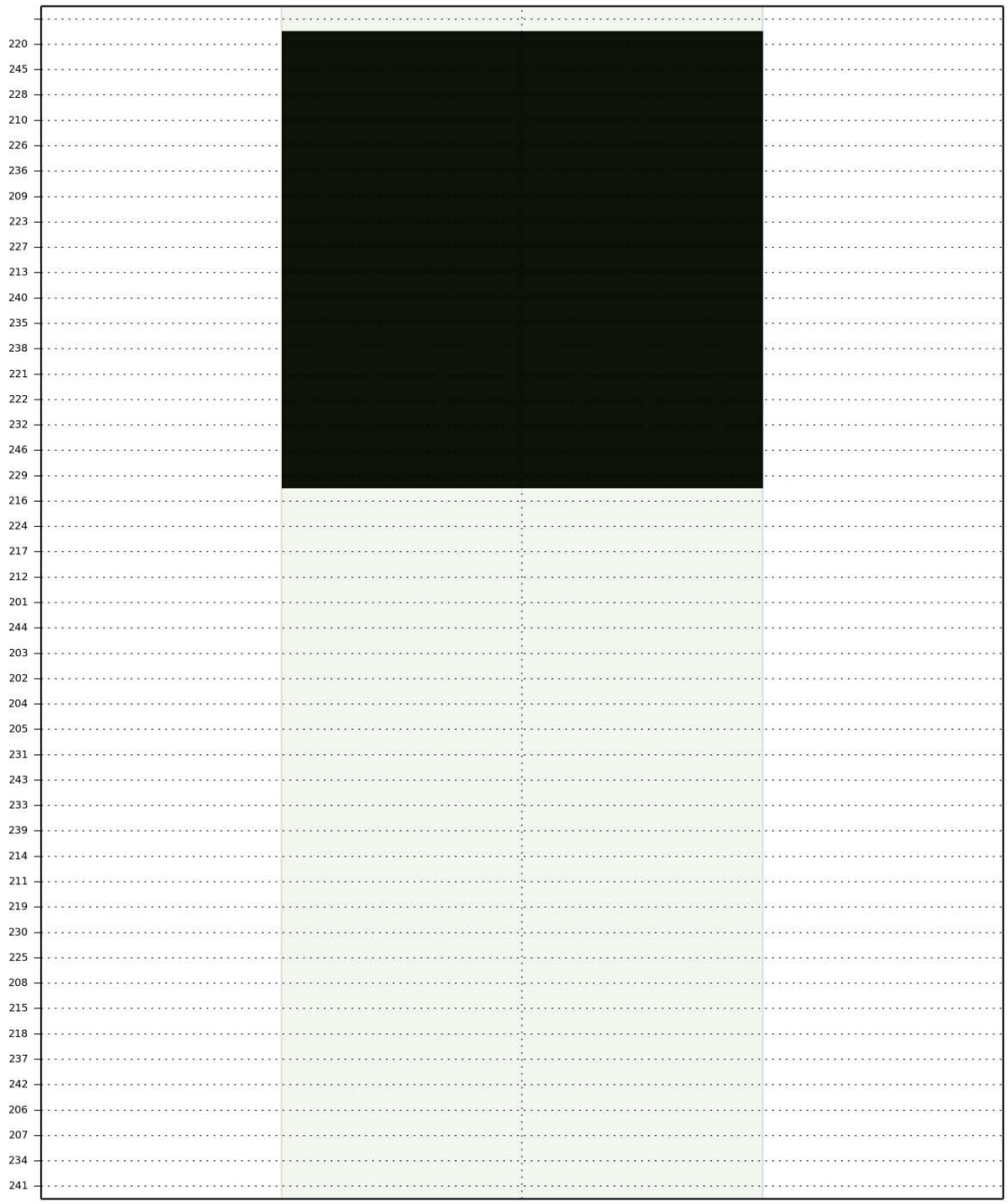
SeqFindR results continued

Run 4



SeqFindR results continued

Run 5



References

Abby, S. S. & Rocha, E. P. C. (2012) 'The Non-Flagellar Type III Secretion System Evolved from the Bacterial Flagellum and Diversified into Host-Cell Adapted Systems'. *PLoS Genet*, 8 (9). pp e1002983.

ACOG (2008) 'ACOG Practice Bulletin No. 91: Treatment of urinary tract infections in nonpregnant women'. *Obstet Gynecol*, 111 (3). pp 785-794.

Adams-Sapper, S., Diep, B. A., Perdreau-Remington, F. & Riley, L. W. (2013) 'Clonal composition and community clustering of drug-susceptible and -resistant Escherichia coli isolates from bloodstream infections'. *Antimicrob Agents Chemother*, 57 (1). pp 490-497.

Ahmad, N. M. & Ahmad, K. M. (2005) 'Corynebacterium minutissimum pyelonephritis with associated bacteraemia: a case report and review of literature'. *J Infect*, 51 (5). pp e299-303.

Aizawa, S. I. & Kubori, T. (1998) 'Bacterial flagellation and cell division'. *Genes Cells*, 3 (10). pp 625-634.

Alexander, C. & Rietschel, E. T. (2001) 'Bacterial lipopolysaccharides and innate immunity'. *J Endotoxin Res*, 7 (3). pp 167-202.

Alghoribi, M. F., Gibreel, T. M., Dodgson, A. R., Beatson, S. A. & Upton, M. (2014) 'Galleria mellonella infection model demonstrates high lethality of ST69 and ST127 uropathogenic E. coli'. *PLoS One*, 9 (7). pp e101547.

Alghoribi, M. F., Gibreel, T. M., Farnham, G., Al Johani, S. M., Balkhy, H. H. & Upton, M. (2015) 'Antibiotic-resistant ST38, ST131 and ST405 strains are the leading uropathogenic Escherichia coli clones in Riyadh, Saudi Arabia'. *J Antimicrob Chemother*, 70 (10). pp 2757-2762.

Alikhan, N. F., Petty, N. K., Ben Zakour, N. L. & Beatson, S. A. (2011) 'BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons'. *BMC Genomics*, 12 pp 402.

Alkeskas, A., Ogrodzki, P., Saad, M., Masood, N., Rhoma, N. R., Moore, K., Farbos, A., Paszkiewicz, K. & Forsythe, S. (2015) 'The molecular characterisation of Escherichia coli K1 isolated from neonatal nasogastric feeding tubes'. *BMC Infect Dis*, 15 pp 449.

Allen-Vercoe, E., Sayers, A. R. & Woodward, M. J. (1999) 'Virulence of Salmonella enterica serotype Enteritidis aflagellate and afimbriate mutants in a day-old chick model'. *Epidemiol Infect*, 122 (3). pp 395-402.

Allen-Vercoe, E. & Woodward, M. J. (1999) 'The role of flagella, but not fimbriae, in the adherence of Salmonella enterica serotype Enteritidis to chick gut explant'. *J Med Microbiol*, 48 (8). pp 771-780.

Allison, C., Emody, L., Coleman, N. & Hughes, C. (1994) 'The role of swarm cell differentiation and multicellular migration in the uropathogenicity of Proteus mirabilis'. *J Infect Dis*, 169 (5). pp 1155-1158.

Allsopp, L. P., Beloin, C., Moriel, D. G., Totsika, M., Ghigo, J. M. & Schembri, M. A. (2012) 'Functional heterogeneity of the UpaH autotransporter protein from uropathogenic Escherichia coli'. *J Bacteriol*, 194 (21). pp 5769-5782.

Alteri, C. J. & Mobley, H. L. (2015) 'Metabolism and Fitness of Urinary Tract Pathogens'. *Microbiol Spectr*, 3 (3).

Altman, D. G. & Bland, J. M. (1994) 'Diagnostic tests. 1: Sensitivity and specificity'. *Bmj*, 308 (6943). pp 1552.

Andersen, C., Bachmeyer, C., Tauber, H., Benz, R., Wang, J., Michel, V., Newton, S. M., Hofnung, M. & Charbit, A. (1999) 'In vivo and in vitro studies of major surface loop deletion mutants of the Escherichia coli K-12 maltoporin: contribution to maltose and maltooligosaccharide transport and binding'. *Mol Microbiol*, 32 (4). pp 851-867.

Anderson, G. G., Palermo, J. J., Schilling, J. D., Roth, R., Heuser, J. & Hultgren, S. J. (2003) 'Intracellular bacterial biofilm-like pods in urinary tract infections'. *Science*, 301 (5629). pp 105-107.

Arias, C. A. & Murray, B. E. (2012) 'The rise of the Enterococcus: beyond vancomycin resistance'. *Nat Rev Microbiol*, 10 (4). pp 266-278.

Asakura, S., Eguchi, G. & Iino, T. (1964) 'Reconstitution of bacterial flagella in vitro'. *J Mol Biol*, 10 (1). pp 42-IN49.

Asscher, A. W., Sussman, M., Waters, W. E., Davis, R. H. & Chick, S. (1966) 'URINE AS A MEDIUM FOR BACTERIAL GROWTH'. *The Lancet*, 288 (7472). pp 1037-1041.

Atlas, R. M. (1996) *Handbook of Microbiological Media: Second Edition.* CRC-Press.

Backhed, F., Alsen, B., Roche, N., Angstrom, J., von Euler, A., Breimer, M. E., Westerlund-Wikstrom, B., Teneberg, S. & Richter-Dahlfors, A. (2002) 'Identification of target tissue glycosphingolipid receptors for uropathogenic, F1C-fimbriated Escherichia coli and its role in mucosal inflammation'. *J Biol Chem*, 277 (20). pp 18198-18205.

Bailey, J. K., Pinyon, J. L., Anantham, S. & Hall, R. M. (2011) 'Distribution of the blaTEM gene and blaTEM-containing transposons in commensal Escherichia coli'. *J Antimicrob Chemother*, 66 (4). pp 745-751.

Bakshi, U., Sarkar, M., Paul, S. & Dutta, C. (2016) 'Assessment of virulence potential of uncharacterized Enterococcus faecalis strains using pan genomic approach - Identification of pathogen-specific and habitat-specific genes'. *Sci Rep*, 6 pp 38648.

Banerjee, R., Johnston, B., Lohse, C., Chattopadhyay, S., Tchesnokova, V., Sokurenko, E. V. & Johnson, J. R. (2013) 'The clonal distribution and diversity of extraintestinal Escherichia coli isolates vary according to patient characteristics'. *Antimicrob Agents Chemother*, 57 (12). pp 5912-5917.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A. & Pevzner, P. A. (2012) 'SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing'. *J Comput Biol*, 19 (5). pp 455-477.

Barros, M., Martinelli, R. & Rocha, H. (2009) 'Enterococcal urinary tract infections in a university hospital: clinical studies'. *Braz J Infect Dis*, 13 (4). pp 294-296.

Bartual, S. G., Seifert, H., Hippler, C., Luzon, M. A., Wisplinghoff, H. & Rodriguez-Valera, F. (2005) 'Development of a multilocus sequence typing scheme for characterization of clinical isolates of Acinetobacter baumannii'. *J Clin Microbiol*, 43 (9). pp 4382-4390.

Bates, J., Thomas-Jones, E., Pickles, T., Kirby, N., Gal, M., Bongard, E., Hood, K., Francis, N., Little, P., Moore, M., Rumsby, K., Llor, C., Burgman, C., Verheij, T., Cohen, D., Wootton, M., Howe, R. & Butler, C. C. (2014) 'Point of care testing for urinary tract infection in primary care (POETIC): protocol for a randomised controlled trial of the clinical and cost effectiveness of FLEXICULT informed management of uncomplicated UTI in primary care'. *BMC Fam Pract*, 15 pp 187.

Becton-Dickinson (2010) 'BD DMACA Indole Reagent Droppers'.

Becton-Dickinson (2011) 'BD Instructions for use ready to use plated media BD CHROMagar Orientation medium'.

Beghain, J., Bridier-Nahmias, A., Le Nagard, H., Denamur, E. & Clermont, O. (2018) 'ClermonTyping: an easy-to-use and accurate in silico method for Escherichia genus strain phylotyping'. *Microb Genom*, 4 (7).

Ben Zakour, N. L., Alsheikh-Hussain, A. S., Ashcroft, M. M., Khanh Nhu, N. T., Roberts, L. W., Stanton-Cook, M., Schembri, M. A. & Beatson, S. A. (2016) 'Sequential Acquisition of Virulence and Fluoroquinolone Resistance Has Shaped the Evolution of Escherichia coli ST131'. *MBio*, 7 (2). pp e00347-00316.

Bennett, K. M., Gorham, R. D., Jr., Gusti, V., Trinh, L., Morikis, D. & Lo, D. D. (2015) 'Hybrid flagellin as a T cell independent vaccine scaffold'. *BMC Biotechnol*, 15 pp 71.

Bermudez, L. E. & Goodman, J. (1996) 'Mycobacterium tuberculosis invades and replicates within type II alveolar cells'. *Infect Immun*, 64 (4). pp 1400-1406.

Berry, R. E., Klumpp, D. J. & Schaeffer, A. J. (2009) 'Urothelial Cultures Support Intracellular Bacterial Community Formation by Uropathogenic Escherichia coli'. *Infection and Immunity*, 77 (7). pp 2762-2772.

Bettelheim, K. A. & Taylor, J. (1969) 'A study of Escherichia coli isolated from chronic urinary infection'. *J Med Microbiol*, 2 (3). pp 225-236.

Beukers, A. G., Zaheer, R., Goji, N., Amoako, K. K., Chaves, A. V., Ward, M. P. & McAllister, T. A. (2017) 'Comparative genomics of Enterococcus spp. isolated from bovine feces'. *BMC Microbiol*, 17 (1). pp 52.

Beutin, L., Delannoy, S. & Fach, P. (2015) 'Sequence Variations in the Flagellar Antigen Genes fliCH25 and fliCH28 of Escherichia coli and Their Use in Identification and Characterization of Enterohemorrhagic E. coli (EHEC) O145:H25 and O145:H28'. *PLoS One*, 10 (5). pp e0126749.

Beyrouthy, R., Robin, F., Cougnoux, A., Dalmasso, G., Darfeuille-Michaud, A., Mallat, H., Dabboussi, F., Hamze, M. & Bonnet, R. (2013) 'Chromosome-mediated OXA-48 carbapenemase in highly virulent Escherichia coli'. *J Antimicrob Chemother*, 68 (7). pp 1558-1561.

Biedzka-Sarek, M., Venho, R. & Skurnik, M. (2005) 'Role of YadA, Ail, and Lipopolysaccharide in Serum Resistance of Yersinia enterocolitica Serotype O:3'. *Infect Immun*, 73 (4). pp 2232-2244.

Bielaszewska, M., Kock, R., Friedrich, A. W., von Eiff, C., Zimmerhackl, L. B., Karch, H. & Mellmann, A. (2007) 'Shiga toxin-mediated hemolytic uremic syndrome: time to change the diagnostic paradigm?'. *PLoS One*, 2 (10). pp e1024.

Bien, J., Sokolova, O. & Bozko, P. (2012) 'Role of Uropathogenic Escherichia coli Virulence Factors in Development of Urinary Tract Infection and Kidney Damage'. *International Journal of Nephrology*, 2012 pp 681473.

Binnenkade, L., Lassak, J. & Thormann, K. M. (2011) 'Analysis of the BarA/UvrY two-component system in Shewanella oneidensis MR-1'. *PLoS One*, 6 (9). pp e23440.

Blair, D. F. & Berg, H. C. (1990) 'The MotA protein of E. coli is a proton-conducting component of the flagellar motor'. *Cell*, 60 (3). pp 439-449.

Blount, Z. D. (2015) 'The unexhausted potential of E. coli'. *Elife*, 4

Blum, G., Falbo, V., Caprioli, A. & Hacker, J. (1995) 'Gene clusters encoding the cytotoxic necrotizing factor type 1, Prs-fimbriae and alpha-hemolysin form the pathogenicity island II of the uropathogenic Escherichia coli strain J96'. *FEMS Microbiol Lett*, 126 (2). pp 189-195.

Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschape, H. & Hacker, J. (1994) 'Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an Escherichia coli wild-type pathogen'. *Infect Immun*, 62 (2). pp 606-614.

Boehm, A. B. & Sassoubre, L. M. (2014) 'Enterococci as Indicators of Environmental Fecal Contamination'. in Gilmore, M.S., Clewell, D.B., Ike, Y. and Shankar, N. (eds.) *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection.* Boston: Massachusetts Eye and Ear Infirmary.

Bogdan, M., Zujic Atalic, V., Hecimovic, I. & Vukovic, D. (2015) 'Brain abscess due to Aggregatibacter aphrophilus and Bacteroides uniformis'. *Acta Med Acad*, 44 (2). pp 181-185.

Bolin, I., Norlander, L. & Wolf-Watz, H. (1982) 'Temperature-inducible outer membrane protein of Yersinia pseudotuberculosis and Yersinia enterocolitica is associated with the virulence plasmid'. *Infect Immun*, 37 (2). pp 506-512.

Bolin, I. & Wolf-Watz, H. (1984) 'Molecular cloning of the temperature-inducible outer membrane protein 1 of Yersinia pseudotuberculosis'. *Infect Immun*, 43 (1). pp 72-78.

Boudeau, J., Glasser, A. L., Masseret, E., Joly, B. & Darfeuille-Michaud, A. (1999) 'Invasive ability of an Escherichia coli strain isolated from the ileal mucosa of a patient with Crohn's disease'. *Infect Immun*, 67 (9). pp 4499-4509.

Bower, J. M., Eto, D. S. & Mulvey, M. A. (2005) 'Covert Operations of Uropathogenic Escherichia coli within the Urinary Tract'. *Traffic (Copenhagen, Denmark)*, 6 (1). pp 18-31.

Brealey, J. C., Sly, P. D., Young, P. R. & Chappell, K. J. (2015) 'Viral bacterial co-infection of the respiratory tract during early childhood'. *FEMS Microbiol Lett*, 362 (10).

Brenner, S., Johnson, M., Bridgham, J., Golda, G., Lloyd, D. H., Johnson, D., Luo, S., McCurdy, S., Foy, M., Ewan, M., Roth, R., George, D., Eletr, S., Albrecht, G., Vermaas, E., Williams, S. R., Moon, K., Burcham, T., Pallas, M., DuBridge, R. B., Kirchner, J., Fearon, K., Mao, J. & Corcoran, K. (2000) 'Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays'. *Nat Biotechnol*, 18 (6). pp 630-634.

Brogden, K. A., Guthmiller, J. M. & Taylor, C. E. (2005) 'Human polymicrobial infections'. *The Lancet*, 365 (9455). pp 253-255.

Brzuszkiewicz, E., Bruggemann, H., Liesegang, H., Emmerth, M., Olschlager, T., Nagy, G., Albermann, K., Wagner, C., Buchrieser, C., Emody, L., Gottschalk, G., Hacker, J. & Dobrindt, U. (2006) 'How to become a uropathogen: comparative genomic analysis of extraintestinal pathogenic Escherichia coli strains'. *Proc Natl Acad Sci U S A*, 103 (34). pp 12879-12884.

Buckles, E. L., Wang, X., Lane, M. C., Lockatell, C. V., Johnson, D. E., Rasko, D. A., Mobley, H. L. & Donnenberg, M. S. (2009) 'Role of the K2 capsule in Escherichia coli urinary tract infection and serum resistance'. *J Infect Dis*, 199 (11). pp 1689-1697.

Burns, S. M. & Hull, S. I. (1998) 'Comparison of loss of serum resistance by defined lipopolysaccharide mutants and an acapsular mutant of uropathogenic Escherichia coli O75:K5'. *Infect Immun*, 66 (9). pp 4244-4253.

Burns, S. M. & Hull, S. I. (1999) 'Loss of resistance to ingestion and phagocytic killing by O(-) and K(-) mutants of a uropathogenic Escherichia coli O75:K5 strain'. *Infect Immun*, 67 (8). pp 3757-3762.

Busch, A. & Waksman, G. (2012) 'Chaperone-usher pathways: diversity and pilus assembly mechanism'. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 367 (1592). pp 1112-1122.

Butler, C. C., Hawking, M. K. D., Quigley, A. & McNulty, C. A. M. (2015) 'Incidence, severity, help seeking, and management of uncomplicated urinary tract infection: a population-based survey'. *The British Journal of General Practice*, 65 (639). pp e702-e707.

Cagnacci, S., Gualco, L., Debbia, E., Schito, G. C. & Marchese, A. (2008) 'European emergence of ciprofloxacin-resistant Escherichia coli clonal groups O25:H4-ST 131 and O15:K52:H1 causing community-acquired uncomplicated cystitis'. *J Clin Microbiol*, 46 (8). pp 2605-2612.

Calzada, M., Roig, M., Martinez-Toldos, M. C. & Segovia, M. (2015) '[Urinary tract infection associated with Delftia acidovorans]'. *Rev Esp Quimioter*, 28 (6). pp 326-327.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. & Madden, T. L. (2009) 'BLAST+: architecture and applications'. *BMC Bioinformatics*, 10 pp 421.

Carlos, C., Pires, M. M., Stoppe, N. C., Hachich, E. M., Sato, M. I. Z., Gomes, T. A. T., Amaral, L. A. & Ottoboni, L. M. M. (2010) 'Escherichia coli phylogenetic group determination and its application in the identification of the major animal source of fecal contamination'. *BMC Microbiol*, 10 pp 161-161.

Carver, T. J., Rutherford, K. M., Berriman, M., Rajandream, M. A., Barrell, B. G. & Parkhill, J. (2005) 'ACT: the Artemis Comparison Tool'. *Bioinformatics*, 21 (16). pp 3422-3423.

'Centres for Disease Control and Prevention: E.coli' (2015).

Ch'ng, J. H., Chong, K. K. L., Lam, L. N., Wong, J. J. & Kline, K. A. (2019) 'Biofilm-associated infection by enterococci'. *Nat Rev Microbiol*, 17 (2). pp 82-94.

Cha, M. K., Kang, C. I., Kim, S. H., Thamlikitkul, V., So, T. M., Ha, Y. E., Chung, D. R., Peck, K. R. & Song, J. H. (2016) 'Emergence and Dissemination of

ST131 Escherichia coli Isolates Among Patients with Hospital-Acquired Pneumonia in Asian Countries'. *Microb Drug Resist*,

Chahales, P. & Thanassi, D. G. (2015) 'Structure, Function, and Assembly of Adhesive Organelles by Uropathogenic Bacteria'. *Microbiol Spectr*, 3 (5).

Chen, S. L., Hung, C. S., Pinkner, J. S., Walker, J. N., Cusumano, C. K., Li, Z., Bouckaert, J., Gordon, J. I. & Hultgren, S. J. (2009) 'Positive selection identifies an in vivo role for FimH during urinary tract infection in addition to mannose binding'. *Proc Natl Acad Sci U S A*, 106 (52). pp 22439-22444.

Chen, S. L., Hung, C. S., Xu, J., Reigstad, C. S., Magrini, V., Sabo, A., Blasiar, D., Bieri, T., Meyer, R. R., Ozersky, P., Armstrong, J. R., Fulton, R. S., Latreille, J. P., Spieth, J., Hooton, T. M., Mardis, E. R., Hultgren, S. J. & Gordon, J. I. (2006) 'Identification of genes subject to positive selection in uropathogenic strains of Escherichia coli: a comparative genomics approach'. *Proc Natl Acad Sci U S A*, 103 (15). pp 5977-5982.

Chia-Suei, H., Julie, B., Danielle, H., Jerome, P., Charlotte, W., Anthony, D., Gale, A. C., Robert, S., Solomon, L., Gabriel, W. & J., H. S. (2002) 'Structural basis of tropism of Escherichia coli to the bladder during urinary tract infection'. *Molecular Microbiology*, 44 (4). pp 903-915.

Chotikanatis, K., Backer, M., Rosas-Garcia, G. & Hammerschlag, M. R. (2011) 'Recurrent intravascular-catheter-related bacteremia caused by Delftia acidovorans in a hemodialysis patient'. *J Clin Microbiol*, 49 (9). pp 3418-3421.

Chuang, L. & Ratnayake, L. (2018) 'Overcoming challenges of treating extensively drug-resistant Acinetobacter baumannii bacteraemic urinary tract infection'. *Int J Antimicrob Agents*, 52 (4). pp 521-522.

Chung, J. W., Hong, S. J., Kim, K. J., Goti, D., Stins, M. F., Shin, S., Dawson, V. L., Dawson, T. M. & Kim, K. S. (2003) '37-kDa laminin receptor precursor modulates cytotoxic necrotizing factor 1-mediated RhoA activation and bacterial uptake'. *J Biol Chem*, 278 (19). pp 16857-16862.

Ciesielczuk, H., Hornsey, M., Choi, V., Woodford, N. & Wareham, D. W. (2013) 'Development and evaluation of a multiplex PCR for eight plasmid-mediated quinolone-resistance determinants'. *J Med Microbiol*, 62 (Pt 12). pp 1823-1827.

Ciesielczuk, H., Jenkins, C., Chattaway, M., Doumith, M., Hope, R., Woodford, N. & Wareham, D. W. (2016) 'Trends in ExPEC serogroups in the UK and their significance'. *Eur J Clin Microbiol Infect Dis*,

Clarke, S. C., Haigh, R. D., Freestone, P. P. E. & Williams, P. H. (2003) 'Virulence of Enteropathogenic Escherichia coli, a Global Pathogen'. *Clinical Microbiology Reviews*, 16 (3). pp 365-378.

Clermont, O., Christenson, J. K., Denamur, E. & Gordon, D. M. (2013) 'The Clermont Escherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups'. *Environ Microbiol Rep*, 5 (1). pp 58-65.

Clermont, O., Dhanji, H., Upton, M., Gibreel, T., Fox, A., Boyd, D., Mulvey, M. R., Nordmann, P., Ruppe, E., Sarthou, J. L., Frank, T., Vimont, S., Arlet, G., Branger, C., Woodford, N. & Denamur, E. (2009) 'Rapid detection of the O25b-ST131 clone of Escherichia coli encompassing the CTX-M-15-producing strains'. *J Antimicrob Chemother*, 64 (2). pp 274-277.

Clermont, O., Gordon, D. & Denamur, E. (2015) 'Guide to the various phylogenetic classification schemes for Escherichia coli and the correspondence among schemes'. *Microbiology*, 161 (Pt 5). pp 980-988.

Coburn, B., Sekirov, I. & Finlay, B. B. (2007) 'Type III Secretion Systems and Disease'. *Clinical Microbiology Reviews*, 20 (4). pp 535-549.

Cossart, P. & Sansonetti, P. J. (2004) 'Bacterial Invasion: The Paradigms of Enteroinvasive Pathogens'. *Science*, 304 (5668). pp 242.

Cove-Smith, A. & Almond, M. K. (2007) 'Management of urinary tract infections in the elderly'. *Trends in Urology, Gynaecology & Sexual Health*, 12 (4). pp 31-34.

Craig, J., Grigor, W., Doyle, B. & Arnold, D. (1994) 'Pyelonephritis caused by Corynebacterium minutissimum'. *The Pediatric Infectious Disease Journal*, 13 (12). pp 1151.

Croxall, G., Hale, J., Weston, V., Manning, G., Cheetham, P., Achtman, M. & McNally, A. (2011a) 'Molecular epidemiology of extraintestinal pathogenic Escherichia coli isolates from a regional cohort of elderly patients highlights the prevalence of ST131 strains with increased antimicrobial resistance in both community and hospital care settings'. *J Antimicrob Chemother*, 66 (11). pp 2501-2508.

Croxall, G., Weston, V., Joseph, S., Manning, G., Cheetham, P. & McNally, A. (2011b) 'Increased human pathogenic potential of Escherichia coli from

polymicrobial urinary tract infections in comparison to isolates from monomicrobial culture samples'. *J Med Microbiol*, 60 (Pt 1). pp 102-109.

Cusumano, C. K., Pinkner, J. S., Han, Z., Greene, S. E., Ford, B. A., Crowley, J. R., Henderson, J. P., Janetka, J. W. & Hultgren, S. J. (2011) 'Treatment and prevention of urinary tract infection with orally active FimH inhibitors'. *Sci Transl Med*, 3 (109). pp 109ra115.

Dale, J. L., Nilson, J. L., Barnes, A. M. T. & Dunny, G. M. (2017) 'Restructuring of Enterococcus faecalis biofilm architecture in response to antibiotic-induced stress'. *NPJ Biofilms Microbiomes*, 3 pp 15.

Darling, A. E., McKinnon, J., Worden, P., Santos, J., Charles, I. G., Chowdhury, P. R. & Djordjevic, S. P. (2014) 'A draft genome of Escherichia coli sequence type 127 strain 2009-46'. *Gut Pathog*, 6 pp 32.

Darouiche, R. O., Donovan, W. H., Del Terzo, M., Thornby, J. I., Rudy, D. C. & Hull, R. A. (2001) 'Pilot trial of bacterial interference for preventing urinary tract infection'. *Urology*, 58 (3). pp 339-344.

Darouiche, R. O., Green, B. G., Donovan, W. H., Chen, D., Schwartz, M., Merritt, J., Mendez, M. & Hull, R. A. (2011) 'Multicenter randomized controlled trial of bacterial interference for prevention of urinary tract infection in patients with neurogenic bladder'. *Urology*, 78 (2). pp 341-346.

Darouiche, R. O., Thornby, J. I., Cerra-Stewart, C., Donovan, W. H. & Hull, R. A. (2005) 'Bacterial interference for prevention of urinary tract infection: a prospective, randomized, placebo-controlled, double-blind pilot trial'. *Clin Infect Dis*, 41 (10). pp 1531-1534.

de Ree, J. M. & van den Bosch, J. F. (1987) 'Serological response to the P fimbriae of uropathogenic Escherichia coli in pyelonephritis'. *Infect Immun*, 55 (9). pp 2204-2207.

Deng, F. M., Liang, F. X., Tu, L., Resing, K. A., Hu, P., Supino, M., Hu, C. C., Zhou, G., Ding, M., Kreibich, G. & Sun, T. T. (2002) 'Uroplakin IIIb, a urothelial differentiation marker, dimerizes with uroplakin Ib as an early step of urothelial plaque assembly'. *J Cell Biol*, 159 (4). pp 685-694.

DeRosier, D. J. (1998) 'The Turn of the Screw: The Bacterial Flagellar Motor'. *Cell*, 93 (1). pp 17-20.

Diamant, E., Palti, Y., Gur-Arie, R., Cohen, H., Hallerman, E. M. & Kashi, Y. (2004) 'Phylogeny and strain typing of Escherichia coli, inferred from variation at mononucleotide repeat loci'. *Appl Environ Microbiol*, 70 (4). pp 2464-2473.

Dibb-Fuller, M. P., Allen-Vercoe, E., Thorns, C. J. & Woodward, M. J. (1999) 'Fimbriae- and flagella-mediated association with and invasion of cultured epithelial cells by Salmonella enteritidis'. *Microbiology*, 145 (Pt 5) pp 1023-1031.

Dobrindt, U., Blum-Oehler, G., Nagy, G., Schneider, G., Johann, A., Gottschalk, G. & Hacker, J. (2002) 'Genetic structure and distribution of four pathogenicity islands (PAI I(536) to PAI IV(536)) of uropathogenic Escherichia coli strain 536'. *Infect Immun*, 70 (11). pp 6365-6372.

Doumith, M., Day, M., Ciesielczuk, H., Hope, R., Underwood, A., Reynolds, R., Wain, J., Livermore, D. M. & Woodford, N. (2015) 'Rapid identification of major Escherichia coli sequence types causing urinary tract and bloodstream infections'. *J Clin Microbiol*, 53 (1). pp 160-166.

Doumith, M., Day, M. J., Hope, R., Wain, J. & Woodford, N. (2012) 'Improved Multiplex PCR Strategy for Rapid Assignment of the Four Major Escherichia coli Phylogenetic Groups'. *J Clin Microbiol*, 50 (9). pp 3108-3110.

Duan, Q., Zhou, M., Liang, H., Zhu, X., Guo, Z., Li, Y., Hardwidge, P. R. & Zhu, G. (2013) 'Contribution of flagellin subunit FliC to piglet epithelial cells invasion by F18ab E. coli'. *Vet Microbiol*, 166 (1-2). pp 220-224.

Duignan, B. (2017) 'Occam's razor'. *Encyclopædia Britannica*,

Duncan, M. J., Li, G., Shin, J. S., Carson, J. L. & Abraham, S. N. (2004) 'Bacterial penetration of bladder epithelium through lipid rafts'. *J Biol Chem*, 279 (18). pp 18944-18951.

Dupont, H., Montravers, P., Mohler, J. & Carbon, C. (1998) 'Disparate findings on the role of virulence factors of Enterococcus faecalis in mouse and rat models of peritonitis'. *Infection and Immunity*, 66 (6). pp 2570-2575.

Durmaz, A. A., Karaca, E., Demkow, U., Toruner, G., Schoumans, J. & Cogulu, O. (2015) 'Evolution of genetic techniques: past, present, and beyond'. *Biomed Res Int*, 2015 pp 461524.

Eaton, T. J. & Gasson, M. J. (2001) 'Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates'. *Appl Environ Microbiol*, 67 (4). pp 1628-1635.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. (2005) 'Diversity of the human intestinal microbial flora'. *Science*, 308 (5728). pp 1635-1638.

Eitel, J. & Dersch, P. (2002) 'The YadA protein of Yersinia pseudotuberculosis mediates high-efficiency uptake into human cells under environmental conditions in which invasin is repressed'. *Infect Immun*, 70 (9). pp 4880-4891.

El Tahir, Y. & Skurnik, M. (2001) 'YadA, the multifaceted Yersinia adhesin'. *Int J Med Microbiol*, 291 (3). pp 209-218.

Ellington, M. J., Ekelund, O., Aarestrup, F. M., Canton, R., Doumith, M., Giske, C., Grundman, H., Hasman, H., Holden, M. T., Hopkins, K. L., Iredell, J., Kahlmeter, G., Koser, C. U., MacGowan, A., Mevius, D., Mulvey, M., Naas, T., Peto, T., Rolain, J. M., Samuelsen, O. & Woodford, N. (2017) 'The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee'. *Clin Microbiol Infect*, 23 (1). pp 2-22.

Ellis, E. L. & Delbruck, M. (1939) 'THE GROWTH OF BACTERIOPHAGE'. *J Gen Physiol*, 22 (3). pp 365-384.

Elsinghorst, E. A. (1994) 'Measurement of invasion by gentamicin resistance'. *Methods in Enzymology.* Academic Press, pp 405-420.

Elsinghorst, E. A. & Kopecko, D. J. (1992) 'Molecular cloning of epithelial cell invasion determinants from enterotoxigenic Escherichia coli'. *Infect Immun*, 60 (6). pp 2409-2417.

Emody, L., Kerenyi, M. & Nagy, G. (2003) 'Virulence factors of uropathogenic Escherichia coli'. *Int J Antimicrob Agents*, 22 Suppl 2 pp 29-33.

Endrullat, C., Glökler, J., Franke, P. & Frohme, M. (2016) 'Standardization and quality management in next-generation sequencing'. *Applied & Translational Genomics*, 10 pp 2-9.

England, P. H. (2017) *Urinary tract infection guidance summary*. [Online]. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/619772/Urinary_tract_infection_UTI_guidance.pdf.

Er, D. K., Dundar, D., Uzuner, H. & Osmani, A. (2015) 'Relationship between phylogenetic groups, antibiotic resistance and patient characteristics in terms of

adhesin genes in cystitis and pyelonephritis isolates of Escherichia coli'. *Microb Pathog*, 89 pp 188-194.

Eto, D. S., Jones, T. A., Sundsbak, J. L. & Mulvey, M. A. (2007) 'Integrin-Mediated Host Cell Invasion by Type 1–Piliated Uropathogenic Escherichia coli'. *PLoS Pathogens*, 3 (7). pp e100.

Evans, L. D., Poulter, S., Terentjev, E. M., Hughes, C. & Fraser, G. M. (2013) 'A chain mechanism for flagellum growth'. *Nature*, 504 (7479). pp 287-290.

Evans, L. D. B., Hughes, C. & Fraser, G. M. (2014) 'Building a flagellum outside the bacterial cell'. *Trends Microbiol*, 22 (10). pp 566-572.

Falkenhagen, U., Zingler, G. & Naumann, G. (1991) 'Serum resistance in different serotypes of Escherichia coli'. *Zentralbl Bakteriol*, 275 (2). pp 216-222.

Ferjani, S., Saidani, M., Hamzaoui, Z., Alonso, C. A., Torres, C., Maamar, E., Slim, A. F. & Boutiba, B. B. (2017) 'Community fecal carriage of broad-spectrum cephalosporin-resistant Escherichia coli in Tunisian children'. *Diagn Microbiol Infect Dis*, 87 (2). pp 188-192.

Finn, R. D., Clements, J. & Eddy, S. R. (2011) 'HMMER web server: interactive sequence similarity searching'. *Nucleic Acids Res*, 39 (Web Server issue). pp W29-37.

Fishbain, J. & Peleg, A. Y. (2010) 'Treatment of Acinetobacter infections'. *Clin Infect Dis*, 51 (1). pp 79-84.

Fisher, K. & Phillips, C. (2009) 'The ecology, epidemiology and virulence of Enterococcus'. *Microbiology*, 155 (Pt 6). pp 1749-1757.

Fling, M. E. & Richards, C. (1983) 'The nucleotide sequence of the trimethoprim-resistant dihydrofolate reductase gene harbored by Tn7'. *Nucleic Acids Res*, 11 (15). pp 5147-5158.

Flores-Mireles, A. L., Walker, J. N., Bauman, T. M., Potretzke, A. M., Schreiber, H. L. t., Park, A. M., Pinkner, J. S., Caparon, M. G., Hultgren, S. J. & Desai, A. (2016) 'Fibrinogen Release and Deposition on Urinary Catheters Placed during Urological Procedures'. *J Urol*, 196 (2). pp 416-421.

Flores-Mireles, A. L., Walker, J. N., Caparon, M. & Hultgren, S. J. (2015) 'Urinary tract infections: epidemiology, mechanisms of infection and treatment options'. *Nat Rev Microbiol*, 13 (5). pp 269-284.

Floyd, R. V., Upton, M., Hultgren, S. J., Wray, S., Burdyga, T. V. & Winstanley, C. (2012) 'Escherichia coli-mediated impairment of ureteric contractility is uropathogenic E. coli specific'. *J Infect Dis*, 206 (10). pp 1589-1596.

Ford, M. (2014) 'Medical Microbiology, Fundamentals of Biomedical Science'. Oxford University Press, 2nd edition pp P390.

Fourel, D., Mizushima, S., Bernadac, A. & Pages, J. M. (1993) 'Specific regions of Escherichia coli OmpF protein involved in antigenic and colicin receptor sites and in stable trimerization'. *J Bacteriol*, 175 (9). pp 2754-2757.

Foxman, B. (2002) 'Epidemiology of urinary tract infections: incidence, morbidity, and economic costs'. *Am J Med*, 113 Suppl 1A pp 5s-13s.

Foxman, B. (2010) 'The epidemiology of urinary tract infection'. *Nat Rev Urol*, 7 (12). pp 653-660.

Foxman, B. & Frerichs, R. R. (1985) 'Epidemiology of urinary tract infection: II. Diet, clothing, and urination habits'. *Am J Public Health*, 75 (11). pp 1314-1317.

Foxman, B., Manning, S. D., Tallman, P., Bauer, R., Zhang, L., Koopman, J. S., Gillespie, B., Sobel, J. D. & Marrs, C. F. (2002) 'Uropathogenic Escherichia coli are more likely than commensal E. coli to be shared between heterosexual sex partners'. *Am J Epidemiol*, 156 (12). pp 1133-1140.

Foxman, B., Zhang, L., Tallman, P., Andree, B. C., Geiger, A. M., Koopman, J. S., Gillespie, B. W., Palin, K. A., Sobel, J. D., Rode, C. K., Bloch, C. A. & Marrs, C. F. (1997) 'Transmission of uropathogens between sex partners'. *J Infect Dis*, 175 (4). pp 989-992.

Franks, A. H., Harmsen, H. J., Raangs, G. C., Jansen, G. J., Schut, F. & Welling, G. W. (1998) 'Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes'. *Appl Environ Microbiol*, 64 (9). pp 3336-3345.

Fricke, W. F. & Rasko, D. A. (2014) 'Bacterial genome sequencing in the clinic: bioinformatic challenges and solutions'. *Nat Rev Genet*, 15 (1). pp 49-55.

Friedlander, R. S., Vlamakis, H., Kim, P., Khan, M., Kolter, R. & Aizenberg, J. (2013) 'Bacterial flagella explore microscale hummocks and hollows to increase adhesion'. *Proc Natl Acad Sci U S A*, 110 (14). pp 5624-5629.

Fukushi, Y., Orikasa, S. & Kagayama, M. (1979) 'An electron microscopic study of the interaction between vesical epitherlium and E. Coli'. *Invest Urol*, 17 (1). pp 61-68.

Garg, S., Mohan, B. & Taneja, N. (2017) 'Biofilm formation capability of enterococcal strains causing urinary tract infection vis-a-vis colonisation and correlation with enterococcal surface protein gene'. *Indian J Med Microbiol*, 35 (1). pp 48-52.

Gawryszewska, I., Malinowska, K., Kuch, A., Chrobak-Chmiel, D., Trokenheim, L. L., Hryniewicz, W. & Sadowy, E. (2017) 'Distribution of antimicrobial resistance determinants, virulence-associated factors and clustered regularly interspaced palindromic repeats loci in isolates of Enterococcus faecalis from various settings and genetic lineages'. *Pathog Dis*, 75 (2).

Gibreel, T. M., Dodgson, A. R., Cheesbrough, J., Bolton, F. J., Fox, A. J. & Upton, M. (2012a) 'High metabolic potential may contribute to the success of ST131 uropathogenic Escherichia coli'. *J Clin Microbiol*, 50 (10). pp 3202-3207.

Gibreel, T. M., Dodgson, A. R., Cheesbrough, J., Fox, A. J., Bolton, F. J. & Upton, M. (2012b) 'Population structure, virulence potential and antibiotic susceptibility of uropathogenic Escherichia coli from Northwest England'. *J Antimicrob Chemother*, 67 (2). pp 346-356.

Girija, S. A., Jayaseelan, V. P. & Arumugam, P. (2018) 'Prevalence of VIM- and GIM-producing Acinetobacter baumannii from patients with severe urinary tract infection'. *Acta Microbiol Immunol Hung*, pp 1-12.

Giron, J. A., Torres, A. G., Freer, E. & Kaper, J. B. (2002) 'The flagella of enteropathogenic Escherichia coli mediate adherence to epithelial cells'. *Mol Microbiol*, 44 (2). pp 361-379.

Gisela, O., Torsten, S., Marklund, B.-I., Peter, U. & Svanborg, C. (1993) 'Virulence Factors and pap Genotype in Escherichia coli Isolates from Women with Acute Pyelonephritis, with or without Bacteremia'. *Clinical Infectious Diseases*, 17 (3). pp 448-456.

Goldstein, E. J., Harvey, W. T., Wilkie, G. S., Shepherd, S. J., MacLean, A. R., Murcia, P. R. & Gunson, R. N. (2018) 'Integrating patient and whole-genome sequencing data to provide insights into the epidemiology of seasonal influenza A(H3N2) viruses'. *Microb Genom*, 4 (1).

Gomi, R., Matsuda, T., Matsumura, Y., Yamamoto, M., Tanaka, M., Ichiyama, S. & Yoneda, M. (2017) 'Whole-Genome Analysis of Antimicrobial-Resistant and

Extraintestinal Pathogenic Escherichia coli in River Water'. *Appl Environ Microbiol*, 83 (5).

Goodier, R. I. & Ahmer, B. M. (2001) 'SirA orthologs affect both motility and virulence'. *J Bacteriol*, 183 (7). pp 2249-2258.

Gould, S. J. & Eldredge, N. (1977) 'Punctuated equilibria: the tempo and mode of evolution reconsidered'. *Paleobiology*, 3 (2). pp 115-151.

Granok, A. B., Benjamin, P. & Garrett, L. S. (2002) 'Corynebacterium minutissimum bacteremia in an immunocompetent host with cellulitis'. *Clin Infect Dis*, 35 (4). pp e40-42.

Gur-Arie, R., Cohen, C. J., Eitan, Y., Shelef, L., Hallerman, E. M. & Kashi, Y. (2000) 'Simple sequence repeats in Escherichia coli: abundance, distribution, composition, and polymorphism'. *Genome Res*, 10 (1). pp 62-71.

Gurevich, A., Saveliev, V., Vyahhi, N. & Tesler, G. (2013) 'QUAST: quality assessment tool for genome assemblies'. *Bioinformatics*, 29 (8). pp 1072-1075.

Guyer, D. M., Radulovic, S., Jones, F. E. & Mobley, H. L. (2002) 'Sat, the secreted autotransporter toxin of uropathogenic Escherichia coli, is a vacuolating cytotoxin for bladder and kidney epithelial cells'. *Infect Immun*, 70 (8). pp 4539-4546.

Habibi, M., Asadi Karam, M. R. & Bouzari, S. (2015) 'In silico design of fusion protein of FimH from uropathogenic Escherichia coli and MrpH from Proteus mirabilis against urinary tract infections'. *Adv Biomed Res*, 4 pp 217.

Habibi, M., Asadi Karam, M. R. & Bouzari, S. (2016) 'Transurethral instillation with fusion protein MrpH.FimH induces protective innate immune responses against uropathogenic Escherichia coli and Proteus mirabilis'. *Apmis*, 124 (6). pp 444-452.

Hacker, J. (1990) 'Genetic determinants coding for fimbriae and adhesins of extraintestinal Escherichia coli'. *Curr Top Microbiol Immunol*, 151 pp 1-27.

Hacker, J., Blum-Oehler, G., Muhldorfer, I. & Tschape, H. (1997) 'Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution'. *Mol Microbiol*, 23 (6). pp 1089-1097.

Hacker, J. & Kaper, J. B. (2000) 'Pathogenicity islands and the evolution of microbes'. *Annu Rev Microbiol*, 54 pp 641-679.

Haiko, J. & Westerlund-Wikstrom, B. (2013) 'The role of the bacterial flagellum in adhesion and virulence'. *Biology (Basel)*, 2 (4). pp 1242-1267.

Hall-Stoodley, L., Costerton, J. W. & Stoodley, P. (2004) 'Bacterial biofilms: from the natural environment to infectious diseases'. *Nat Rev Microbiol*, 2 (2). pp 95-108.

Hall, L. M., Duke, B., Urwin, G. & Guiney, M. (1992) 'Epidemiology of Enterococcus faecalis urinary tract infection in a teaching hospital in London, United Kingdom'. *J Clin Microbiol*, 30 (8). pp 1953-1957.

Hamady, M., Walker, J. J., Harris, J. K., Gold, N. J. & Knight, R. (2008) 'Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex'. *Nat Methods*, 5 (3). pp 235-237.

Hansson, S., Jodal, U., Lincoln, K. & Svanborg-Eden, C. (1989) 'Untreated asymptomatic bacteriuria in girls: II--Effect of phenoxymethylpenicillin and erythromycin given for intercurrent infections'. *Bmj*, 298 (6677). pp 856-859.

Hardwick, S. A., Deveson, I. W. & Mercer, T. R. (2017) 'Reference standards for next-generation sequencing'. *Nature Reviews Genetics*, 18 pp 473.

Harmon, R. C., Rutherford, R. L., Wu, H. M. & Collins, M. S. (1989) 'Monoclonal antibody-mediated protection and neutralization of motility in experimental Proteus mirabilis infection'. *Infect Immun*, 57 (7). pp 1936-1941.

Herren, C. D., Mitra, A., Palaniyandi, S. K., Coleman, A., Elankumaran, S. & Mukhopadhyay, S. (2006) 'The BarA-UvrY two-component system regulates virulence in avian pathogenic Escherichia coli O78:K80:H9'. *Infect Immun*, 74 (8). pp 4900-4909.

Hertz, F. B., Nielsen, J. B., Schonning, K., Littauer, P., Knudsen, J. D., Lobner-Olesen, A. & Frimodt-Moller, N. (2016) '"Population structure of drug-susceptible,-resistant and ESBL-producing Escherichia coli from community-acquired urinary tract"'. *BMC Microbiol*, 16 pp 63.

Hickling, D. R., Sun, T. T. & Wu, X. R. (2015) 'Anatomy and Physiology of the Urinary Tract: Relation to Host Defense and Microbial Infection'. *Microbiol Spectr*, 3 (4).

Hidalgo, G., Chan, M. & Tufenkji, N. (2011) 'Inhibition of Escherichia coli CFT073 fliC expression and motility by cranberry materials'. *Appl Environ Microbiol*, 77 (19). pp 6852-6857.

Hochhut, B., Wilde, C., Balling, G., Middendorf, B., Dobrindt, U., Brzuszkiewicz, E., Gottschalk, G., Carniel, E. & Hacker, J. (2006) 'Role of pathogenicity island-associated integrases in the genome plasticity of uropathogenic Escherichia coli strain 536'. *Mol Microbiol*, 61 (3). pp 584-595.

Hooton, T. M., Roberts, P. L., Cox, M. E. & Stapleton, A. E. (2013) 'Voided midstream urine culture and acute cystitis in premenopausal women'. *N Engl J Med*, 369 (20). pp 1883-1891.

Horvath, D. J., Jr., Li, B., Casper, T., Partida-Sanchez, S., Hunstad, D. A., Hultgren, S. J. & Justice, S. S. (2011) 'Morphological plasticity promotes resistance to phagocyte killing of uropathogenic Escherichia coli'. *Microbes Infect*, 13 (5). pp 426-437.

How, K. Y., Song, K. P. & Chan, K. G. (2016) 'Porphyromonas gingivalis: An Overview of Periodontopathic Pathogen below the Gum Line'. *Frontiers in microbiology*, 7 pp 53-53.

Huang, M. M., Arnheim, N. & Goodman, M. F. (1992) 'Extension of base mispairs by Taq DNA polymerase: implications for single nucleotide discrimination in PCR'. *Nucleic Acids Res*, 20 (17). pp 4567-4573.

Hull, R., Rudy, D., Donovan, W., Svanborg, C., Wieser, I., Stewart, C. & Darouiche, R. (2000) 'Urinary tract infection prophylaxis using Escherichia coli 83972 in spinal cord injured patients'. *J Urol*, 163 (3). pp 872-877.

Hull, R. A., Rudy, D. C., Donovan, W. H., Wieser, I. E., Stewart, C. & Darouiche, R. O. (1999) 'Virulence properties of Escherichia coli 83972, a prototype strain associated with asymptomatic bacteriuria'. *Infect Immun*, 67 (1). pp 429-432.

Hullegie, S., Wootton, M., Verheij, T. J. M., Thomas-Jones, E., Bates, J., Hood, K., Gal, M., Francis, N. A., Little, P., Moore, M., Llor, C., Pickles, T., Gillespie, D., Kirby, N., Brugman, C. & Butler, C. C. (2017) 'Clinicians' interpretations of point of care urine culture versus laboratory culture results: analysis from the four-country POETIC trial of diagnosis of uncomplicated urinary tract infection in primary care'. *Fam Pract*, 34 (4). pp 392-399.

Huovinen, P. (1987) 'Trimethoprim resistance'. *Antimicrob Agents Chemother*, 31 (10). pp 1451-1456.

Huovinen, P. (2001) 'Resistance to trimethoprim-sulfamethoxazole'. *Clin Infect Dis*, 32 (11). pp 1608-1614.

Huovinen, P., Sundström, L., Swedberg, G. & Sköld, O. (1995) 'Trimethoprim and sulfonamide resistance'. *Antimicrob Agents Chemother*, 39 (2). pp 279-289.

Huycke, M. M., Sahm, D. F. & Gilmore, M. S. (1998) 'Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future'. *Emerg Infect Dis*, 4 (2). pp 239-249.

Hyatt, D., Chen, G. L., Locascio, P. F., Land, M. L., Larimer, F. W. & Hauser, L. J. (2010) 'Prodigal: prokaryotic gene recognition and translation initiation site identification'. *BMC Bioinformatics*, 11 pp 119.

Iguchi, A., Iyoda, S., Seto, K., Morita-Ishihara, T., Scheutz, F. & Ohnishi, M. (2015) 'Escherichia coli O-Genotyping PCR: a Comprehensive and Practical Platform for Molecular O Serogrouping'. *J Clin Microbiol*, 53 (8). pp 2427-2432.

Imani Fooladi, A. A., Bagherpour, G., Khoramabadi, N., Fallah Mehrabadi, J., Mahdavi, M., Halabian, R., Amin, M., Izadi Mobarakeh, J. & Einollahi, B. (2014) 'Cellular immunity survey against urinary tract infection using pVAX/fimH cassette with mammalian and wild type codon usage as a DNA vaccine'. *Clin Exp Vaccine Res*, 3 (2). pp 185-193.

Ingham, H. R. & Sisson, P. R. (1984) 'Pathogenic synergism'. *Microbiol Sci*, 1 (8). pp 206-208.

Isaacs, D. (2014) 'Evidence Based Neonatal Infections'.[Book] in Wiley-Blackwell. (86). (Accessed:Isaacs, D.

Jacob, F. & Monod, J. (1961) 'Genetic regulatory mechanisms in the synthesis of proteins'. *J Mol Biol*, 3 pp 318-356.

Jacob, F., Perrin, D., Sanchez, C. & Monod, J. (1960) '[Operon: a group of genes with the expression coordinated by an operator]'. *C R Hebd Seances Acad Sci*, 250 pp 1727-1729.

Jaiswal, S., Pati, N. B., Dubey, M., Padhi, C., Sahoo, P. K., Ray, S., Arunima, A., Mohakud, N. K. & Suar, M. (2015) 'The O-antigen negative wbaV mutant of Salmonella enterica serovar Enteritidis shows adaptive resistance to antimicrobial peptides and elicits colitis in streptomycin pretreated mouse model'. *Gut Pathog*, 7 pp 24.

Jann, K., Schmidt, G., Blumenstock, E. & Vosbeck, K. (1981) 'Escherichia coli adhesion to Saccharomyces cerevisiae and mammalian cells: role of piliation and surface hydrophobicity'. *Infect Immun*, 32 (2). pp 484-489.

Joensen, K. G., Scheutz, F., Lund, O., Hasman, H., Kaas, R. S., Nielsen, E. M. & Aarestrup, F. M. (2014) 'Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli'. *J Clin Microbiol*, 52 (5). pp 1501-1510.

Joensen, K. G., Tetzschner, A. M., Iguchi, A., Aarestrup, F. M. & Scheutz, F. (2015) 'Rapid and Easy In Silico Serotyping of Escherichia coli Isolates by Use of Whole-Genome Sequencing Data'. *J Clin Microbiol*, 53 (8). pp 2410-2426.

Johnson, J. R. (1991) 'Virulence factors in Escherichia coli urinary tract infection'. *Clin Microbiol Rev*, 4 (1). pp 80-128.

Johnson, J. R., Johnston, B., Clabots, C. R., Kuskowski, M. A., Roberts, E. & DebRoy, C. (2008) 'Virulence genotypes and phylogenetic background of Escherichia coli serogroup O6 isolates from humans, dogs, and cats'. *J Clin Microbiol*, 46 (2). pp 417-422.

Johnson, J. R. & Stell, A. L. (2000) 'Extended virulence genotypes of Escherichia coli strains from patients with urosepsis in relation to phylogeny and host compromise'. *J Infect Dis*, 181 (1). pp 261-272.

Johnson, J. R., Stell, A. L., O'Bryan, T. T., Kuskowski, M., Nowicki, B., Johnson, C., Maslow, J. N., Kaul, A., Kavle, J. & Prats, G. (2002) 'Global molecular epidemiology of the O15:K52:H1 extraintestinal pathogenic Escherichia coli clonal group: evidence of distribution beyond Europe'. *J Clin Microbiol*, 40 (6). pp 1913-1923.

Jones, G. W., Richardson, L. A. & Uhlman, D. (1981) 'The invasion of HeLa cells by Salmonella typhimurium: reversible and irreversible bacterial attachment and the role of bacterial motility'. *J Gen Microbiol*, 127 (2). pp 351-360.

Justice, S. S., Hung, C., Theriot, J. A., Fletcher, D. A., Anderson, G. G., Footer, M. J. & Hultgren, S. J. (2004) 'Differentiation and developmental pathways of uropathogenic Escherichia coli in urinary tract pathogenesis'. *Proc Natl Acad Sci U S A*, 101 (5). pp 1333-1338.

Justice, S. S., Hunstad, D. A., Seed, P. C. & Hultgren, S. J. (2006) 'Filamentation by Escherichia coli subverts innate defenses during urinary tract infection'. *Proc Natl Acad Sci U S A*, 103 (52). pp 19884-19889.

Kaas, R. S., Leekitcharoenphon, P., Aarestrup, F. M. & Lund, O. (2014) 'Solving the problem of comparing whole bacterial genomes across different sequencing platforms'. *PLoS One*, 9 (8). pp e104984.

Kakkanat, A., Totsika, M., Schaale, K., Duell, B. L., Lo, A. W., Phan, M. D., Moriel, D. G., Beatson, S. A., Sweet, M. J., Ulett, G. C. & Schembri, M. A. (2015) 'The role of H4 flagella in Escherichia coli ST131 virulence'. *Sci Rep*, 5 pp 16149.

Kallonen, T., Brodrick, H. J., Harris, S. R., Corander, J., Brown, N. M., Martin, V., Peacock, S. J. & Parkhill, J. (2017) 'Systematic longitudinal survey of invasive Escherichia coli in England demonstrates a stable population structure only transiently disturbed by the emergence of ST131'. *Genome Res*,

Kam, S.-K., Lee, W.-S., Ou, T.-Y., Teng, S.-O. & Chen, F.-L. (2012) 'Delftia acidovorans Bacteremia Associated with Ascending Urinary Tract Infections Proved by Molecular Method'. *Journal of Experimental & Clinical Medicine*, 4 (3). pp 180-182.

Kaper, J. B. (2005) 'Pathogenic Escherichia coli'. *Int J Med Microbiol*, 295 (6-7). pp 355-356.

Kaper, J. B., Nataro, J. P. & Mobley, H. L. (2004) 'Pathogenic Escherichia coli'. *Nat Rev Microbiol*, 2 (2). pp 123-140.

Kart, D., Kustimur, A. S., Sagiroglu, M. & Kalkanci, A. (2017) 'Evaluation of Antimicrobial Durability and Anti-Biofilm Effects in Urinary Catheters Against Enterococcus faecalis Clinical Isolates and Reference Strains'. *Balkan Med J*, 34 (6). pp 546-552.

Kass, E. H. (1956) 'Asymptomatic infections of the urinary tract'. *Trans Assoc Am Physicians*, 69 pp 56-64.

Kass, E. H. (1957) 'Bacteriuria and the diagnosis of infections of the urinary tract; with observations on the use of methionine as a urinary antiseptic'. *AMA Arch Intern Med*, 100 (5). pp 709-714.

Kauffmann, F. (1946) 'Studies on the serology of the Escherichia coli group'. *J Bacteriol*, 51 pp 126.

Kauffmann, F. (1947) 'The serology of the coli group'. *J Immunol*, 57 (1). pp 71-100.

Kauffmann, F. (1964) '[SEROLOGY AND CHEMISTRY OF ENTEROBACTERIACEAE]'. *Arb Paul Ehrlich Inst Georg Speyer Haus Ferdinand Blum Inst Frankf A M*, 61 pp 17-28.

Kearns, D. B. (2010) 'A field guide to bacterial swarming motility'. *Nat Rev Microbiol*, 8 (9). pp 634-644.

Kelly, A. T. & Fulton, M. (1953) 'Use of triphenyl tetrazolium in motility test medium'. *Am J Clin Pathol*, 23 (5). pp 512.

Khan, A. S., Kniep, B., Oelschlaeger, T. A., Van Die, I., Korhonen, T. & Hacker, J. (2000) 'Receptor structure for F1C fimbriae of uropathogenic Escherichia coli'. *Infect Immun*, 68 (6). pp 3541-3547.

Khan, N. A., Kim, Y., Shin, S. & Kim, K. S. (2007) 'FimH-mediated Escherichia coli K1 invasion of human brain microvascular endothelial cells'. *Cell Microbiol*, 9 (1). pp 169-178.

Khandelwal, P., Abraham, S. N. & Apodaca, G. (2009) 'Cell biology and physiology of the uroepithelium'. *Am J Physiol Renal Physiol*, 297 (6). pp F1477-1501.

Khandelwal, P., Ruiz, W. G. & Apodaca, G. (2010) 'Compensatory endocytosis in bladder umbrella cells occurs through an integrin-regulated and RhoA- and dynamin-dependent pathway'. *Embo j*, 29 (12). pp 1961-1975.

Kolbe, D. L. & Eddy, S. R. (2011) 'Fast filtering for RNA homology search'. *Bioinformatics*, 27 (22). pp 3102-3109.

Komatsu, H., Hayashi, F., Sasa, M., Shikata, K., Yamaguchi, S., Namba, K. & Oosawa, K. (2016) 'Genetic analysis of revertants isolated from the rod-fragile fliF mutant of Salmonella'. *Biophys Physicobiol*, 13 pp 13-25.

Kommedal, O., Wilhelmsen, M. T., Skrede, S., Meisal, R., Jakovljev, A., Gaustad, P., Hermansen, N. O., Vik-Mo, E., Solheim, O., Ambur, O. H., Saebo, O., Hostmaelingen, C. T. & Helland, C. (2014) 'Massive parallel sequencing provides new perspectives on bacterial brain abscesses'. *J Clin Microbiol*, 52 (6). pp 1990-1997.

Kommineni, S., Bretl, D. J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., Cao, Y., Bousounis, P., Kristich, C. J. & Salzman, N. H. (2015) 'Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract'. *Nature*, 526 (7575). pp 719-722.

Korea, C. G., Badouraly, R., Prevost, M. C., Ghigo, J. M. & Beloin, C. (2010) 'Escherichia coli K-12 possesses multiple cryptic but functional chaperone-usher fimbriae with distinct surface specificities'. *Environ Microbiol*, 12 (7). pp 1957-1977.

Koren, S., Harhay, G. P., Smith, T. P., Bono, J. L., Harhay, D. M., McVey, S. D., Radune, D., Bergman, N. H. & Phillippy, A. M. (2013) 'Reducing assembly complexity of microbial genomes with single-molecule sequencing'. *Genome Biol*, 14 (9). pp R101.

Korhonen, T. K., Parkkinen, J., Hacker, J., Finne, J., Pere, A., Rhen, M. & Holthöfer, H. (1986) 'Binding of Escherichia coli S fimbriae to human kidney epithelium'. *Infection and Immunity*, 54 (2). pp 322-327.

Kram, K. E., Geiger, C., Ismail, W. M., Lee, H., Tang, H., Foster, P. L. & Finkel, S. E. (2017) 'Adaptation of Escherichia coli to Long-Term Serial Passage in Complex Medium: Evidence of Parallel Evolution'. *mSystems*, 2 (2).

Kreft, B., Placzek, M., Doehn, C., Hacker, J., Schmidt, G., Wasenauer, G., Daha, M. R., van der Woude, F. J. & Sack, K. (1995) 'S fimbriae of uropathogenic Escherichia coli bind to primary human renal proximal tubular epithelial cells but do not induce expression of intercellular adhesion molecule 1'. *Infect Immun*, 63 (8). pp 3235-3238.

Kreft, M. E., Sterle, M., Veranic, P. & Jezernik, K. (2005) 'Urothelial injuries and the early wound healing response: tight junctions and urothelial cytodifferentiation'. *Histochem Cell Biol*, 123 (4-5). pp 529-539.

Kubori, T., Shimamoto, N., Yamaguchi, S., Namba, K. & Aizawa, S. (1992) 'Morphological pathway of flagellar assembly in Salmonella typhimurium'. *J Mol Biol*, 226 (2). pp 433-446.

Kuehl, C. J., Dragoi, A.-M., Talman, A. & Agaisse, H. (2015) 'Bacterial spread from cell to cell: beyond actin-based motility'. *Trends Microbiol*, 23 (9). pp 558-566.

Kuhnert, P., Boerlin, P. & Frey, J. (2000) 'Target genes for virulence assessment of Escherichia coli isolates from water, food and the environment'. *FEMS Microbiol Rev*, 24 (1). pp 107-117.

Kukkonen, M., Raunio, T., Virkola, R., Lahteenmaki, K., Makela, P. H., Klemm, P., Clegg, S. & Korhonen, T. K. (1993) 'Basement membrane carbohydrate as a target for bacterial adhesion: binding of type I fimbriae of Salmonella enterica and Escherichia coli to laminin'. *Mol Microbiol*, 7 (2). pp 229-237.

Kunin, C. M., White, L. V. & Hua, T. H. (1993) 'A reassessment of the importance of "low-count" bacteriuria in young women with acute urinary symptoms'. *Ann Intern Med*, 119 (6). pp 454-460.

Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C. & Salzberg, S. L. (2004) 'Versatile and open software for comparing large genomes'. *Genome Biol*, 5 (2). pp R12.

Ladomenou, F., Moschandreas, J., Kafatos, A., Tselentis, Y. & Galanakis, E. (2010) 'Protective effect of exclusive breastfeeding against infections during infancy: a prospective study'. *Arch Dis Child*, 95 (12). pp 1004-1008.

Lagesen, K., Hallin, P., Rodland, E. A., Staerfeldt, H. H., Rognes, T. & Ussery, D. W. (2007) 'RNAmmer: consistent and rapid annotation of ribosomal RNA genes'. *Nucleic Acids Res*, 35 (9). pp 3100-3108.

Lam, O., Wheeler, J. & Tang, C. M. (2014) 'Thermal control of virulence factors in bacteria: a hot topic'. *Virulence*, 5 (8). pp 852-862.

Land, M., Hauser, L., Jun, S. R., Nookaew, I., Leuze, M. R., Ahn, T. H., Karpinets, T., Lund, O., Kora, G., Wassenaar, T., Poudel, S. & Ussery, D. W. (2015) 'Insights from 20 years of bacterial genome sequencing'. *Funct Integr Genomics*, 15 (2). pp 141-161.

Landraud, L., Pulcini, C., Gounon, P., Flatau, G., Boquet, P. & Lemichez, E. (2004) 'E. coli CNF1 toxin: a two-in-one system for host-cell invasion'. *Int J Med Microbiol*, 293 (7-8). pp 513-518.

Lane, M. C., Alteri, C. J., Smith, S. N. & Mobley, H. L. (2007) 'Expression of flagella is coincident with uropathogenic Escherichia coli ascension to the upper urinary tract'. *Proc Natl Acad Sci U S A*, 104 (42). pp 16669-16674.

Lane, M. C., Lockatell, V., Monterosso, G., Lamphier, D., Weinert, J., Hebel, J. R., Johnson, D. E. & Mobley, H. L. T. (2005) 'Role of Motility in the Colonization of Uropathogenic Escherichia coli in the Urinary Tract'. *Infection and Immunity*, 73 (11). pp 7644-7656.

Lane, M. C. & Mobley, H. L. (2007) 'Role of P-fimbrial-mediated adherence in pyelonephritis and persistence of uropathogenic Escherichia coli (UPEC) in the mammalian kidney'. *Kidney Int*, 72 (1). pp 19-25.

Larsen, B. & Monif, G. R. (2001) 'Understanding the bacterial flora of the female genital tract'. *Clin Infect Dis*, 32 (4). pp e69-77.

Laslett, D. & Canback, B. (2004) 'ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences'. *Nucleic Acids Res*, 32 (1). pp 11-16.

Lau, S. H., Kaufmann, M. E., Livermore, D. M., Woodford, N., Willshaw, G. A., Cheasty, T., Stamper, K., Reddy, S., Cheesbrough, J., Bolton, F. J., Fox, A. J. & Upton, M. (2008a) 'UK epidemic Escherichia coli strains A-E, with CTX-M-15 beta-lactamase, all belong to the international O25:H4-ST131 clone'. *J Antimicrob Chemother*, 62 (6). pp 1241-1244.

Lau, S. H., Reddy, S., Cheesbrough, J., Bolton, F. J., Willshaw, G., Cheasty, T., Fox, A. J. & Upton, M. (2008b) 'Major uropathogenic Escherichia coli strain isolated in the northwest of England identified by multilocus sequence typing'. *J Clin Microbiol*, 46 (3). pp 1076-1080.

Lau, S. K., Woo, P. C., Li, N. K., Teng, J. L., Leung, K. W., Ng, K. H., Que, T. L. & Yuen, K. Y. (2006) 'Globicatella bacteraemia identified by 16S ribosomal RNA gene sequencing'. *J Clin Pathol*, 59 (3). pp 303-307.

Lavigne, J. P., Nicolas-Chanoine, M. H., Bourg, G., Moreau, J. & Sotto, A. (2008) 'Virulent synergistic effect between Enterococcus faecalis and Escherichia coli assayed by using the Caenorhabditis elegans model'. *PLoS One*, 3 (10). pp e3370.

Lazdunski, C. J., Bouveret, E., Rigal, A., Journet, L., Lloubes, R. & Benedetti, H. (1998) 'Colicin import into Escherichia coli cells'. *J Bacteriol*, 180 (19). pp 4993-5002.

Lee, H., Popodi, E., Tang, H. & Foster, P. L. (2012) 'Rate and molecular spectrum of spontaneous mutations in the bacterium Escherichia coli as determined by whole-genome sequencing'. *Proc Natl Acad Sci U S A*, 109 (41). pp E2774-2783.

Leonard, A. F. C., Zhang, L., Balfour, A. J., Garside, R., Hawkey, P. M., Murray, A. K., Ukoumunne, O. C. & Gaze, W. H. (2018) 'Exposure to and colonisation by antibiotic-resistant E. coli in UK coastal water users: Environmental

surveillance, exposure assessment, and epidemiological study (Beach Bum Survey)'. *Environ Int*, 114 pp 326-333.

Lerouge, I. & Vanderleyden, J. (2002) 'O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions'. *FEMS Microbiol Rev*, 26 (1). pp 17-47.

Levy, I., Comarsca, J., Davidovits, M., Klinger, G., Sirota, L. & Linder, N. (2009) 'Urinary tract infection in preterm infants: the protective role of breastfeeding'. *Pediatr Nephrol*, 24 (3). pp 527-531.

Lewis, A. J., Richards, A. C. & Mulvey, M. A. (2016) 'Invasion of Host Cells and Tissues by Uropathogenic Bacteria'. *Microbiology spectrum*, 4 (6). pp 10.1128/microbiolspec.UTI-0026-2016.

Li, D., Liu, B., Chen, M., Guo, D., Guo, X., Liu, F., Feng, L. & Wang, L. (2010) 'A multiplex PCR method to detect 14 Escherichia coli serogroups associated with urinary tract infections'. *J Microbiol Methods*, 82 (1). pp 71-77.

Li, H. & Durbin, R. (2010) 'Fast and accurate long-read alignment with Burrows-Wheeler transform'. *Bioinformatics*, 26 (5). pp 589-595.

Liang, F., Kachar, B., Ding, M., Zhai, Z., Wu, X. R. & Sun, T. T. (1999) 'Urothelial hinge as a highly specialized membrane: detergent-insolubility, urohingin association, and in vitro formation'. *Differentiation*, 65 (1). pp 59-69.

Liang, F. X., Riedel, I., Deng, F. M., Zhou, G., Xu, C., Wu, X. R., Kong, X. P., Moll, R. & Sun, T. T. (2001) 'Organization of uroplakin subunits: transmembrane topology, pair formation and plaque composition'. *Biochem J*, 355 (Pt 1). pp 13-18.

Lijek, R. S. & Weiser, J. N. (2012) 'Co-infection subverts mucosal immunity in the upper respiratory tract'. *Curr Opin Immunol*, 24 (4). pp 417-423.

Lillehoj, E. P., Kim, B. T. & Kim, K. C. (2002) 'Identification of Pseudomonas aeruginosa flagellin as an adhesin for Muc1 mucin'. *Am J Physiol Lung Cell Mol Physiol*, 282 (4). pp L751-756.

Lin, J., Lee, I. S., Frey, J., Slonczewski, J. L. & Foster, J. W. (1995) 'Comparative analysis of extreme acid survival in Salmonella typhimurium, Shigella flexneri, and Escherichia coli'. *J Bacteriol*, 177 (14). pp 4097-4104.

Lindberg, U., Hanson, L. A., Jodal, U., Lidin-Janson, G., Lincoln, K. & Olling, S. (1975) 'Asymptomatic bacteriuria in schoolgirls. II. Differences in escherichia coli causing asymptomatic bacteriuria'. *Acta Paediatr Scand*, 64 (3). pp 432-436.

Linke, D., Riess, T., Autenrieth, I. B., Lupas, A. & Kempf, V. A. (2006) 'Trimeric autotransporter adhesins: variable structure, common function'. *Trends Microbiol*, 14 (6). pp 264-270.

Liu, S. L., Ezaki, T., Miura, H., Matsui, K. & Yabuuchi, E. (1988) 'Intact motility as a Salmonella typhi invasion-related factor'. *Infect Immun*, 56 (8). pp 1967-1973.

Lo, A. W., Moriel, D. G., Phan, M. D., Schulz, B. L., Kidd, T. J., Beatson, S. A. & Schembri, M. A. (2017) ''Omic' Approaches to Study Uropathogenic Escherichia coli Virulence'. *Trends Microbiol*, 25 (9). pp 729-740.

Loveday, H. P., Wilson, J. A., Pratt, R. J., Golsorkhi, M., Tingle, A., Bak, A., Browne, J., Prieto, J., Wilcox, M. & Health, U. K. D. o. (2014) 'epic3: national evidence-based guidelines for preventing healthcare-associated infections in NHS hospitals in England'. *J Hosp Infect*, 86 Suppl 1 pp S1-70.

Luck, S. N., Badea, L., Bennett-Wood, V., Robins-Browne, R. & Hartland, E. L. (2006) 'Contribution of FliC to epithelial cell invasion by enterohemorrhagic Escherichia coli O113:H21'. *Infect Immun*, 74 (12). pp 6999-7004.

Lun, A., Ziebig, R., Priem, F., Filler, G. & Sinha, P. (1999) 'Routine workflow for use of urine strips and urine flow cytometer UF-100 in the hospital laboratory'. *Clin Chem*, 45 (8 Pt 1). pp 1305-1307.

Lund, B., Lindberg, F., Marklund, B. I. & Normark, S. (1988) 'Tip proteins of pili associated with pyelonephritis: new candidates for vaccine development'. *Vaccine*, 6 (2). pp 110-112.

Madelung, M., Kronborg, T., Doktor, T. K., Struve, C., Krogfelt, K. A. & Møller-Jensen, J. (2017) 'DFI-seq identification of environment-specific gene expression in uropathogenic Escherichia coli'. *BMC Microbiol*, 17 pp 99.

Madsen, K. T., Skov, M. N., Gill, S. & Kemp, M. (2017) 'Virulence Factors Associated with Enterococcus Faecalis Infective Endocarditis: A Mini Review'. *Open Microbiol J*, 11 pp 1-11.

Magariyama, Y., Sugiyama, S., Muramoto, K., Maekawa, Y., Kawagishi, I., Imae, Y. & Kudo, S. (1994) 'Very fast flagellar rotation'. *Nature*, 371 pp 752.

Mahajan, A., Currie, C. G., Mackie, S., Tree, J., McAteer, S., McKendrick, I., McNeilly, T. N., Roe, A., La Ragione, R. M., Woodward, M. J., Gally, D. L. & Smith, D. G. (2009) 'An investigation of the expression and adhesin function of H7 flagella in the interaction of Escherichia coli O157 : H7 with bovine intestinal epithelium'. *Cell Microbiol*, 11 (1). pp 121-137.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M. & Spratt, B. G. (1998) 'Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms'. *Proc Natl Acad Sci U S A*, 95 (6). pp 3140-3145.

Malagolini, N., Cavallone, D., Wu, X. R. & Serafini-Cessi, F. (2000) 'Terminal glycosylation of bovine uroplakin III, one of the major integral-membrane glycoproteins of mammalian bladder'. *Biochim Biophys Acta*, 1475 (3). pp 231-237.

Manges, A. R., Johnson, J. R., Foxman, B., O'Bryan, T. T., Fullerton, K. E. & Riley, L. W. (2001) 'Widespread distribution of urinary tract infections caused by a multidrug-resistant Escherichia coli clonal group'. *N Engl J Med*, 345 (14). pp 1007-1013.

Mansour, L. & Mansour, A. (1993) 'Breast feeding protects infants against urinary tract infection'. *New Egypt J Med*, 8 (2). pp 463-464.

Marild, S., Hansson, S., Jodal, U., Oden, A. & Svedberg, K. (2004) 'Protective effect of breastfeeding against urinary tract infection'. *Acta Paediatr*, 93 (2). pp 164-168.

Martin-Sosa, S., Martin, M. J. & Hueso, P. (2002) 'The sialylated fraction of milk oligosaccharides is partially responsible for binding to enterotoxigenic and uropathogenic Escherichia coli human strains'. *J Nutr*, 132 (10). pp 3067-3072.

Martinez, J. J. & Hultgren, S. J. (2002) 'Requirement of Rho-family GTPases in the invasion of Type 1-piliated uropathogenic Escherichia coli'. *Cell Microbiol*, 4 (1). pp 19-28.

Martinez, J. J., Mulvey, M. A., Schilling, J. D., Pinkner, J. S. & Hultgren, S. J. (2000) 'Type 1 pilus-mediated bacterial invasion of bladder epithelial cells'. *Embo j*, 19 (12). pp 2803-2812.

Matsumoto, H. & Young, G. M. (2009) 'Translocated effectors of Yersinia'. *Curr Opin Microbiol*, 12 (1). pp 94-100.

Matsunami, M., Otsuka, Y., Ohkusu, K., Sogi, M., Kitazono, H. & Hosokawa, N. (2012) 'Urosepsis caused by Globicatella sanguinis and Corynebacterium riegelii in an adult: case report and literature review'. *J Infect Chemother*, 18 (4). pp 552-554.

Mattick, J. S. (2002) 'Type IV pili and twitching motility'. *Annu Rev Microbiol*, 56 pp 289-314.

McCarter, L. L. (2005) 'Multiple Modes of Motility: a Second Flagellar System in Escherichia coli'. *J Bacteriol*, 187 (4). pp 1207-1209.

McMurry, J. L., Van Arnam, J. S., Kihara, M. & Macnab, R. M. (2004) 'Analysis of the cytoplasmic domains of Salmonella FlhA and interactions with components of the flagellar export machinery'. *J Bacteriol*, 186 (22). pp 7586-7592.

McNally, A., Alhashash, F., Collins, M., Alqasim, A., Paszckiewicz, K., Weston, V. & Diggle, M. (2013) 'Genomic analysis of extra-intestinal pathogenic Escherichia coli urosepsis'. *Clin Microbiol Infect*, 19 (8). pp E328-334.

McNulty, C. (2017) 'Public Health England, Management and treatment of common infections: guidance for consultation and adaptation'.

McTaggart, L. A., Rigby, R. C. & Elliott, T. S. (1990) 'The pathogenesis of urinary tract infections associated with Escherichia coli, Staphylococcus saprophyticus and S. epidermidis'. *J Med Microbiol*, 32 (2). pp 135-141.

Mendelman, L. V., Boosalis, M. S., Petruska, J. & Goodman, M. F. (1989) 'Nearest neighbor influences on DNA polymerase insertion fidelity'. *J Biol Chem*, 264 (24). pp 14415-14423.

Merino, S., Rubires, X., Aguilar, A. & Tomas, J. M. (1997) 'The role of flagella and motility in the adherence and invasion to fish cell lines by Aeromonas hydrophila serogroup O:34 strains'. *FEMS Microbiol Lett*, 151 (2). pp 213-217.

Mignot, T. (2007) 'The elusive engine in Myxococcus xanthus gliding motility'. *Cell Mol Life Sci*, 64 (21). pp 2733-2745.

Miles, A. A., Misra, S. S. & Irwin, J. O. (1938) 'The estimation of the bactericidal power of the blood'. *J Hyg (Lond)*, 38 (6). pp 732-749.

Minamino, T., Imada, K. & Namba, K. (2008) 'Mechanisms of type III protein export for bacterial flagellar assembly'. *Mol Biosyst*, 4 (11). pp 1105-1115.

Minardi, D., d'Anzeo, G., Cantoro, D., Conti, A. & Muzzonigro, G. (2011) 'Urinary tract infections in women: etiology and treatment options'. *Int J Gen Med*, 4 pp 333-343.

Mitra, A., Palaniyandi, S., Herren, C. D., Zhu, X. & Mukhopadhyay, S. (2013) 'Pleiotropic roles of uvrY on biofilm formation, motility and virulence in uropathogenic Escherichia coli CFT073'. *PLoS One*, 8 (2). pp e55492.

Mol, O. & Oudega, B. (1996) 'Molecular and structural aspects of fimbriae biosynthesis and assembly in Escherichia coli'. *FEMS Microbiol Rev*, 19 (1). pp 25-52.

Montravers, P., Mohler, J., Saint Julien, L. & Carbon, C. (1997) 'Evidence of the proinflammatory role of Enterococcus faecalis in polymicrobial peritonitis in rats'. *Infect Immun*, 65 (1). pp 144-149.

Moore, W. E. & Moore, L. V. (1994) 'The bacteria of periodontal diseases'. *Periodontol 2000*, 5 pp 66-77.

Moran-Gilad, J., Rokney, A., Danino, D., Ferdous, M., Alsana, F., Baum, M., Dukhan, L., Agmon, V., Anuka, E., Valinsky, L., Yishay, R., Grotto, I., Rossen, J. W. A. & Gdalevich, M. (2017) 'Real-time genomic investigation underlying the public health response to a Shiga toxin-producing Escherichia coli O26:H11 outbreak in a nursery'. *Epidemiol Infect*, 145 (14). pp 2998-3006.

Moriel, D. G., Tan, L., Goh, K. G., Phan, M. D., Ipe, D. S., Lo, A. W., Peters, K. M., Ulett, G. C., Beatson, S. A. & Schembri, M. A. (2016) 'A Novel Protective Vaccine Antigen from the Core Escherichia coli Genome'. *mSphere*, 1 (6).

Morschhauser, J., Hoschutzky, H., Jann, K. & Hacker, J. (1990) 'Functional analysis of the sialic acid-binding adhesin SfaS of pathogenic Escherichia coli by site-specific mutagenesis'. *Infect Immun*, 58 (7). pp 2133-2138.

Moulin-Schouleur, M., Reperant, M., Laurent, S., Bree, A., Mignon-Grasteau, S., Germon, P., Rasschaert, D. & Schouler, C. (2007) 'Extraintestinal pathogenic Escherichia coli strains of avian and human origin: link between phylogenetic relationships and common virulence patterns'. *J Clin Microbiol*, 45 (10). pp 3366-3376.

Muhlenkamp, M., Oberhettinger, P., Leo, J. C., Linke, D. & Schutz, M. S. (2015) 'Yersinia adhesin A (YadA)--beauty & beast'. *Int J Med Microbiol*, 305 (2). pp 252-258.

Mulvey, M. A. (2002) 'Adhesion and entry of uropathogenic Escherichia coli'. *Cell Microbiol*, 4 (5). pp 257-271.

Mulvey, M. A., Lopez-Boado, Y. S., Wilson, C. L., Roth, R., Parks, W. C., Heuser, J. & Hultgren, S. J. (1998) 'Induction and evasion of host defenses by type 1-piliated uropathogenic Escherichia coli'. *Science*, 282 (5393). pp 1494-1497.

Mulvey, M. A., Schilling, J. D. & Hultgren, S. J. (2001) 'Establishment of a persistent Escherichia coli reservoir during the acute phase of a bladder infection'. *Infect Immun*, 69 (7). pp 4572-4579.

Mydock-McGrane, L. K., Hannan, T. J. & Janetka, J. W. (2017) 'Rational design strategies for FimH antagonists: new drugs on the horizon for urinary tract infection and Crohn's disease'. *Expert Opin Drug Discov*, 12 (7). pp 711-731.

Najar, M. S., Saldanha, C. L. & Banday, K. A. (2009) 'Approach to urinary tract infections'. *Indian J Nephrol*, 19 (4). pp 129-139.

National Human Genomic Research Institute (2003) 'The Human Genome Project Complettion: Frequently Asked Questions'. (https://www.genome.gov/11006943/human-genome-project-completion-frequently-asked-questions/)

Nichols, G. L. (1975) 'Variants of Escherichia coli giving the appearance of mixed growths in urine'. *J Clin Pathol*, 28 (9). pp 728-730.

Nicolle, L. E. (2001) 'Urinary tract pathogens in complicated infection and in elderly individuals'. *J Infect Dis*, 183 Suppl 1 pp S5-8.

Nicolle, L. E. (2015) 'Asymptomatic Bacteriuria and Bacterial Interference'. *Microbiol Spectr*, 3 (5).

Nielsen, K. L., Dynesen, P., Larsen, P. & Frimodt-Moller, N. (2014) 'Faecal Escherichia coli from patients with E. coli urinary tract infection and healthy controls who have never had a urinary tract infection'. *J Med Microbiol*, 63 (Pt 4). pp 582-589.

Norton, C., Whitehead, W. E., Bliss, D. Z., Harari, D. & Lang, J. (2010) 'Management of fecal incontinence in adults'. *Neurourol Urodyn*, 29 (1). pp 199-206.

Nowicki, B., Moulds, J., Hull, R. & Hull, S. (1988) 'A hemagglutinin of uropathogenic Escherichia coli recognizes the Dr blood group antigen'. *Infect Immun*, 56 (5). pp 1057-1060.

Nowicki, B., Selvarangan, R. & Nowicki, S. (2001) 'Family of Escherichia coli Dr adhesins: decay-accelerating factor receptor recognition and invasiveness'. *J Infect Dis*, 183 Suppl 1 pp S24-27.

Nuesch-Inderbinen, M. T., Baschera, M., Zurfluh, K., Hachler, H., Nuesch, H. & Stephan, R. (2017) 'Clonal Diversity, Virulence Potential and Antimicrobial Resistance of Escherichia coli Causing Community Acquired Urinary Tract Infection in Switzerland'. *Front Microbiol*, 8 pp 2334.

Nummelin, H., Merckel, M. C., Leo, J. C., Lankinen, H., Skurnik, M. & Goldman, A. (2004) 'The Yersinia adhesin YadA collagen-binding domain structure is a novel left-handed parallel beta-roll'. *Embo j*, 23 (4). pp 701-711.

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A. A., Korobeynikov, A., Lapidus, A., Prjibelski, A. D., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., Clingenpeel, S. R., Woyke, T., McLean, J. S., Lasken, R., Tesler, G., Alekseyev, M. A. & Pevzner, P. A. (2013) 'Assembling single-cell genomes and mini-metagenomes from chimeric MDA products'. *J Comput Biol*, 20 (10). pp 714-737.

Ochi, H., Ohtsuka, H., Yokota, S., Uezumi, I., Terashima, M., Irie, K. & Noguchi, H. (1991) 'Inhibitory activity on bacterial motility and in vivo protective activity of human monoclonal antibodies against flagella of Pseudomonas aeruginosa'. *Infect Immun*, 59 (2). pp 550-554.

Ochman, H., Elwyn, S. & Moran, N. A. (1999) 'Calibrating bacterial evolution'. *Proc Natl Acad Sci U S A*, 96 (22). pp 12638-12643.

Ochman, H. & Selander, R. K. (1984) 'Standard reference strains of Escherichia coli from natural populations'. *J Bacteriol*, 157 (2). pp 690-693.

Oelschlaeger, T. A., Barrett, T. J. & Kopecko, D. J. (1994) 'Some structures and processes of human epithelial cells involved in uptake of enterohemorrhagic Escherichia coli O157:H7 strains'. *Infect Immun*, 62 (11). pp 5142-5150.

Olesen, B., Kolmos, H. J., Orskov, F. & Orskov, I. (1998) 'Escherichia coli bacteraemia in patients with and without haematological malignancies: a study of strain characters and recurrent episodes'. *J Infect*, 36 (1). pp 93-100.

Onderdonk, A. B., Bartlett, J. G., Louie, T., Sullivan-Seigler, N. & Gorbach, S. L. (1976) 'Microbial synergy in experimental intra-abdominal abscess'. *Infect Immun*, 13 (1). pp 22-26.

Orenstein, R. & Wong, E. S. (1999) 'Urinary tract infections in adults'. *Am Fam Physician*, 59 (5). pp 1225-1234, 1237.

Osawa, K., Shigemura, K., Iguchi, A., Shirai, H., Imayama, T., Seto, K., Raharjo, D., Fujisawa, M., Osawa, R. & Shirakawa, T. (2013) 'Modulation of O-antigen chain length by the wzz gene in Escherichia coli O157 influences its sensitivities to serum complement'. *Microbiol Immunol*, 57 (9). pp 616-623.

Ott, M., Hoschutzky, H., Jann, K., Van Die, I. & Hacker, J. (1988) 'Gene clusters for S fimbrial adhesin (sfa) and F1C fimbriae (foc) of Escherichia coli: comparative aspects of structure and function'. *J Bacteriol*, 170 (9). pp 3983-3990.

Pak, J., Pu, Y., Zhang, Z. T., Hasty, D. L. & Wu, X. R. (2001) 'Tamm-Horsfall protein binds to type 1 fimbriated Escherichia coli and prevents E. coli from binding to uroplakin Ia and Ib receptors'. *J Biol Chem*, 276 (13). pp 9924-9930.

Palaniyandi, S., Mitra, A., Herren, C. D., Lockatell, C. V., Johnson, D. E., Zhu, X. & Mukhopadhyay, S. (2012) 'BarA-UvrY Two-Component System Regulates Virulence of Uropathogenic E. coli CFT073'. *PLoS One*, 7 (2). pp e31348.

Pallen, M. J., Beatson, S. A. & Bailey, C. M. (2005) 'Bioinformatics, genomics and evolution of non-flagellar type-III secretion systems: a Darwinian perpective⋆'. *FEMS Microbiol Rev*, 29 (2). pp 201-229.

Pankhurst, L. J., Del Ojo Elias, C., Votintseva, A. A., Walker, T. M., Cole, K., Davies, J., Fermont, J. M., Gascoyne-Binzi, D. M., Kohl, T. A., Kong, C., Lemaitre, N., Niemann, S., Paul, J., Rogers, T. R., Roycroft, E., Smith, E. G., Supply, P., Tang, P., Wilcox, M. H., Wordsworth, S., Wyllie, D., Xu, L. & Crook, D. W. (2016) 'Rapid, comprehensive, and affordable mycobacterial diagnosis with whole-genome sequencing: a prospective study'. *Lancet Respir Med*, 4 (1). pp 49-58.

Paradis, G., Chevance, F. F. V., Liou, W., Renault, T. T., Hughes, K. T., Rainville, S. & Erhardt, M. (2017) 'Variability in bacterial flagella re-growth patterns after breakage'. *Sci Rep*, 7 (1). pp 1282.

Parkinson, J. S. (1976) 'cheA, cheB, and cheC genes of Escherichia coli and their role in chemotaxis'. *J Bacteriol*, 126 (2). pp 758-770.

Parkkinen, J., Hacker, J. & Korhonen, T. K. (1991) 'Enhancement of tissue plasminogen activator-catalyzed plasminogen activation by Escherichia coli S fimbriae associated with neonatal septicaemia and meningitis'. *Thromb Haemost*, 65 (5). pp 483-486.

Pernestig, A.-K., Georgellis, D., Romeo, T., Suzuki, K., Tomenius, H., Normark, S. & Melefors, Ö. (2003) 'The Escherichia coli BarA-UvrY Two-Component System Is Needed for Efficient Switching between Glycolytic and Gluconeogenic Carbon Sources'. *J Bacteriol*, 185 (3). pp 843-853.

Pernestig, A. K., Melefors, O. & Georgellis, D. (2001) 'Identification of UvrY as the cognate response regulator for the BarA sensor kinase in Escherichia coli'. *J Biol Chem*, 276 (1). pp 225-231.

Peters, B. M. & Noverr, M. C. (2013) 'Candida albicans-Staphylococcus aureus polymicrobial peritonitis modulates host innate immunity'. *Infect Immun*, 81 (6). pp 2178-2189.

Petersen, L., Bollback, J. P., Dimmic, M., Hubisz, M. & Nielsen, R. (2007) 'Genes under positive selection in Escherichia coli'. *Genome Res*, 17 (9). pp 1336-1343.

Petersen, T. N., Brunak, S., von Heijne, G. & Nielsen, H. (2011) 'SignalP 4.0: discriminating signal peptides from transmembrane regions'. *Nat Methods*, 8 (10). pp 785-786.

Phan, M. D., Peters, K. M., Sarkar, S., Lukowski, S. W., Allsopp, L. P., Gomes Moriel, D., Achard, M. E., Totsika, M., Marshall, V. M., Upton, M., Beatson, S. A. & Schembri, M. A. (2013) 'The serum resistome of a globally disseminated multidrug resistant uropathogenic Escherichia coli clone'. *PLoS Genet*, 9 (10). pp e1003834.

Pichon, C., Hechard, C., du Merle, L., Chaudray, C., Bonne, I., Guadagnini, S., Vandewalle, A. & Le Bouguenec, C. (2009) 'Uropathogenic Escherichia coli AL511 requires flagellum to enter renal collecting duct cells'. *Cell Microbiol*, 11 (4). pp 616-628.

Pisacane, A., Graziano, L., Mazzarella, G., Scarpellino, B. & Zona, G. (1992) 'Breast-feeding and urinary tract infection'. *J Pediatr*, 120 (1). pp 87-89.

Platell, J. L., Cobbold, R. N., Johnson, J. R. & Trott, D. J. (2010) 'Clonal group distribution of fluoroquinolone-resistant Escherichia coli among humans and companion animals in Australia'. *J Antimicrob Chemother*, 65 (9). pp 1936-1938.

Pluschke, G., Mayden, J., Achtman, M. & Levine, R. P. (1983) 'Role of the capsule and the O antigen in resistance of O18:K1 Escherichia coli to complement-mediated killing'. *Infect Immun*, 42 (3). pp 907-913.

Pouttu, R., Puustinen, T., Virkola, R., Hacker, J., Klemm, P. & Korhonen, T. K. (1999) 'Amino acid residue Ala-62 in the FimH fimbrial adhesin is critical for the adhesiveness of meningitis-associated Escherichia coli to collagens'. *Mol Microbiol*, 31 (6). pp 1747-1757.

Prasadarao, N. V., Wass, C. A., Hacker, J., Jann, K. & Kim, K. S. (1993) 'Adhesion of S-fimbriated Escherichia coli to brain glycolipids mediated by sfaA gene-encoded protein of S-fimbriae'. *J Biol Chem*, 268 (14). pp 10356-10363.

Pratt, L. A. & Kolter, R. (1998) 'Genetic analysis of Escherichia coli biofilm formation: roles of flagella, motility, chemotaxis and type I pili'. *Mol Microbiol*, 30 (2). pp 285-293.

Puhar, A. & Sansonetti, P. J. (2014) 'Type III secretion system'. *Current Biology*, 24 (17). pp R784-R791.

Pybus, V. & Onderdonk, A. B. (1997) 'Evidence for a commensal, symbiotic relationship between Gardnerella vaginalis and Prevotella bivia involving ammonia: potential significance for bacterial vaginosis'. *J Infect Dis*, 175 (2). pp 406-413.

Pybus, V. & Onderdonk, A. B. (1998) 'A commensal symbiosis between Prevotella bivia and Peptostreptococcus anaerobius involves amino acids: potential significance to the pathogenesis of bacterial vaginosis'. *FEMS Immunol Med Microbiol*, 22 (4). pp 317-327.

Qin, Y., Lin, G., Chen, W., Huang, B., Huang, W. & Yan, Q. (2014) 'Flagellar motility contributes to the invasion and survival of Aeromonas hydrophila in Anguilla japonica macrophages'. *Fish Shellfish Immunol*, 39 (2). pp 273-279.

Ramos, H. C., Rumbo, M. & Sirard, J.-C. (2004) 'Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa'. *Trends Microbiol*, 12 (11). pp 509-517.

Rasko, D. A., Rosovitz, M. J., Myers, G. S., Mongodin, E. F., Fricke, W. F., Gajer, P., Crabtree, J., Sebaihia, M., Thomson, N. R., Chaudhuri, R., Henderson, I. R., Sperandio, V. & Ravel, J. (2008) 'The pangenome structure of Escherichia coli: comparative genomic analysis of E. coli commensal and pathogenic isolates'. *J Bacteriol*, 190 (20). pp 6881-6893.

Rasko, D. A., Webster, D. R., Sahl, J. W., Bashir, A., Boisen, N., Scheutz, F., Paxinos, E. E., Sebra, R., Chin, C. S., Iliopoulos, D., Klammer, A., Peluso, P., Lee, L., Kislyuk, A. O., Bullard, J., Kasarskis, A., Wang, S., Eid, J., Rank, D., Redman, J. C., Steyert, S. R., Frimodt-Moller, J., Struve, C., Petersen, A. M., Krogfelt, K. A., Nataro, J. P., Schadt, E. E. & Waldor, M. K. (2011) 'Origins of the E. coli strain causing an outbreak of hemolytic-uremic syndrome in Germany'. *N Engl J Med*, 365 (8). pp 709-717.

Reid, G., Howard, J. & Gan, B. S. (2001) 'Can bacterial interference prevent infection?'. *Trends Microbiol*, 9 (9). pp 424-428.

Reid, S. D., Herbelin, C. J., Bumbaugh, A. C., Selander, R. K. & Whittam, T. S. (2000) 'Parallel evolution of virulence in pathogenic Escherichia coli'. *Nature*, 406 (6791). pp 64-67.

Reynolds, E. S. (1963) 'The use of lead citrate at high pH as an electron-opaque stain in electron microscopy'. *J Cell Biol*, 17 pp 208-212.

Reynolds, R., Hope, R. & Williams, L. (2008) 'Survey, laboratory and statistical methods for the BSAC Resistance Surveillance Programmes'. *J Antimicrob Chemother*, 62 Suppl 2 pp ii15-28.

Riegman, N., Kusters, R., Van Veggel, H., Bergmans, H., Van Bergen en Henegouwen, P., Hacker, J. & Van Die, I. (1990) 'F1C fimbriae of a uropathogenic Escherichia coli strain: genetic and functional organization of the foc gene cluster and identification of minor subunits'. *J Bacteriol*, 172 (2). pp 1114-1120.

Rissman, A. I., Mau, B., Biehl, B. S., Darling, A. E., Glasner, J. D. & Perna, N. T. (2009) 'Reordering contigs of draft genomes using the Mauve aligner'. *Bioinformatics*, 25 (16). pp 2071-2073.

Rivers, C. A., Adaramola, O. O. & Schwebke, J. R. (2011) 'Prevalence of bacterial vaginosis and vulvovaginal candidiasis mixed infection in a southeastern american STD clinic'. *Sex Transm Dis*, 38 (7). pp 672-674.

Roberts, J. A., Marklund, B. I., Ilver, D., Haslam, D., Kaack, M. B., Baskin, G., Louis, M., Mollby, R., Winberg, J. & Normark, S. (1994) 'The Gal(alpha 1-4)Gal-

specific tip adhesin of Escherichia coli P-fimbriae is needed for pyelonephritis to occur in the normal urinary tract'. *Proc Natl Acad Sci U S A*, 91 (25). pp 11889-11893.

Roberts, K. B. (2015) 'The diagnosis of UTI: liquid gold and the problem of gold standards'. *Pediatrics*, 135 (6). pp 1126-1127.

Rosa Eugenia Reyes, C. R. G. l., Rafael Coria Jiménez, Maribel Ortiz Herrera and Alejandra Aquino Andrade (2012) 'Mechanisms of O-Antigen Structural Variation of Bacterial Lipopolysaccharide (LPS), The Complex World of Polysaccharides'. *IntechOpen, DOI: 10.5772/48147.*,

Rossez, Y., Wolfson, E. B., Holmes, A., Gally, D. L. & Holden, N. J. (2015) 'Bacterial Flagella: Twist and Stick, or Dodge across the Kingdoms'. *PLoS Pathogens*, 11 (1). pp e1004483.

Roy, K., Hilliard, G. M., Hamilton, D. J., Luo, J., Ostmann, M. M. & Fleckenstein, J. M. (2009) 'Enterotoxigenic Escherichia coli EtpA mediates adhesion between flagella and host cells'. *Nature*, 457 (7229). pp 594-598.

Roy, S., Coldren, C., Karunamurthy, A., Kip, N. S., Klee, E. W., Lincoln, S. E., Leon, A., Pullambhatla, M., Temple-Smolkin, R. L., Voelkerding, K. V., Wang, C. & Carter, A. B. (2018) 'Standards and Guidelines for Validating Next-Generation Sequencing Bioinformatics Pipelines: A Joint Recommendation of the Association for Molecular Pathology and the College of American Pathologists'. *J Mol Diagn*, 20 (1). pp 4-27.

Ruiz-Garbajosa, P., Bonten, M. J., Robinson, D. A., Top, J., Nallapareddy, S. R., Torres, C., Coque, T. M., Canton, R., Baquero, F., Murray, B. E., del Campo, R. & Willems, R. J. (2006) 'Multilocus sequence typing scheme for Enterococcus faecalis reveals hospital-adapted genetic complexes in a background of high rates of recombination'. *J Clin Microbiol*, 44 (6). pp 2220-2228.

Rump, L. V., Beutin, L., Fischer, M. & Feng, P. C. H. (2010a) 'Characterization of a gne::IS629 O Rough:H7 Escherichia coli Strain from a Hemorrhagic Colitis Patient'. *Appl Environ Microbiol*, 76 (15). pp 5290-5291.

Rump, L. V., Feng, P. C. H., Fischer, M. & Monday, S. R. (2010b) 'Genetic Analysis for the Lack of Expression of the O157 Antigen in an O Rough:H7 Escherichia coli Strain'. *Appl Environ Microbiol*, 76 (3). pp 945-947.

Russo, T. A. & Johnson, J. R. (2000) 'Proposal for a new inclusive designation for extraintestinal pathogenic isolates of Escherichia coli: ExPEC'. *J Infect Dis*, 181 (5). pp 1753-1754.

Russo, T. A., Sharma, G., Brown, C. R. & Campagnari, A. A. (1995a) 'Loss of the O4 antigen moiety from the lipopolysaccharide of an extraintestinal isolate of Escherichia coli has only minor effects on serum sensitivity and virulence in vivo'. *Infect Immun*, 63 (4). pp 1263-1269.

Russo, T. A., Stapleton, A., Wenderoth, S., Hooton, T. M. & Stamm, W. E. (1995b) 'Chromosomal restriction fragment length polymorphism analysis of Escherichia coli strains causing recurrent urinary tract infections in young women'. *J Infect Dis*, 172 (2). pp 440-445.

Saier, M. H. (2004) 'Evolution of bacterial type III protein secretion systems'. *Trends Microbiol*, 12 (3). pp 113-115.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) 'Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia'. *Science*, 230 (4732). pp 1350-1354.

Salipante, S. J., Roach, D. J., Kitzman, J. O., Snyder, M. W., Stackhouse, B., Butler-Wu, S. M., Lee, C., Cookson, B. T. & Shendure, J. (2015) 'Large-scale genomic sequencing of extraintestinal pathogenic Escherichia coli strains'. *Genome Res*, 25 (1). pp 119-128.

Samuel, G. & Reeves, P. (2003) 'Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly'. *Carbohydr Res*, 338 (23). pp 2503-2519.

Samuelsson, P., Hang, L., Wullt, B., Irjala, H. & Svanborg, C. (2004) 'Toll-like receptor 4 expression and cytokine responses in the human urinary tract mucosa'. *Infect Immun*, 72 (6). pp 3179-3186.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977) 'DNA sequencing with chain-terminating inhibitors'. *Proc Natl Acad Sci U S A*, 74 (12). pp 5463-5467.

Sanya Tahmina Jhora, S. P. (2011) 'Urinary Tract Infections Caused by Staphylococcus saprophyticus and their antimicrobial sensitivity pattern in Young Adult Women'. *Bangladesh J Med Microbiol*, 05 (01). pp 21-25.

Sarkar, S., Ulett, G. C., Totsika, M., Phan, M. D. & Schembri, M. A. (2014) 'Role of capsule and O antigen in the virulence of uropathogenic Escherichia coli'. *PLoS One*, 9 (4). pp e94786.

Saukkonen, K. M., Nowicki, B. & Leinonen, M. (1988) 'Role of type 1 and S fimbriae in the pathogenesis of Escherichia coli O18:K1 bacteremia and meningitis in the infant rat'. *Infection and Immunity*, 56 (4). pp 892-897.

Savar, N. S., Jahanian-Najafabadi, A., Mahdavi, M., Shokrgozar, M. A., Jafari, A. & Bouzari, S. (2014) 'In silico and in vivo studies of truncated forms of flagellin (FliC) of enteroaggregative Escherichia coli fused to FimH from uropathogenic Escherichia coli as a vaccine candidate against urinary tract infections'. *J Biotechnol*, 175 pp 31-37.

Schembri, M. A., Sokurenko, E. V. & Klemm, P. (2000) 'Functional flexibility of the FimH adhesin: insights from a random mutant library'. *Infect Immun*, 68 (5). pp 2638-2646.

Schilling, J. D. & Hultgren, S. J. (2002) 'Recent advances into the pathogenesis of recurrent urinary tract infections: the bladder as a reservoir for uropathogenic Escherichia coli'. *Int J Antimicrob Agents*, 19 (6). pp 457-460.

Schirmer, T. (1998) 'General and specific porins from bacterial outer membranes'. *J Struct Biol*, 121 (2). pp 101-109.

Seemann, T. (2014) 'Prokka: rapid prokaryotic genome annotation'. *Bioinformatics*, 30 (14). pp 2068-2069.

Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986) 'Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics'. *Appl Environ Microbiol*, 51 (5). pp 873-884.

Serra, D. O., Richter, A. M., Klauck, G., Mika, F. & Hengge, R. (2013) 'Microanatomy at cellular resolution and spatial order of physiological differentiation in a bacterial biofilm'. *MBio*, 4 (2). pp e00103-00113.

Seveau, S., Tham, T. N., Payrastre, B., Hoppe, A. D., Swanson, J. A. & Cossart, P. (2007) 'A FRET analysis to unravel the role of cholesterol in Rac1 and PI 3-kinase activation in the InlB/Met signalling pathway'. *Cell Microbiol*, 9 (3). pp 790-803.

Shankar, N., Lockatell, C. V., Baghdayan, A. S., Drachenberg, C., Gilmore, M. S. & Johnson, D. E. (2001) 'Role of Enterococcus faecalis surface protein Esp in the pathogenesis of ascending urinary tract infection'. *Infect Immun*, 69 (7). pp 4366-4372.

Shao, J., Cheng, H., Wang, C., Wu, D., Zhu, X., Zhu, L. & Sun, Z. (2013) 'Sodium houttuyfonate, a potential phytoanticipin derivative of antibacterial agent, inhibits bacterial attachment and pyocyanine secretion of Pseudomonas aeruginosa by attenuating flagella-mediated swimming motility'. *World J Microbiol Biotechnol*, 29 (12). pp 2373-2378.

Shulman, S. T., Friedmann, H. C. & Sims, R. H. (2007) 'Theodor Escherich: the first pediatric infectious diseases physician?'. *Clin Infect Dis*, 45 (8). pp 1025-1029.

Siegman-Igra, Y., Kulka, T., Schwartz, D. & Konforti, N. (1994) 'Polymicrobial and monomicrobial bacteraemic urinary tract infection'. *J Hosp Infect*, 28 (1). pp 49-56.

Silverman, M., Matsumura, P. & Simon, M. (1976) 'The identification of the mot gene product with Escherichia coli-lambda hybrids'. *Proc Natl Acad Sci U S A*, 73 (9). pp 3126-3130.

Smith, K. D., Andersen-Nissen, E., Hayashi, F., Strobe, K., Bergman, M. A., Barrett, S. L., Cookson, B. T. & Aderem, A. (2003) 'Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility'. *Nat Immunol*, 4 (12). pp 1247-1253.

Soheili, S., Ghafourian, S., Sekawi, Z., Neela, V., Sadeghifard, N., Ramli, R. & Hamat, R. A. (2014) 'Wide distribution of virulence genes among Enterococcus faecium and Enterococcus faecalis clinical isolates'. *ScientificWorldJournal*, 2014 pp 623174.

Sokurenko, E. (2016) 'Pathoadaptive Mutations in Uropathogenic Escherichia coli'. *Microbiol Spectr*, 4 (2).

Sokurenko, E. V., Hasty, D. L. & Dykhuizen, D. E. (1999) 'Pathoadaptive mutations: gene loss and variation in bacterial pathogens'. *Trends Microbiol*, 7 (5). pp 191-195.

Song, J. & Abraham, S. N. (2008) 'Innate and adaptive immune responses in the urinary tract'. *Eur J Clin Invest*, 38 Suppl 2 pp 21-28.

Spencer, R. C. & Nicol, C. D. (1986) 'Increased detection of polymicrobial septicaemia by repeat subculture'. *J Med Microbiol*, 22 (1). pp 85-87.

Srinivasan, U., Foxman, B. & Marrs, C. F. (2003) 'Identification of a gene encoding heat-resistant agglutinin in Escherichia coli as a putative virulence factor in urinary tract infection'. *J Clin Microbiol*, 41 (1). pp 285-289.

Stajich, J. E., Block, D., Boulez, K., Brenner, S. E., Chervitz, S. A., Dagdigian, C., Fuellen, G., Gilbert, J. G., Korf, I., Lapp, H., Lehvaslaiho, H., Matsalla, C., Mungall, C. J., Osborne, B. I., Pocock, M. R., Schattner, P., Senger, M., Stein, L. D., Stupka, E., Wilkinson, M. D. & Birney, E. (2002) 'The Bioperl toolkit: Perl modules for the life sciences'. *Genome Res*, 12 (10). pp 1611-1618.

Stamm, W. E. & Norrby, S. R. (2001) 'Urinary tract infections: disease panorama and challenges'. *J Infect Dis*, 183 Suppl 1 pp S1-4.

Stapleton, A. & Stamm, W. E. (1997) 'Prevention of urinary tract infection'. *Infect Dis Clin North Am*, 11 (3). pp 719-733.

Stewart, R. C., Roth, A. F. & Dahlquist, F. W. (1990) 'Mutations that affect control of the methylesterase activity of CheB, a component of the chemotaxis adaptation system in Escherichia coli'. *J Bacteriol*, 172 (6). pp 3388-3399.

Stromberg, N., Marklund, B. I., Lund, B., Ilver, D., Hamers, A., Gaastra, W., Karlsson, K. A. & Normark, S. (1990) 'Host-specificity of uropathogenic Escherichia coli depends on differences in binding specificity to Gal alpha 1-4Gal-containing isoreceptors'. *Embo j*, 9 (6). pp 2001-2010.

Subashchandrabose, S. & Mobley, H. L. (2015) 'Virulence and Fitness Determinants of Uropathogenic Escherichia coli'. *Microbiol Spectr*, 3 (4).

Suerbaum, S., Friedrich, S., Leying, H. & Opferkuch, W. (1994) 'Expression of capsular polysaccharide determines serum resistance in Escherichia coli K92'. *Zentralbl Bakteriol*, 281 (2). pp 146-157.

Sug Kim, J., Won Lee, T., Gyoo Ihm, C., Jin Kim, Y., Mi Moon, S., Joo Lee, H. & Hwan Jeong, K. (2015) 'CAPD peritonitis caused by co-infection with Cellulosimicrobium cellulans (Oerskovia xanthineolytica) and Enterobacter cloacae: a case report and literature review'. *Intern Med*, 54 (6). pp 627-630.

Sulaiman, H., Ponnampalavanar, S., Mun, K. S. & Italiano, C. M. (2013) 'Cervical abscesses due to co-infection with Burkholderia pseudomallei, Salmonella enterica serovar Stanley and Mycobacterium tuberculosis in a patient with diabetes mellitus'. *BMC Infect Dis*, 13 pp 527.

Sun, T. T. (2006) 'Altered phenotype of cultured urothelial and other stratified epithelial cells: implications for wound healing'. *Am J Physiol Renal Physiol*, 291 (1). pp F9-21.

Sunden, F., Hakansson, L., Ljunggren, E. & Wullt, B. (2010) 'Escherichia coli 83972 bacteriuria protects against recurrent lower urinary tract infections in patients with incomplete bladder emptying'. *J Urol*, 184 (1). pp 179-185.

Sussman, M. & Gally, D. L. (1999) 'The biology of cystitis: host and bacterial factors'. *Annu Rev Med*, 50 pp 149-158.

Szczesny, P. & Lupas, A. (2008) 'Domain annotation of trimeric autotransporter adhesins--daTAA'. *Bioinformatics*, 24 (10). pp 1251-1256.

Takahashi, S., Xu, C., Sakai, T., Fujii, K. & Nakamura, M. (2017) 'Infective endocarditis following urinary tract infection caused by Globicatella sanguinis'. *IDCases*, 11 pp 18-21.

Tang, Y.-W. (2015) 'Molecular Medical Microbiology 2nd edition'. in Sussman, M., Liu, D., Poxton, I. and Schwartzman, J. (eds.) *Molecular Medical Microbiology (Second Edition).* Boston: Academic Press, 16 16 pp P303-314.

Tasteyre, A., Barc, M.-C., Collignon, A., Boureau, H. & Karjalainen, T. (2001) 'Role of FliC and FliD Flagellar Proteins of Clostridium difficile in Adherence and Gut Colonization'. *Infection and Immunity*, 69 (12). pp 7937-7940.

Tendolkar, P. M., Baghdayan, A. S., Gilmore, M. S. & Shankar, N. (2004) 'Enterococcal surface protein, Esp, enhances biofilm formation by Enterococcus faecalis'. *Infect Immun*, 72 (10). pp 6032-6039.

Thumbikat, P., Berry, R. E., Zhou, G., Billips, B. K., Yaggie, R. E., Zaichuk, T., Sun, T. T., Schaeffer, A. J. & Klumpp, D. J. (2009) 'Bacteria-induced uroplakin signaling mediates bladder response to infection'. *PLoS Pathog*, 5 (5). pp e1000415.

Tobe, T., Beatson, S. A., Taniguchi, H., Abe, H., Bailey, C. M., Fivian, A., Younis, R., Matthews, S., Marches, O., Frankel, G., Hayashi, T. & Pallen, M. J. (2006) 'An extensive repertoire of type III secretion effectors in Escherichia coli O157 and the role of lambdoid phages in their dissemination'. *Proceedings of the National Academy of Sciences of the United States of America*, 103 (40). pp 14941-14946.

Tomenius, H., Pernestig, A. K., Jonas, K., Georgellis, D., Mollby, R., Normark, S. & Melefors, O. (2006) 'The Escherichia coli BarA-UvrY two-component system is a virulence determinant in the urinary tract'. *BMC Microbiol*, 6 pp 27.

Totsika, M., Beatson, S. A., Sarkar, S., Phan, M. D., Petty, N. K., Bachmann, N., Szubert, M., Sidjabat, H. E., Paterson, D. L., Upton, M. & Schembri, M. A. (2011) 'Insights into a multidrug resistant Escherichia coli pathogen of the globally disseminated ST131 lineage: genome analysis and virulence mechanisms'. *PLoS One*, 6 (10). pp e26578.

Totsika, M., Kostakioti, M., Hannan, T. J., Upton, M., Beatson, S. A., Janetka, J. W., Hultgren, S. J. & Schembri, M. A. (2013) 'A FimH inhibitor prevents acute bladder infection and treats chronic cystitis caused by multidrug-resistant uropathogenic Escherichia coli ST131'. *J Infect Dis*, 208 (6). pp 921-928.

Totsika, M., Wells, T. J., Beloin, C., Valle, J., Allsopp, L. P., King, N. P., Ghigo, J. M. & Schembri, M. A. (2012) 'Molecular characterization of the EhaG and UpaG trimeric autotransporter proteins from pathogenic Escherichia coli'. *Appl Environ Microbiol*, 78 (7). pp 2179-2189.

Trautner, B. W., Darouiche, R. O., Hull, R. A., Hull, S. & Thornby, J. I. (2002) 'Pre-inoculation of urinary catheters with Escherichia coli 83972 inhibits catheter colonization by Enterococcus faecalis'. *J Urol*, 167 (1). pp 375-379.

Treangen, T. J. & Salzberg, S. L. (2011) 'Repetitive DNA and next-generation sequencing: computational challenges and solutions'. *Nat Rev Genet*, 13 (1). pp 36-46.

Turner, L., Stern, A. S. & Berg, H. C. (2012) 'Growth of flagellar filaments of Escherichia coli is independent of filament length'. *J Bacteriol*, 194 (10). pp 2437-2442.

Ulett, G. C., Totsika, M., Schaale, K., Carey, A. J., Sweet, M. J. & Schembri, M. A. (2013) 'Uropathogenic Escherichia coli virulence and innate immune responses during urinary tract infection'. *Curr Opin Microbiol*, 16 (1). pp 100-107.

Uliczka, F., Kornprobst, T., Eitel, J., Schneider, D. & Dersch, P. (2009) 'Cell invasion of Yersinia pseudotuberculosis by invasin and YadA requires protein kinase C, phospholipase C-gamma1 and Akt kinase'. *Cell Microbiol*, 11 (12). pp 1782-1801.

Ulleryd, P., Sandberg, T., Scheutz, F., Clabots, C., Johnston, B. D., Thuras, P. & Johnson, J. R. (2015) 'Colonization with Escherichia coli Strains among

Female Sex Partners of Men with Febrile Urinary Tract Infection'. *J Clin Microbiol*, 53 (6). pp 1947-1950.

Umene, Y. D., Wong, L. K., Satoh, T., Yamane, K., Matsui, M., Riley, L. W., Arakawa, Y. & Suzuki, S. (2015) 'Molecular epidemiological characterization of uropathogenic escherichia coli from an outpatient urology clinic in rural Japan'. *J Clin Microbiol*, 53 (2). pp 681-683.

Underwood, A. P., Dallman, T., Thomson, N. R., Williams, M., Harker, K., Perry, N., Adak, B., Willshaw, G., Cheasty, T., Green, J., Dougan, G., Parkhill, J. & Wain, J. (2013) 'Public health value of next-generation DNA sequencing of enterohemorrhagic Escherichia coli isolates from an outbreak'. *J Clin Microbiol*, 51 (1). pp 232-237.

Valle, J., Mabbett, A. N., Ulett, G. C., Toledo-Arana, A., Wecker, K., Totsika, M., Schembri, M. A., Ghigo, J. M. & Beloin, C. (2008) 'UpaG, a new member of the trimeric autotransporter family of adhesins in uropathogenic Escherichia coli'. *J Bacteriol*, 190 (12). pp 4147-4161.

Varga, Z. G., Armada, A., Cerca, P., Amaral, L., Mior Ahmad Subki, M. A., Savka, M. A., Szegedi, E., Kawase, M., Motohashi, N. & Molnar, J. (2012) 'Inhibition of quorum sensing and efflux pump system by trifluoromethyl ketone proton pump inhibitors'. *In Vivo*, 26 (2). pp 277-285.

Veranic, P., Erman, A., Kerec-Kos, M., Bogataj, M., Mrhar, A. & Jezernik, K. (2009) 'Rapid differentiation of superficial urothelial cells after chitosan-induced desquamation'. *Histochem Cell Biol*, 131 (1). pp 129-139.

Wakefield, J. S. & Hicks, R. M. (1974) 'Erythrophagocytosis by the epithelial cells of the bladder'. *J Cell Sci*, 15 (3). pp 555-573.

Wandersman, C. & Delepelaire, P. (1990) 'TolC, an Escherichia coli outer membrane protein required for hemolysin secretion'. *Proc Natl Acad Sci U S A*, 87 (12). pp 4776-4780.

Wang, H., Liang, F.-X. & Kong, X.-P. (2008) 'Characteristics of the Phagocytic Cup Induced by Uropathogenic Escherichia coli'. *Journal of Histochemistry and Cytochemistry*, 56 (6). pp 597-604.

Wang, H., Min, G., Glockshuber, R., Sun, T. T. & Kong, X. P. (2009) 'Uropathogenic E. coli adhesin-induced host cell receptor conformational changes: implications in transmembrane signaling transduction'. *J Mol Biol*, 392 (2). pp 352-361.

Wang, L., Rothemund, D., Curd, H. & Reeves, P. R. (2003) 'Species-wide variation in the Escherichia coli flagellin (H-antigen) gene'. *J Bacteriol*, 185 (9). pp 2936-2943.

Wang, L., Wang, Q. & Reeves, P. R. (2010) 'The variation of O antigens in gram-negative bacteria'. *Subcell Biochem*, 53 pp 123-152.

Ward, D. V., Scholz, M., Zolfo, M., Taft, D. H., Schibler, K. R., Tett, A., Segata, N. & Morrow, A. L. (2016) 'Metagenomic Sequencing with Strain-Level Resolution Implicates Uropathogenic E. coli in Necrotizing Enterocolitis and Mortality in Preterm Infants'. *Cell Rep*, 14 (12). pp 2912-2924.

Webb, L., Goodwin, C. S. & Green, J. (1982) 'O antigen loss by semi-rough E. coli causing recurrent urinary infections, analysed by gel column filtration and gas-liquid chromatography'. *Pathology*, 14 (1). pp 17-24.

Weissman, S. J., Beskhlebnaya, V., Chesnokova, V., Chattopadhyay, S., Stamm, W. E., Hooton, T. M. & Sokurenko, E. V. (2007) 'Differential stability and trade-off effects of pathoadaptive mutations in the Escherichia coli FimH adhesin'. *Infect Immun*, 75 (7). pp 3548-3555.

Weissman, S. J., Moseley, S. L., Dykhuizen, D. E. & Sokurenko, E. V. (2003) 'Enterobacterial adhesins and the case for studying SNPs in bacteria'. *Trends Microbiol*, 11 (3). pp 115-117.

Welch, R. A., Burland, V., Plunkett, G., 3rd, Redford, P., Roesch, P., Rasko, D., Buckles, E. L., Liou, S. R., Boutin, A., Hackett, J., Stroud, D., Mayhew, G. F., Rose, D. J., Zhou, S., Schwartz, D. C., Perna, N. T., Mobley, H. L., Donnenberg, M. S. & Blattner, F. R. (2002) 'Extensive mosaic structure revealed by the complete genome sequence of uropathogenic Escherichia coli'. *Proc Natl Acad Sci U S A*, 99 (26). pp 17020-17024.

Weng, P. L., Ramli, R., Shamsudin, M. N., Cheah, Y. K. & Hamat, R. A. (2013) 'High genetic diversity of Enterococcus faecium and Enterococcus faecalis clinical isolates by pulsed-field gel electrophoresis and multilocus sequence typing from a hospital in Malaysia'. *Biomed Res Int*, 2013 pp 938937.

Whiteside, S. A., Dave, S., Seney, S. L., Wang, P., Reid, G. & Burton, J. P. (2018) 'Enterococcus faecalis persistence in pediatric patients treated with antibiotic prophylaxis for recurrent urinary tract infections'. *Future Microbiol*, 13 pp 1095-1115.

Wiedemann, A., Linder, S., Grassl, G., Albert, M., Autenrieth, I. & Aepfelbacher, M. (2001) 'Yersinia enterocolitica invasin triggers phagocytosis via beta1 integrins, CDC42Hs and WASp in macrophages'. *Cell Microbiol*, 3 (10). pp 693-702.

Wiffen, P. (2018) 'Apocalypse: the end of antibiotics?'. *European Journal of Hospital Pharmacy*, 25 (1). pp 1-1.

Wijetunge, D. S., Gongati, S., DebRoy, C., Kim, K. S., Couraud, P. O., Romero, I. A., Weksler, B. & Kariyawasam, S. (2015) 'Characterizing the pathotype of neonatal meningitis causing Escherichia coli (NMEC)'. *BMC Microbiol*, 15 pp 211.

Wiksten, J. E., Laakso, S., Maki, M., Makitie, A. A., Pitkaranta, A. & Blomgren, K. (2015) 'Microarray identification of bacterial species in peritonsillar abscesses'. *Eur J Clin Microbiol Infect Dis*, 34 (5). pp 905-911.

Wiles, T. J., Kulesus, R. R. & Mulvey, M. A. (2008) 'Origins and virulence mechanisms of uropathogenic Escherichia coli'. *Exp Mol Pathol*, 85 (1). pp 11-19.

Williams, A. W., Yamaguchi, S., Togashi, F., Aizawa, S. I., Kawagishi, I. & Macnab, R. M. (1996) 'Mutations in fliK and flhB affecting flagellar hook and filament assembly in Salmonella typhimurium'. *J Bacteriol*, 178 (10). pp 2960-2970.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L. H., Karch, H., Reeves, P. R., Maiden, M. C., Ochman, H. & Achtman, M. (2006) 'Sex and virulence in Escherichia coli: an evolutionary perspective'. *Mol Microbiol*, 60 (5). pp 1136-1151.

Wood, D. E. & Salzberg, S. L. (2014) 'Kraken: ultrafast metagenomic sequence classification using exact alignments'. *Genome Biol*, 15 (3). pp R46.

Wright, K. J., Seed, P. C. & Hultgren, S. J. (2005) 'Uropathogenic Escherichia coli flagella aid in efficient urinary tract colonization'. *Infect Immun*, 73 (11). pp 7657-7668.

Wu, X.-R., Kong, X.-P., Pellicer, A., Kreibich, G. & Sun, T.-T. (2009) 'Uroplakins in urothelial biology, function, and disease'. *Kidney International*, 75 (11). pp 1153-1165.

Wu, X. R., Manabe, M., Yu, J. & Sun, T. T. (1990) 'Large scale purification and immunolocalization of bovine uroplakins I, II, and III. Molecular markers of urothelial differentiation'. *J Biol Chem*, 265 (31). pp 19170-19179.

Wullt, B. (2003) 'The role of P fimbriae for Escherichia coli establishment and mucosal inflammation in the human urinary tract'. *Int J Antimicrob Agents*, 21 (6). pp 605-621.

Wurpel, D. J., Beatson, S. A., Totsika, M., Petty, N. K. & Schembri, M. A. (2013) 'Chaperone-Usher Fimbriae of Escherichia coli'. *PLoS One*, 8 (1). pp e52835.

Xing, Q., Shi, K., Portaliou, A., Rossi, P., Economou, A. & Kalodimos, C. G. (2018) 'Structures of chaperone-substrate complexes docked onto the export gate in a type III secretion system'. *Nat Commun*, 9 (1). pp 1773.

Yamaji, R., Rubin, J., Thys, E., Friedman, C. R. & Riley, L. W. (2018) 'Persistent pandemic lineages of uropathogenic Escherichia coli in a college community-1999-2017'. *J Clin Microbiol*,

Yamamoto, S., Tsukamoto, T., Terai, A., Kurazono, H., Takeda, Y. & Yoshida, O. (1997) 'Genetic evidence supporting the fecal-perineal-urethral hypothesis in cystitis caused by Escherichia coli'. *J Urol*, 157 (3). pp 1127-1129.

Yun, K. W., Kim, D. S., Kim, W. & Lim, I. S. (2015) 'Molecular typing of uropathogenic Escherichia coli isolated from Korean children with urinary tract infection'. *Korean J Pediatr*, 58 (1). pp 20-27.

Zdziarski, J., Brzuszkiewicz, E., Wullt, B., Liesegang, H., Biran, D., Voigt, B., Gronberg-Hernandez, J., Ragnarsdottir, B., Hecker, M., Ron, E. Z., Daniel, R., Gottschalk, G., Hacker, J., Svanborg, C. & Dobrindt, U. (2010) 'Host imprints on bacterial genomes--rapid, divergent evolution in individual patients'. *PLoS Pathog*, 6 (8). pp e1001078.

Zere, T. R., Vakulskas, C. A., Leng, Y., Pannuri, A., Potts, A. H., Dias, R., Tang, D., Kolaczkowski, B., Georgellis, D., Ahmer, B. M. & Romeo, T. (2015) 'Genomic Targets and Features of BarA-UvrY (-SirA) Signal Transduction Systems'. *PLoS One*, 10 (12). pp e0145035.

Zhang, Z., Schwartz, S., Wagner, L. & Miller, W. (2000) 'A greedy algorithm for aligning DNA sequences'. *J Comput Biol*, 7 (1-2). pp 203-214.

Zhao, L., Gao, S., Huan, H., Xu, X., Zhu, X., Yang, W., Gao, Q. & Liu, X. (2009) 'Comparison of virulence factors and expression of specific genes between

uropathogenic Escherichia coli and avian pathogenic E. coli in a murine urinary tract infection model and a chicken challenge model'. *Microbiology*, 155 (Pt 5). pp 1634-1644.

Zheng, J. X., Bai, B., Lin, Z. W., Pu, Z. Y., Yao, W. M., Chen, Z., Li, D. Y., Deng, X. B., Deng, Q. W. & Yu, Z. J. (2017) 'Characterization of biofilm formation by Enterococcus faecalis isolates derived from urinary tract infections in China'. *J Med Microbiol*,

Zhou, G., Mo, W. J., Sebbel, P., Min, G., Neubert, T. A., Glockshuber, R., Wu, X. R., Sun, T. T. & Kong, X. P. (2001) 'Uroplakin Ia is the urothelial receptor for uropathogenic Escherichia coli: evidence from in vitro FimH binding'. *J Cell Sci*, 114 (Pt 22). pp 4095-4103.

Zschuttig, A., Zimmermann, K., Blom, J., Goesmann, A., Pohlmann, C. & Gunzer, F. (2012) 'Identification and characterization of microcin S, a new antibacterial peptide produced by probiotic Escherichia coli G3/10'. *PLoS One*, 7 (3). pp e33351.

Zurfluh, K., Hachler, H., Stephan, R. & Nuesch-Inderbinen, M. (2016) 'Long-term shedding of CTX-M-15-producing Escherichia coli B2:ST127 by a healthy asymptomatic carrier'. *Int J Antimicrob Agents*,