Molecular Epidemiology, Virulence Potential and Antibiotic Susceptibility of the Major Lineages of Uropathogenic *Escherichia coli*

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

2015

MAJED F. ALGHORIBI

School of Medicine

List of contents

Total word Count: 52064 words

List of figures

List of tables

List of abbreviations

ABU	Asymptomatic Bacteriuria
AES	Advanced Expert System
bla	β -lactamase
Bp	Base pair
BURST	Based Upon Related Sequence Types
CFU	Colony Forming Unit
CGA	Clonal Group A
CMFT	Central Manchester Foundation Trust
CPE	Carbapenemase Producing Enterobacteriaceae
DLV	Double Locus Variant
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended Spectrum β-Lactamase
ExPEC	Extra-intestinal pathogenic *E. coli*
FimH	Type 1 Fimbrial Adhesin
FQ	Fluoroquinolone
FQ-R	Fluoroquinolone-Resistant
GCC	Gulf Cooperation Council
HGT	Horizontal Gene Transfer
IBCs	Intercellular Biofilm-like Communities
KAMC	King Abdulaziz Medical City
KPC	*K. pneumoniae* Carbapenemase
LD_{50}	Median Lethal Dose
LPS	Lipopolysaccharide
MDR	Multiple Drug Resistance

MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Typing
MRI	Manchester Royal Infirmary
MSTree	Minimum Spanning Tree
NDM	New Delhi Metallo-β -lactamase
NGS	Next Generation Sequencing
NHS	National Health Service
nt	nontypable
OXA	Oxacillinase-β-lactamases
PAIs	Pathogenicity Islands
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
QRDRs	Quinolone Resistance Determining Regions
RAPD	Randomly Amplification Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SEPEC	Sepsis-associated *Escherichia coli*
SLV	Single Locus Variant
SNP	Single Nucleotide Polymorphism
ST	Sequence Type
T1F	Type 1 Fimbrae
TLR4	Toll-Like Receptor 4
TLV	Triple Locus Variant
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary Tract Infection
VF	Virulence Factor
VIM	Verona Integron-Encoded Metallo-β-lactamase

Abstract

The University of Manchester, 2015

For the degree of Doctor of Philosophy - Majed Alghoribi

Molecular Epidemiology, Virulence Potential and Antibiotic Susceptibility of the Major Lineages of Uropathogenic *Escherichia coli*

Uropathogenic *E. coli* (UPEC) is the most frequent cause of urinary tract infection (UTI), being responsible for up to 85% of community acquired and 40% of nosocomial cases. UPEC strains harbour various virulence factors that contribute to their ability to cause disease. The high prevalence across the globe of multidrug resistant UPEC is a significant threat to therapy. Virulent and resistant UPEC strains have been recognised as belonging to major lineages and we have only recently begun to understand the factors contributing to their successful global dissemination.

Work in this thesis was carried out to identify the population structure of *E. coli* isolates recovered from urosepsis and biliary sepsis, to reveal any differences in genetic background. A total of 100 isolates from the blood and urine of 50 patients presenting with urosepsis and 27 isolates from cases of biliary sepsis were subjected to genotypic and phenotypic analysis, including MLST, virulence gene detection and antibiogram and metabolic profiling. Urosepsis paired isolates showed identical genotypes and antimicrobial resistance profiles. However, several pairs of isolates showed discrepant metabolic activity profiles suggesting niche specific regulation of metabolism. Members of the ST131 clone were significantly associated with antibiotic resistance and ST38 isolates were associated with the highest level of metabolic activity.

An *in vivo* infection model was used to investigate the virulence potential of isolates from the major UPEC lineages. *Galleria mellonella* larvae inoculated with ST69 and ST127 isolates showed significantly higher mortality rates than those infected with other strains. However, one isolate of ST127 (strain EC18) was avirulent and comparative genomic analyses with a single virulent ST127 strain revealed an IS1 mediated deletion in the O-antigen cluster in strain EC18, which is likely to explain the lack of virulence in the larvae and demonstrates the importance of this cell surface molecule in the model system.

Finally, a total of 202 UPEC isolates were recovered from community and hospital urine samples from a tertiary care hospital in Riyadh, Saudi Arabia. Molecular epidemiological investigation of the strains was carried out to examine the overall UPEC population structure, for the first time in any part of Saudi Arabia. The most common lineages were ST131 (17.3%), ST73 (11.4%), ST38 (7.4%), ST69 (7.4%) and ST10 (6.4%). The findings highlight the successful spread of multidrug resistant, CTX-M positive ST38, ST131 and ST405 UPEC in Saudi Arabia. The high proportion (35%) of ESBL producing *E. coli* isolates is a particular concern and is driving frequent prescription of carbapenem antibiotics. A total of four isolates of ST38 were positive for *aggR*, which is a virulence marker of enteroaggregative *E. coli* (EAEC); ST38 strains that cause UTI but have an EAEC genetic background are becoming recognised as novel UPEC and this clonal group warrants further study.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Majed F. Alghoribi

Copyright statement

I. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the "Copyright") and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

II. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

III. The ownership of certain Copyright, patents, designs, trade marks and other intellectual property (the "Intellectual Property") and any reproductions of copyright works in the thesis, for example graphs and tables ("Reproductions"), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

IV. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DocuInfo.aspx?DocID=487), in any relevant Thesis restriction declarations deposited in the University Library, The University Library's regulations (see http://www.manchester.ac.uk/library/aboutus/regulations) and in The University's policy on Presentation of Theses

Dedication

To my beloved Mum and Dad,

my lovely wife, and my son

Acknowledgements

First and foremost, I would like to express my deep and sincere gratitude to my supervisor Dr. Mathew Upton for all his contributions of time, ideas, and support throughout this study.

I am deeply grateful to the supervision team Dr. Paul Dark, Dr. Andrew Dodgson and Dr. Andrew McBain for their advices and constructive comments, and for their important support during my study.

I would also like to express my sincere thanks to Miss Audrey Coke and Mr. John Moat for technical assistance and support they have provided during the years.

During this work I had assistance and support from many colleagues especially Dr. Hanan Balkhy, Dr. Sameera Al-johani, Dr. Tarek Gibreel, Dr. Garry Farnham and Dr. Sarah Maxwell for whom I have great regard and I wish to extend my warmest thanks to my friend Arif Felek for his friendship and the pleasant times we shared.

I wish to express my warm and sincere thanks to Dr. Scott Beatson and his research group for hosting me during my research visit at the University of Queensland, Brisbane Australia.

And lovingly, I would like to express my appreciation, gratitude and thanks to my family; my mum, Khadija, who has suffered to give me happiness and has supported me with her prayers, my dad, Faisal, who is my support and my role model, my sisters and brothers for their emotional support and prayers.

I owe my loving thanks to my wife, Sundos and my son, Alfaisal, I greatly appreciate your love and support throughout this journey. It would have been impossible for me to finish this work without you encouragement and understanding and to whom this thesis is dedicated.

The administrative and financial support was provided by King Abdullah International Medical Research Center, King Saud bin Abdulaziz University for Health Sciences, Saudi Arabia–Riyadh and the Saudi Arabia Cultural Bureau, London, UK.

Publications arising from this work

This project gave rise to the following published work, which includes conference presentations (oral and poster), manuscripts to be submitted for publication and published papers.

- ## Oral Presentations

Majed F. Alghoribi (2012), Understanding the Virulence of Major Lineages of Uropathogenic *E. coli*, The 2012 Interdisciplinary PhD conference, 11 May 2012, The University of Manchester, United Kingdom.

Majed F. Alghoribi (2012), Investigating the virulence of Uropathogenic *E. coli* using *Galleria mellonella* larvae as infection model, North West Microbiology Group Meeting, 20 September 2012, The University of Liverpool, United Kingdom.

- ## Poster presentations

Majed F. Alghoribi, Tarek M. Gibreel, Andrew R. Dodgson and Mathew Upton (2013), *Galleria mellonella* Larvae Infection Model Indicates that ST69 and ST127 Strains are More Pathogenic than other Uropathogenic *E. coli*, The Society for General Microbiology, Spring Conference 2013, Manchester-UK, 25-28 March 2013.

Majed F. Alghoribi, Tarek M. Gibreel, Andrew R. Dodgson and Mathew Upton (2013), Investigating the virulence of Uropathogenic E. coli (UPEC) using *Galleria mellonella* larvae, 23rd European Society of Clinical Microbiology and Infectious Diseases, Berlin- Germany, 27-30 April 2013.

Majed F. Alghoribi, Scott Beatson, and Mathew Upton (2013), Genome sequence determination and comparative genomics of Uropathogenic *E. coli* ST127, 9th Annual SCMB Research Students Symposium 2013 Queensland, Australia.

Majed F. Alghoribi, Scott Beatson, and Mathew Upton (2014), Comparative genomics reveals that O-antigen is essential for virulence of ST127 UPEC in *Galleria mellonella* larvae, 24th European Congress of Clinical Microbiology and Infectious Diseases 2014, 10 - 13 May 2014 Barcelona, Spain.

Majed F. Alghoribi, Scott Beatson, and Mathew Upton (2014), Genome Sequence Determination and Comparative Genomics of Uropathogenic *E. coli* ST127, The Society for General Microbiology Annual Conference 2014, Liverpool, UK,

- **Journal publications**

Alghoribi MF, Gibreel TM, Dodgson AR, Beatson SA, Upton M. *Galleria mellonella* infection model demonstrates high lethality of ST69 and ST127 uropathogenic *E. coli*. *PLoS One* 2014; **9**: 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. dkv188–.

Alghoribi MF, Dodgson AR, Maxwell S and Upton M. Molecular Epidemiological and Phylogenetic analysis of *E. coli* Strains from Patients with Urosepsis and Biliary Sepsis.
(In preparation).

Chapter One

1 General Introduction

In 1885, a German paediatric infectious diseases physician, Theodor Escherich, discovered *Escherichia coli* (*E. coli*). *E. coli* is considered one of the most important bacterial agents in medicine and biological research and is subsequently one of the best-studied and most highly characterised microorganisms (Shulman et al., 2007). For more than half a century after the discovery of *E. coli*, it was believed to be a major part of the normal human and mammalian intestinal flora with a minimum level of pathogenicity (Ferguson and June, 1952). This thought changed however after comprehensive studies and recognition of frequent reports of outbreaks of *E. coli* infections, which are a major cause of morbidity and mortality in humans with considerable associated health care costs (DuPont et al., 1971; Russo and Johnson, 2003; Staley et al., 1972).

Eventually, *E. coli* was found to be a ubiquitous pathogen and it is now considered to be one of the most frequent causes of common bacterial infections in humans. Pathogenic strains of *E. coli* are known to be a major cause of both community- and hospital-acquired infection. In addition, it is predominantly responsible for four main types of infection in humans according to both the virulence determinants and clinical criteria: urinary tract infections (UTI); blood infection or sepsis; neonatal meningitis; and gastroenteritis (Katouli, 2010; Russo and Johnson, 2003; Weintraub, 2007).

When a potentially pathogenic strain encounters new environmental conditions within a host, the array of virulence determinants carried by the strain, often on mobile elements, allows them to cause infections. Recombination plays a central role in evolution of *E. coli* (Petty et al., 2014) and strains will readily acquire plasmids and other mobile elements leading to development of resistance to antimicrobial agents (Diard et al., 2007) .

Since the first occurrence of antimicrobial-resistant *E. coli* strains, highly significant health concerns have emerged and driven a need to understand the resistance mechanisms responsible for reduced susceptibility. There is also a pressing need to identify and understand the virulence factors that are key in pathogenesis.

Antimicrobial resistant *E. coli* strains have frequently been reported all over the world causing sporadic outbreaks (Croxall et al., 2011; Lavollay et al., 2006). Some of this disease is associated with of emergence of *E. coli* clonal groups that are resistant to numerous antimicrobial agents, largely as a result of their ability to exchange genetic material (Petty et al., 2014; Rogers et al., 2011).

A study conducted between 2003 and 2008 in Chicago evaluated the frequency of extended spectrum β-lactamase (ESBL) producing *E. coli* isolates and showed a significant increase of CTX-M type ESBL producing *E. coli*, with prevalence rising from 0.21% in 2003 to 2.99% in 2008 (Qi et al., 2010). In light of such studies, it is clear that decreasing the dissemination of multidrug-resistant *E. coli* requires on-going monitoring and further studies to understand the phylogenetic background, virulence spectrum and the transmission mechanisms of these pathogens should be conducted. The lack of knowledge relating to these factors makes it difficult to achieve effective infection control (Jadhav et al., 2011).

1.1. What is *Escherichia coli?* – A commentary on the evolution and diversity of this important microorganism

The early description of *E. coli* was initially performed by Theodor Escherich in 1885 who named the organism *Bacterium coli commune* (Escherich, 1989). A little later, *Shigella dysenteriae* (*Bacillus dysentericus*) was identified by the Japanese bacteriologist Kiyoshi as a related organism (Shiga, 1897). Subsequently, these two organisms were renamed as *Escherichia* and *Shigella* after their respective discoverers (Castellani, 1919) as they are distinguished on the basis of motility, metabolic profiles and clinical manifestation. Before the availability of DNA sequence data, the study of *E. coli* diversity was based on serotyping studies to distinguishing strains of *E. coli* (Ørskov et al., 1976). However, DNA sequencing data is rapidly changing the landscape of genetics, providing the ability to generate vast sequencing data sets and enhance the understanding of the population biology and genetic diversity of these organisms. *E. coli* is the classical model system to biologists and is the first bacterium that was used to introduce population genetics tools (Wirth et al., 2006). A number of studies have used these population genetics tools to investigate the evolutionary pathways of *E. coli* and observe their genetic diversity (Clermont et al., 2013; Wirth et al., 2006). A study by Chaudhuri and Henderson discussed the understanding of genome evolution and the phylogenetic relationships between *E. coli* strains (Chaudhuri and Henderson, 2012). This study illustrated the importance of genome sequence data and emphasized the use of population genetic tools to provide a deeper insight into the evolution of *E. coli.* Multi Locus Sequence Typing (MLST) is a method that has become an essential part of

the population genetics toolbox and has provided a great understanding of bacterial evolution (Maiden et al., 1998; Wirth et al., 2006).

Tests of recombination rate are usually performed during MLST analysis using the ClonalFrame method (Vos and Didelot, 2009). However, previous studies showed that the sequence-type of *E. coli* (EAEC) shows no consensus on recombination rates (Okeke et al., 2010; Wirth et al., 2006). Okeke and others developed a molecular phylogenetic framework for EAEC, which confirms that EAEC are very heterogeneous and demonstrates that the EAEC pathotype does not reflect a common ancestry or even a manageable number of clonal groups (Okeke et al., 2010).

Genetic diversity of *E. coli* and emergence of pathogenic variants results from two evolutionary processes: horizontal exchange and spontaneous mutations (Donnenberg, 2002). Horizontal exchange occurs through different mechanisms including transformation, transduction and conjugation (Arber, 2014). Mutations involve the introduction of single nucleotide polymorphisms (SNPs) or specific patterns of deletions of various sizes throughout the genome, which are transmitted vertically from generation to generation (Dixit et al., 2015; Donnenberg, 2002).

Horizontal gene transfer (HGT) is common between *E. coli* strains and is the primary cause of increasing virulence potential and antimicrobial resistance (Juhas, 2013). This occurs when bacterial cells acquire plasmid-mediated antibiotic resistance and virulence determinants as well as through incorporation of pathogenicity islands (PAIs) (Beceiro et al., 2013). Further studies of these events will enhance our understanding of the genomic evolution of *E. coli* and the phylogenetic relationships within diverse clonal groups.

1.1 The characteristics of *Escherichia coli*

1.1.1 General and cultural characteristics

E. coli is a Gram-negative bacillus capable of facultative-anaerobic respiration and fermentation and is classified under the family *Enterobacteriaceae.* It is a motile non-sporulating bacterium and has the capability to grow and live in a wide variety of natural environments. The optimum temperature range for *E. coli* to be able to grow is between 7°C and 50°C and it can tolerate acidic conditions down to pH 4.4 (Kayser et al., 2011).

As a result of these characteristics, *E. coli* occur in diverse forms in nature, ranging from commensal strains to those pathogenic in human or animal hosts and they can also be detected in a variety of environments such as water, soil and surfaces (van Elsas et al., 2011). In addition, *E. coli* has the ability to grow either *in the presence or absence of oxygen at* 37ºC *incubation temperature* (Kaper et al., 2004). The morphology of *E. coli* on sold media reveals non-pigmented colonies that are identified as either smooth (S) or rough form (R), where the former are smooth, high and circular and the latter are rough, flat and irregular (Hasman et al., 2000).

This phenotypic distinction is based on lipopolysaccharide (LPS) status. The presence of full-length O-antigen chains determines the smooth LPS, whereas the absence or reduction of O-antigen chains results in rough LPS (Hasman et al., 2000).

1.1.2 Serological characteristics

E. coli strains can be classified under their serological characteristics of three markers, depending on the multitude of epitopes present on the cell surface. This classification was first introduced by Kauffmann based on the presence of O-antigens (heat-stable somatic lipopolysaccharide antigens), K-antigens (surface or capsular antigens) and H-antigens (flagellar antigens) (Kauffmann, 1964). These three serological characteristics were identified as an important scheme for *E. coli* serotyping (Orskov and Orskov, 1985).

1.2 Pathotypes of *Escherichia coli*

The pathogenic strains of *E. coli* can be classified into two major groups according to the ability to cause diseases within (enteric pathogenic) and outside (extra-intestinal pathogenic) the gastrointestinal tract. The enteric pathogenic group is sub-classified into six well-described pathotypes: enteropathogenic *E. coli* (EPEC); enterohaemorrhagic *E. coli* (EHEC); enterotoxigenic *E. coli* (ETEC); enteroaggregative *E. coli* (EAEC); enteroinvasive *E. coli* (EIEC); and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004). However, it is the extra-intestinal pathogenic (ExPEC) that are responsible for several infections due to the ability of *E. coli* to spread from the intestine to other parts of the body.

1.2.1 Extra intestinal pathogenic *Escherichia coli*

ExPEC is considered to be a significant heath concern and one of the most predominant causes of both community and hospital acquired infection. Until now, classifications within ExPEC have not been clearly categorised, compared to enteric pathogens (Jadhav et al., 2011).

However, the group can be differentiated according to the type of disease caused into uropathogenic *E. coli* (UPEC), sepsis-associated *E. coli* (SEPEC) and neonatal meningitis-associated *E. coli* (NMEC) (Johnson et al., 2003a; Russo and Johnson, 2003). This study will not consider the two latter groups of *E. coli*.

ExPEC possess specific virulence traits that allow strains to invade, colonize, persist and cause diseases in body sites outside of the gastrointestinal tract (Smith et al., 2007). The phylogeny and epidemiology of ExPEC are distinct compared to commensal and intestinal pathogenic strains. A study by Johnson and others defined ExPEC strains based on the existence of two or more virulence markers that were determined by multiplex PCR, including *papA* (P fimbriae structural subunit) and/or *papC* (P fimbriae assembly), sfa/foc (S and F1C fimbriae subunits), afa/dra (Dr-antigen binding adhesins), *kpsMT* II (group 2 capsular polysaccharide units) and *iutA* (aerobactin receptor) (Johnson et al., 2003b).

1.2.2 Uropathogenic *Escherichia coli* (UPEC)

E. coli is the most common infectious agent that causes urinary tract infection (UTI). As stated above, *E. coli* is a commensal organism in the gastrointestinal tract, which provides a pool of organisms for the initiation of UTI.

UTI will occur at least once during the lifetime of half of all women and the incidence of UTI is observed to be higher in females (64%) than in males (36%) due to the shorter urethra in females than in men (Foxman, 2003; Jadhav et al., 2011). These infections are mainly caused by UPEC, which are responsible for 85-95% of uncomplicated cystitis in premenopausal women and around 90% of uncomplicated pyelonephritis (Russo and Johnson, 2003). Moreover, *E. coli* is responsible for up to 90% of all the UTIs which are noted among ambulatory patients and around 50% of all nosocomial UTIs (Tartof et al., 2005).

During the past two decades, there has been a dramatic increase in research into the virulence factors involved during UTI in order to better understand the mechanisms of pathogenesis of UPEC (Flores-Mireles et al., 2015; Kalita et al., 2014; Wiles et al., 2008). These studies have been conducted to determine the virulence factors present and identify the surface structure and secreted proteins of uropathogenic strains. The results of these studies have identified various factors that make *E. coli* more virulent when present within the urinary tract (Kawamura-Sato et al., 2010; Piatti et al., 2008; Russo and Johnson, 2003). This subject is discussed in more detail in a following section (1.4 Urovirulence factors).

1.3 Clinical relevance of uropathogenic *E. coli*

The majority of the bacterial cells existing in the urinary tract are from the ascending route from the periurethral area through the urethra into the bladder (Figure 1.1) (Walters et al., 2012). In addition, bacteria can be present in the upper urinary tract by further ascent through the ureters to the kidneys (Figure 1.2). The presence of *E. coli* within the urinary tract is not always sufficient to cause symptomatic infection and observed clinical conditions range from asymptomatic bacteriuria (ABU) to chronic pyelonephritis (Ragnarsdóttir and Svanborg, 2012).

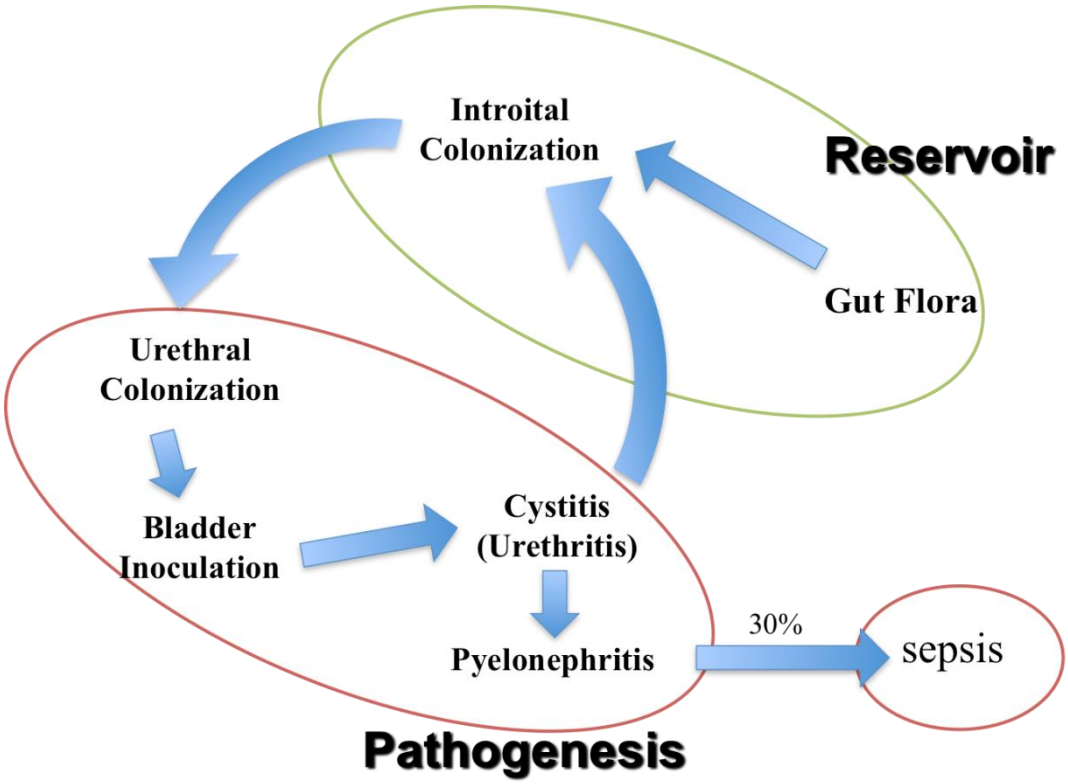

Figure 1.1 Postulated sequence of events in urinary tract infection and urosepsis (Neu, 1992).

Successful ascent of the organism to the urinary tract may lead to invasion and colonisation into host cells due to expression of some or all of the virulence factors carried by a strain. In addition, UPEC has developed several strategies to evade the innate immune response to be more effective in colonising host cells (Wiles et al., 2008). Currently, the mechanism of ascension is unknown but motility may be considered as playing an important role (Lloyd et al., 2007). Previous studies have paid much attention to the ability of UPEC to form biofilms and develop intracellular biofilm-like communities (IBCs) (Ben Mkaddem et al., 2010; Goller and Seed, 2010; Rosen et al., 2007).

When infection occurs in the urinary tract, UPEC multiply and grow in or on the urothelium, which leads to high numbers of bacterial cells. The main indication of bacteriuria is usually considered clinically by the quantitation of the bacteria present. In 1956, a study by Kass suggested that a count of $>10^5$ CFU/ml of UPEC, is considered as significant and indicative of UTI (Kass, 1956; Perlhagen et al., 2007).

However, another study by Stamm and colleagues re-evaluated the quantity criteria for diagnosing coliform infection of the urinary tract in symptomatic women and found that $\geq 10^2$ CFU/ml of a uropathogen appears to be significant and associated with UTI (Rosen et al., 2007; Stamm et al., 1982).

The clinical features of UTI vary depending upon the gender and the age of the patient. In addition, other clinical conditions play a considerably important role in increasing the chances of developing a UTI; these include diabetes, pregnancy, bowel incontinence and insertion of urinary catheters into the urinary tract of a patient undergoing a long period of hospitalisation.

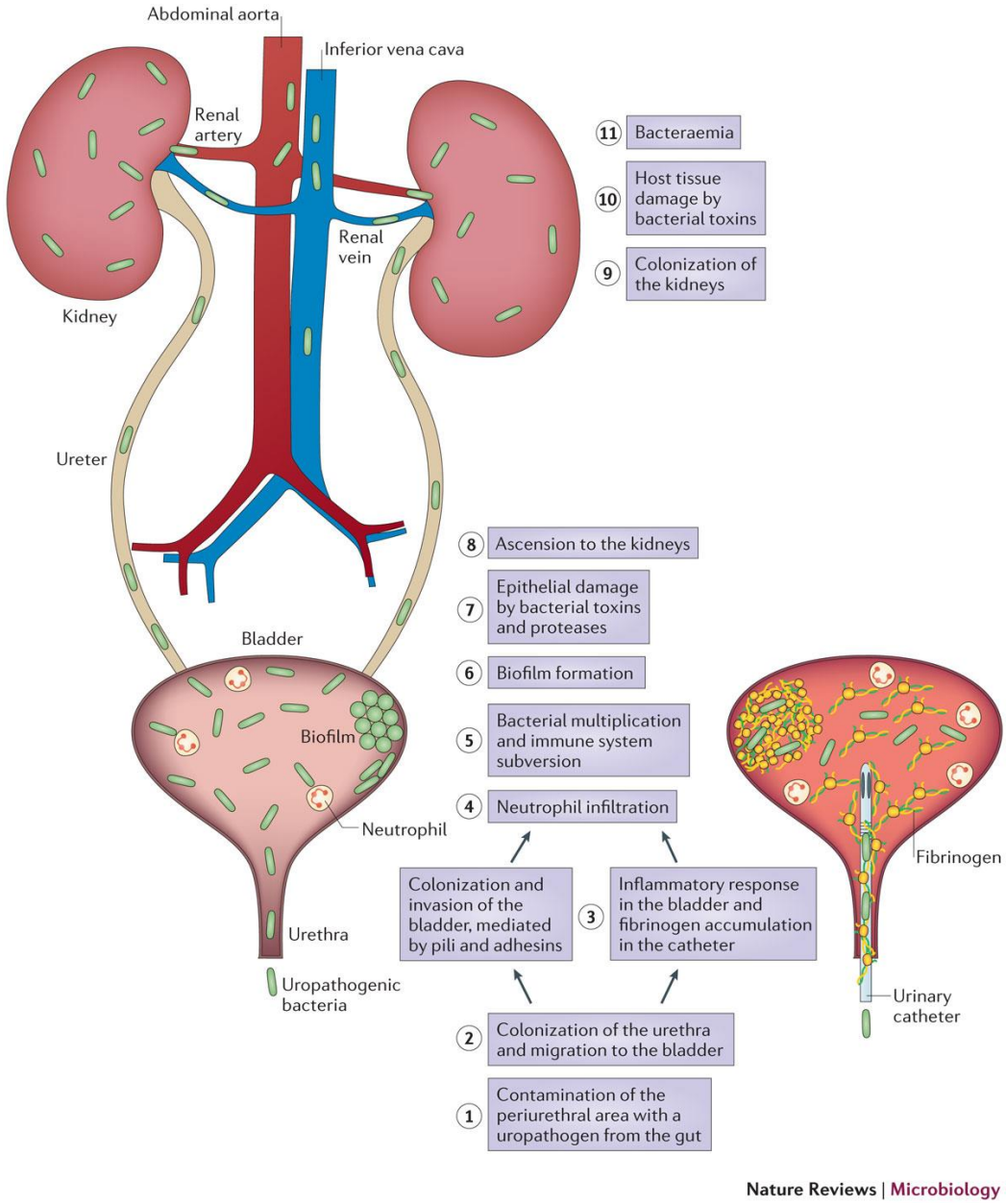

Figure 1.2 Stages of urinary tract infection caused by uropathogenic *E. coli*. Figure adapted from (Flores-Mireles et al., 2015).

1.4 Urovirulence factors

As *E. coli* is considered the most predominant cause of UTI, many studies have suggested that there are special properties (virulence factors; VF) that enhance the ability of *E. coli* to cause disease in a particular host (Emody et al., 2003; Johnson, 1991; Moreno et al., 2006; Wiles et al., 2008). *E. coli* can be an infectious agent in the urinary tract that causes disease by the cumulative impact of one or several virulence factors (Figure 1.3). In fact, investigation of the virulence factors in certain strains is very important to help understand the mechanism of action of the virulence properties and also to develop specific anti-VF interventions to prevent infection (Johnson, 1991; Kawamura-Sato et al., 2010).

At the basic level of epidemiology, virulence factors of UPEC can be categorised by comparing the host population variation (e.g. male vs female, pregnant vs non-pregnant women, patient with normal vs abnormal or instrumented urinary tracts). This comparison will reflect the correlation between a particular virulence factor with the host-parasite interactions (Johnson, 1991). Also, potential findings of virulence factors from UPEC can be identified by comparing the properties of the bacteria, which have been isolated as a main cause of UTI, with faecal strains from healthy subjects (Abe et al., 2008). In addition, the severity of the clinical syndrome of UTI is an important factor in categorising urinary isolates for the purpose of identifying more potential virulence factors. Suspected virulence factors have also been identified using localisation methods to determine the upper (kidney and ureter) and lower (bladder and urethra) uropathogenic isolates (Bien et al., 2012).

However, a true prevalence of virulence factors can be obtained by combined results from a variety of epidemiological studies and is now much more feasible using next-generation sequencing approaches (Totsika et al., 2011).

In fact, human epidemiology and the study of animals have played an important role in determining the mechanisms of action of putative virulence factors. The assessment of certain bacterial properties and their contribution to virulence can be determined in-vitro at the cellular or subcellular level or by using animal models. In addition, identification and understanding the virulence factors of UPEC and their mechanism of action facilitate practical applications for more precise approaches in phenotypic or molecular diagnosis and epidemiology. Several properties of UPEC are well described as virulence factors in UTI and Table 1.1 summaries the most important ones.

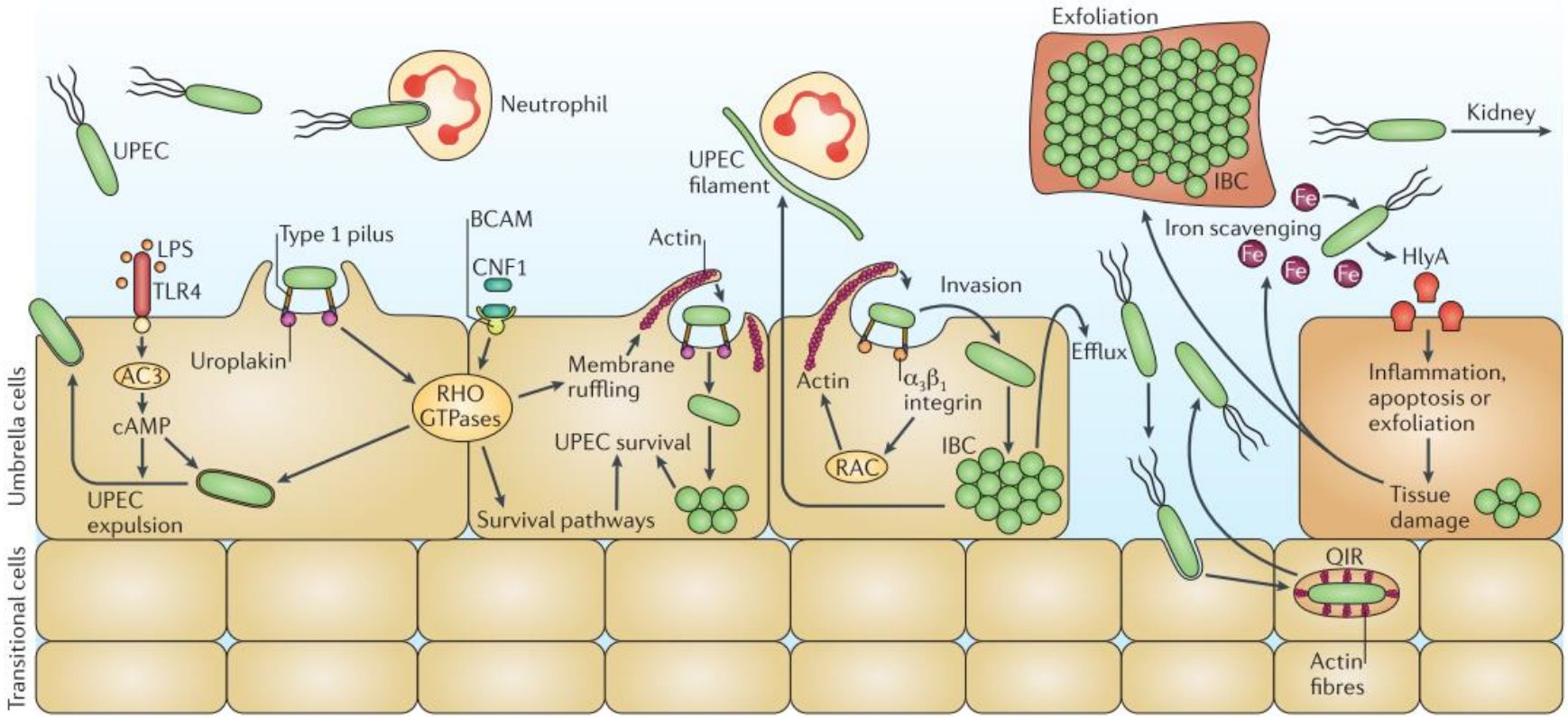

Figure 1.3 Virulence factors that are involved in different stages of the pathogenicity of UPEC. Figure adapted from (Flores-Mireles et al., 2015).

Table 1.1 Virulence factors of *E. coli* involved in urinary tract infection

Virulence factors	Location	Function	Epidemiology	Remarks	Reference
Type 1 fimbriae (*fimA* gene)	Bacterial surface	Adhesion to mucosal epithelium and tissue matrix, invasion, biofilm formation	Present in almost all *E. coli* isolates, but *fimA* gene was observed to be present more in *E. coli* phylogroups A and D	UPEC had the ability to form intracellular bacterial communities (IBCs) due to the high expression of the *fimA* gene, coding for type 1 fimbrae (T1F), which are responsible for adherence to and invasion of luminal facet cells of the bladder	(Johnson, 1991) (Emody et al., 2003) (Piatti et al., 2008) (Berry et al., 2009)
P fimbriae (*papC* gene) (*papGIII* gene)	Bacterial surface	Adhesion to mucosal epithelium and tissue matrix, cytokine induction	Higher rate of detection of *papC* among strains causing pyelonephritis and prostatitis than among those causing cystitis	*papGIII* gene was significantly detected among strains that cause prostatitis more than those causing pyelonephritis	(Emody et al., 2003)
S fimbriae (*sfa* gene)	Bacterial surface	Adhesion to mucosal and endothelial cells, and to tissue matrix	S fimbriae have been identified and expressed by *E. coli* strains causing newborn meningitis or UTI	S fimbriae found to bind to the epithelial cells of the proximal and distal tubules and on renal vascular endothelium	(Korhonen et al., 1986) (Marre et al., 1990)
F1C fimbriae (*foc* gene)	Bacterial surface	Adhesion to mucosal and endothelial cells	14 % of *E. coli*-harbouring F1C fimbriae are responsible for causing UTI	F1C fimbriae have been reported to bind to epithelial cells and endothelial cells of human kidney and bladder	(Khan et al., 2000)
K antigen O antigen	Bacterial surface	Antiphagocytic, anticomplement effect, serum resistance, evasion of immune recognition	Certain types of K antigen are associated with UTI (Kl, K5, K12, K13, and K20). However, K1 and K5 have been detected in 63% *E. coli* isolates from women with pyelonephritis. Certain types of O antigen are associated with *E. coli* isolated from UTIs (such as O1, O2, O4, O6, O16, O18, O22, O25 and O75).	Together, K and O antigens serve as an important tool to differentiate UPEC from other *E. coli* strains.	(Johnson, 1991) (Emody et al., 2003)

Table 1.1 Virulence factors of *E. coli* involved in urinary tract infection (continued)

Virulence factors	Location	Function	Epidemiology	Remarks	Reference
Lipopolysaccharide	Bacterial surface	Endotoxic effects, 'O'-antigen, cytokine induction, serum resistance, immuno-adjuvant	Lipopolysaccharide has been a major factor contributing to the pathogenesis of *E. coli* infection	Induces cytokine expression through toll-like receptor 4 (TLR4)	(Kaper et al., 2004) (Morrison and Ryan, 1987)
α - Haemolysin	Exported	Cytotoxicity, haemolysis Lysis of erythrocytes and leukocytes	One of the common important virulence factors in ExPEC isolated from pyelonephritis, cystitis and asymptomatic bacteriuria and faecal isolates	Mainly associated with invasive UTI. Play complex roles including aiding iron acquisition to disruption of phagocyte function and direct toxicity towards host cells	(Johnson, 1991) (Emody et al., 2003)
Cytotoxic necrotising factor 1		Interference with phagocytosis and apoptosis	Frequently associated with UTI isolates and significantly related to prostatitis		(Dhakal and Mulvey, 2012)
Secreted autotransporter toxin		Cytotoxicity	More frequently associated with pyelonephritis causing isolates		
Enterobactin	Exported	Growth under iron restriction	73%, 49%, 58%, 41% and 38% of isolates from pyelonephritis, cystitis, bacteraemia, asymptomatic bacteriuria and faecal isolates, respectively	Proposed association with *E. coli* causing serious and complicated UTI, as it promotes bacterial growth in conditions limited in iron concentration	(Johnson, 1991) (Emody et al., 2003)

1.5 Antimicrobial resistance and virulence

During recent decades, *E. coli* has developed a high rate of antimicrobial resistance, and researchers have become motivated to understand the mechanism of resistance and how, if at all, it can be correlated with virulence factors. Despite the increase of antimicrobial-resistant *E. coli*, a study by Johnson and colleagues (1987) reported that some *E. coli* strains causing UTI that are P fimbriated were susceptible to antibiotics, which indicates that carriage of virulence factors might not be association with the development of antimicrobial resistance (Johnson et al., 1987).

Another study conducted in 1991 by Johnson and others suggested that there is no association between virulence factors and some UPEC isolates that are resistant to a number of antimicrobial agents such as ampicillin, sulfonamides, tetracycline and streptomycin (Johnson, 1991). The authors of this study suggested that infection in immunocompromised patients frequently using antibiotics may strengthen the resistance of UPEC, irrespective of their virulence potential. More recently, several studies have demonstrated a negative association between antimicrobial resistance and virulence potential (Gibreel et al., 2012b; Johnson et al., 2002a; Velasco et al., 2001; Vila et al., 2002).

A recent study in Japan by Kawamura-Sato and colleagues again focused on this issue and investigated the potential relationship between virulence genes, antimicrobial resistance and phylogenetic background of UPEC (Kawamura-Sato et al., 2010).

The results of this study were consistent with previous reports, indicating that a complex relationship exists and the authors suggest more intensive studies are needed to identify the genetic elements that are involved either in virulence or antibiotic resistance.

1.6 Antimicrobial resistance in uropathogenic *E. coli*

Antibiotic resistance is considered to be a major concern worldwide and there is an urgent requirement to control the development and spread of antibiotic resistance. *E. coli*, as one of the main aerobic commensal bacterial species, has been extensively studied with respect to surveillance of antimicrobial resistance. During the last two decades, there has been a substantial increase in antimicrobial resistance in UPEC among human clinical isolates. A recent surveillance report from the European Centre for Disease Prevention and Control (ECDC) assessing antimicrobial resistance, showed a significant decline of antimicrobial susceptibility among *E. coli* isolates collected from different European countries (ECDC, 2013). The report showed that half of *E. coli* isolates reported to EARS-Net in 2013 were resistant to antimicrobials from at least one group. The most common resistance pattern was reportedly towards both to aminopenicillins and fluoroquinolones, both as single resistance phenotypes and in combination with other antimicrobial groups. Between 2010 and 2013, 30 EU/EEA countries reported a low trend of carbapenem resistance in *E. coli*, which remains very uncommon in Europe.

It should be noted that antibiotic resistance is not restricted to pathogenic *E. coli* since the commensal bacterial flora may become a major reservoir of resistant strains/resistance determinants for acquisition by pathogenic strains (Van Den Bogaard and Stobberingh, 2000). It is also possible that reduced susceptibility may increase the potential for commensal strains to become opportunistic pathogens in the urinary tract or bloodstream (Russo and Johnson, 2003).

The increased resistance in UPEC has been globally reported with unexpected trends of resistance to a remarkable range of antimicrobial agents, including the most well known antibiotic groups such as the β-lactam antibiotics, fluoroquinolones, trimethoprim and the aminoglycosides (Pitout, 2012).

1.6.1 β-lactam antibiotics

β-lactam antibiotics are the most commonly-used antimicrobial agents and UTI is often treated using these antibiotics. The first generation cephalosporins and amoxicillins were used to treat uncomplicated UTI, whereas complicated UTI used to be treated with third generation cephalosporins. Numerous reports have documented the high rates of β-lactam antibiotic resistance found in *E. coli* (Furuya and Lowy, 2006; Pitout and Laupland, 2008; Tang et al., 2014; Wright, 2011).

1.6.1.1 β-lactamases

Since the introduction of β-lactam antibiotics, Gram-negative rods have been developing resistance by producing different β-lactamase enzymes to inactivate β-lactam antibiotics. Production of β-lactamase is the most prevalent and evident resistance mechanism in *E. coli* (Tang et al., 2014).

The resistance mechanism is often related to the plasmid-mediated β-lactamases, TEM1 and TEM2, which are widely distributed among Gram-negative bacteria (Jarlier et al., 1988). In addition to these enzymes, another type of β-lactamase called SHV has the ability to hydrolyse penicillins and some cephalosporins (Gutmann et al., 1989).

Many Gram-negative bacteria also harbour β-lactamase genes on their chromosome. These bacteria exhibit low expression of the ESBL phenotype due to the presence of a single *bla* gene copy coupled with a weak promoter (Mulvey et al., 2004; Tracz et al., 2007). These bacteria naturally show resistance to penicillin and first and second-generation cephalosporin antibiotics, but are susceptible to third-generation cephalosporin antibiotics.

1.6.1.2 Extended Spectrum β-lactamases

Extended spectrum β-lactamase (ESBL)-producing isolates mostly express resistance towards multiple antibiotics that are used to treat infections caused by Gram-negative bacteria. In 1989, Philippon and colleagues first named extended spectrum β-lactamases (Cantón and Coque, 2006; Philippon et al., 1989). Most ESBLs can generally be divided into three groups, TEM, SHV and CTX-M, depending on amino acid sequence homology (Figure 1.4). Later, in 1989, another group of ESBLs was detected and named CTX-M because of their increased activity against cefotaxime (and their discovery in Munich) (Bauernfeind et al., 1990).

This was the first report that recognized ESBL from the CTX-M group in a cefotaxime-resistant but ceftazidime susceptible *E. coli* isolate recovered from the ear of 4-month-old child. CTX-M enzymes are considered to be an example in the evolution of a resistance mechanism of *E. coli*. $bla_{\text{CTX-M}}$ related genes spread globally with the participation of the so-called "epidemic resistance plasmids" which are often carried in strains from multi-drug resistant clones (Cantón et al., 2012).

According to a clinical update in 2005, ESBLs developed significantly over the two preceding decades. It was suggested at the time that this may create major therapeutic problems in the future, including the potential for plasmid-mediated quinolone and carbapenem resistance (Paterson and Bonomo, 2005). Figure 1.4 shows the most prevalent ESBL types that have been identified in UPEC strains worldwide (Pitout et al., 2005).

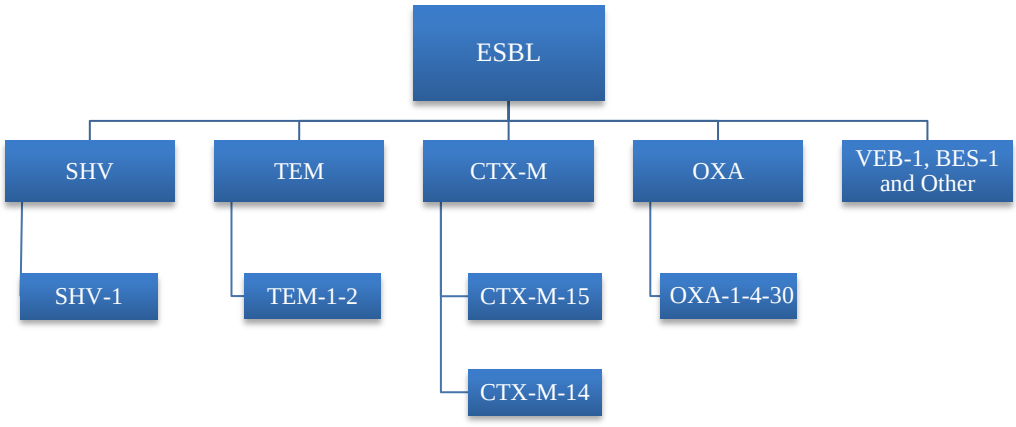

Figure 1.4 Flow chart diagram of the most prevalent ESBL types.

1.6.1.3 AmpC-type β-lactamases

In 1940, an extract of *E. coli* was reported to contain an enzyme which destroyed the growth inhibiting property of penicillin (Abraham and Chain, 1940; Jacoby, 2009). Subsequently, a systematic study of penicillin resistance genes revealed mutations in *ampA* and *ampB,* which enhanced penicillin resistance in *E. coli* (Eriksson-Grennberg et al., 1965). AmpC β-lactamases arose as a result of mutations in *ampA* and the sequence of *ampC* was first reported in 1981 (Jaurin and Grundström, 1981).

Some *E. coli* strains contain a chromosomal *ampC* gene, which has a weak promoter that leads to low transcription of AmpC that makes *E. coli* susceptible to ampicillin. However, mutations were found within the *ampC* promoter sequence that lead to AmpC overproduction, which makes *E. coli* resistant to ampicillin and reduces susceptibilities to extended spectrum β-lactam antibiotics (Caroff et al., 1999; Corvec et al., 2002; Mulvey et al., 2005; Siu et al., 2003). In 1988, *ampC* genes were first captured on a plasmid and described as the novel plasmid-mediated β-lactamase (MIR-1) (Papanicolaou et al., 1990; Philippon et al., 2002). The CMY-2-type AmpC β-lactamase is the most common reported plasmid-mediated AmpC β-lactamase worldwide (Pavez et al., 2008; Sheng et al., 2013). Genomic analysis of a multidrug resistant *E. coli* strain, EC958, from the ST131 clone revealed carriage of the CMY-23 type AmpC cephalosporinase, identified within a putative genomic island (Totsika et al., 2011). It has very recently been demonstrated that the CMY-23 enzyme in EC958 is the predominant driver of resistance to 3rd generation cephalosporins in this strain and its chromosomal location confirmed (Phan et al., 2015).

1.6.1.4 Carbapenemases

The rapid growth of cephalosporin resistance leads to more reliance on carbapenem antibiotics, which have good stability towards both AmpC and ESBL enzymes (Livermore and Woodford, 2006). This increased reliance is based on the fact that many ESBL producers also exhibit multi-resistance to other antibiotic classes including aminoglycosides, trimethoprim, tetracycline and fluoroquinolones. The resistance to carbapenems was reported to be extremely slow in Enterobacteriaceae after 20 years of imipenem use (Livermore, 2003).

Carbapenem resistance can be increased by three routes, as described by Livermore and Woodford (i) permeability lesions in organisms with AmpC enzymes or ESBLs, (ii) acquisition of IMP or VIM metallo-β-lactamases, or (iii) acquisition of non-metallocarbapenemases of the KPC, IMI/NMC, SME or OXA families (Livermore and Woodford, 2006). In addition, New Delhi metallo-β -lactamase (NDM) enzymes are the latest carbapenemases to be recognized and they have been reported globally (Nordmann et al., 2011; Yong et al., 2009) More specifically, several studies have reported a global increase in carbapenemase-producing UPEC, especially from the ST131 lineage (L. Chen et al., 2014; Morris et al., 2012, 2011; Peirano et al., 2014b).

A recent antimicroial resistance surveillance report in Europe showed that carbapenem resistance in *E. coli* in 2013 remained very uncommon, with resistance precentages of < 0.1% reported by the majorty of 30 Europen contries (ECDC, 2013). Dissmentation of carbapenemase-producing *E. coli* clones would create significant public health concerns and limit even further the available treatment options for UPEC infections (Figure 1.5).

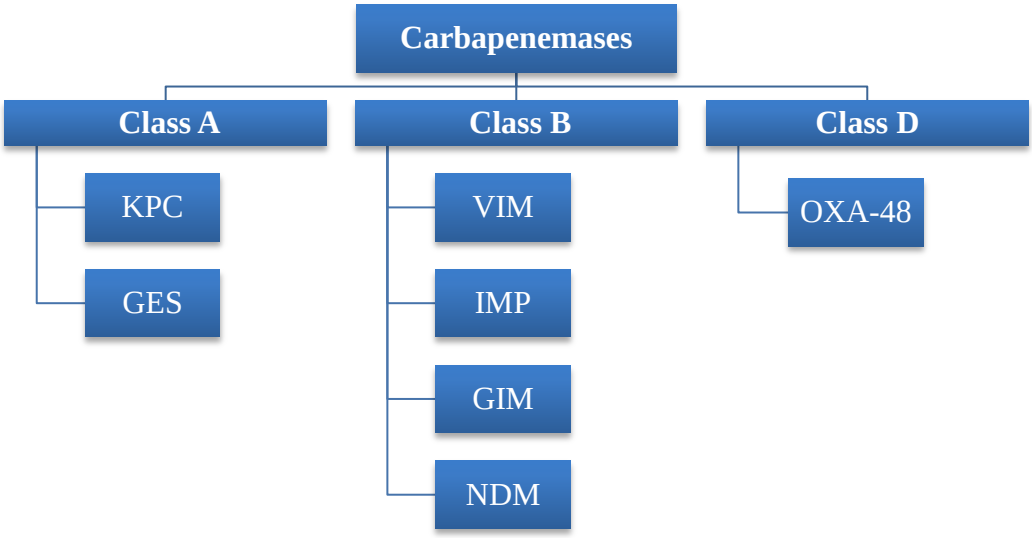

Figure 1.5 Flow chart diagram of the most commonly identified carbapenemases in Enterobacteriaceae (Dortet et al., 2014; Nordmann, 2014).

1.6.2 Fluoroquinolones

In 1962, quinolones were first developed as antibiotics against Gram-negative bacteria (Takahashi et al., 2003). Quinolones can inhibit chromosome re-ligation by attaching to DNA gyrase and topoisomerase (Walker, 1999). Shortly after the introduction of quinolones, fluoroquinolones became favoured for their wide spectrum of activities and were recommended as the drug of choice for treating upper and complicated UTI. A recent survey carried out across the United States and Europe illustrated an increase of quinolone and fluoroquinolone resistance among UPEC isolates, with resistance occurring by chromosomal mutation or acquisition of plasmids carrying resistance genes (Kawamura-Sato et al., 2010).

A retrospective study illustrated a remarkable increase of ciprofloxacin resistance in UPEC in the West of Ireland from 2004 to 2008. The increase is significantly associated with an increase in prescriptions for ciprofloxacin to patients in general practice (Vellinga et al., 2010); the increase in resistance is due to the frequent use of ciprofloxacin in both complicated and uncomplicated UTI.

Resistance to fluoroquniolones is associated with a series of mutation patterns in different target genes. A study by Takahashi and others included an analysis of the mutation patterns of quinolone resistance-determining regions (QRDRs) in *gyrA, gyrB, parC* and *parE* (Takahashi et al., 2009). The result of this analysis revealed at least three points of mutation at Ser83 and Asp87 encoded in *gyrA* and at Ser80 encoded in *parC* that occur among fluoroquinolone-resistant *E. coli* isolates obtained from patients with cystitis. Similar mutations have been reported worldwide (Al-Agamy et al., 2012; Garcı, 2010; Gibreel et al., 2012b; Kawamura-Sato et al., 2010; Matsumura et al., 2013; Yue et al., 2008).

In addition, the study by Takahashi also reported other mutations in the same isolates, which possessed one or two more mutation points at Glu84 or Ala108 encoded in *parC* and/or at Leu445 or Ser458 encoded in *parE*. However, in this study no mutation was found in the target gene *gyrB* (Takahashi et al., 2009).

In 1998, quinolone resistance increased and the known mechanisms for this resistance were shown to involve chromosomal mutations of DNA gyrase or DNA topoisomerase IV genes or result from decreased quinolone accumulation (Martínez-Martínez et al., 1998). However, plasmid-mediated horizontally transferable genes that encode the Qnr protein are another quinolone resistance mechanism, which protects the quinolone target from inhibition (Strahilevitz et al., 2009).

1.6.3 Trimethoprim

Trimethoprim is considered one of the most widely-used antibiotics for inhibiting bacterial growth based on use alone, or in combination with bacterial protein production inhibitors (bacteriostatic agents), such as the sulphonamides (Huovinen et al., 1986). In fact, trimethoprim is one of the first-line antibiotics that is effective for the treatment and prophylaxis of UTI (Chakupurakal et al., 2010).

The mechanism of action of trimethoprim is based on the inhibition of DNA replication by interfering with dihydrofolate reductase and inhibiting synthesis of tetrahydrofolic acid (Sköld, 2001). Recent studies have illustrated an increase of resistance to trimethoprim, especially in *E. coli*, which is associated with a plasmid mediated gene (*dfr*) that is modified to affect the function of the target enzyme dihydrofolate reductase (*dfr*) (Brolund et al., 2010). The study by Brolund and colleagues investigated the distribution of 13 *dfr*-genes among urinary tract isolates of *Enterobacteriaceae* over a four-year period (Brolund et al., 2010). The investigation was mainly associated with screening for integron classes I, II and *dfr*-genes and the epidemiology of trimethoprim resistance within *E. coli* and *Klebsiella pneumoniae.* The results of this study, combined with a comparison with other studies in different clinical and geographical settings to evaluate the overall prevalence of the different *dfr*-genes in *E. coli*, indicated very stable distribution of *dfr*-genes. The prevalent genes detected in these studies are *dfrA1* and *dfrA17* (Blahna et al., 2006; Lee et al., 2001).

1.6.4 Aminoglycosides

Aminoglycosides are highly effective, broad-spectrum antibiotics with several properties relevant to treatment of microbial infections. The first of this group of antibiotics to be introduced was streptomycin, in 1944, which proved to be effective against Gram-negative bacillary infections (Mingeot-Leclercq et al., 1999). However, the aminoglycosides include many antimicrobial agents such as gentamicin, kanamycin, amikacin, tobramycin and neomycin. The basis of aminoglycoside action lies mainly in their ability to inhibit bacterial protein synthesis through targeting the small ribosomal subunit (Davies and Courvalin, 1977; Mingeot-Leclercq et al., 1999).

Aminoglycoside resistance is primarily mediated by plasmid-encoded aminoglycoside-modifying enzymes. A study by Ho and colleagues identified over 85 aminoglycoside-modifying enzymes in a collection of *E. coli* isolates (Ho et al., 2010). There are several mechanisms mediating aminoglycoside resistance and these include use of efflux pumps to decrease the intracellular concentration of the drug, target modification (16S rRNA and ribosomal mutations) and enzymatic drug modification (Magnet and Blanchard, 2005). Several studies have suggested that bacterial isolates from food-producing animals might be an important reservoir for aminoglycoside-resistance genes that have been observed in human isolates (Chaslus-Dancla et al., 1991; Johnson et al., 1995).

1.7 Typing methods for pathogenic *E. coli*

Investigation and surveillance of bacterial pathogens requires strain-typing methods to characterise the population of the pathogens as well as for determining the epidemiological distribution of the infectious agents. In addition, strain-typing methods are essential for surveillance of outbreaks and can improve our understanding of the evolutionary development of bacterial pathogens. UPEC is one of the most common agents causing UTI and strains need to be investigated to determine the epidemiology of the infection. There are two main typing methods for identification and differentiation of pathogenic *E. coli* strains. The flow diagram in Figure 1.6 illustrates typing methods and divides them into two types; phenotyping and genotyping methods. These methods can be characterised in terms of typeability, reproducibility, discriminatory power, ease of performance and ease of interpretation (Köhler and Dobrindt, 2011; Maslow et al., 1993; Tenover et al., 1997).

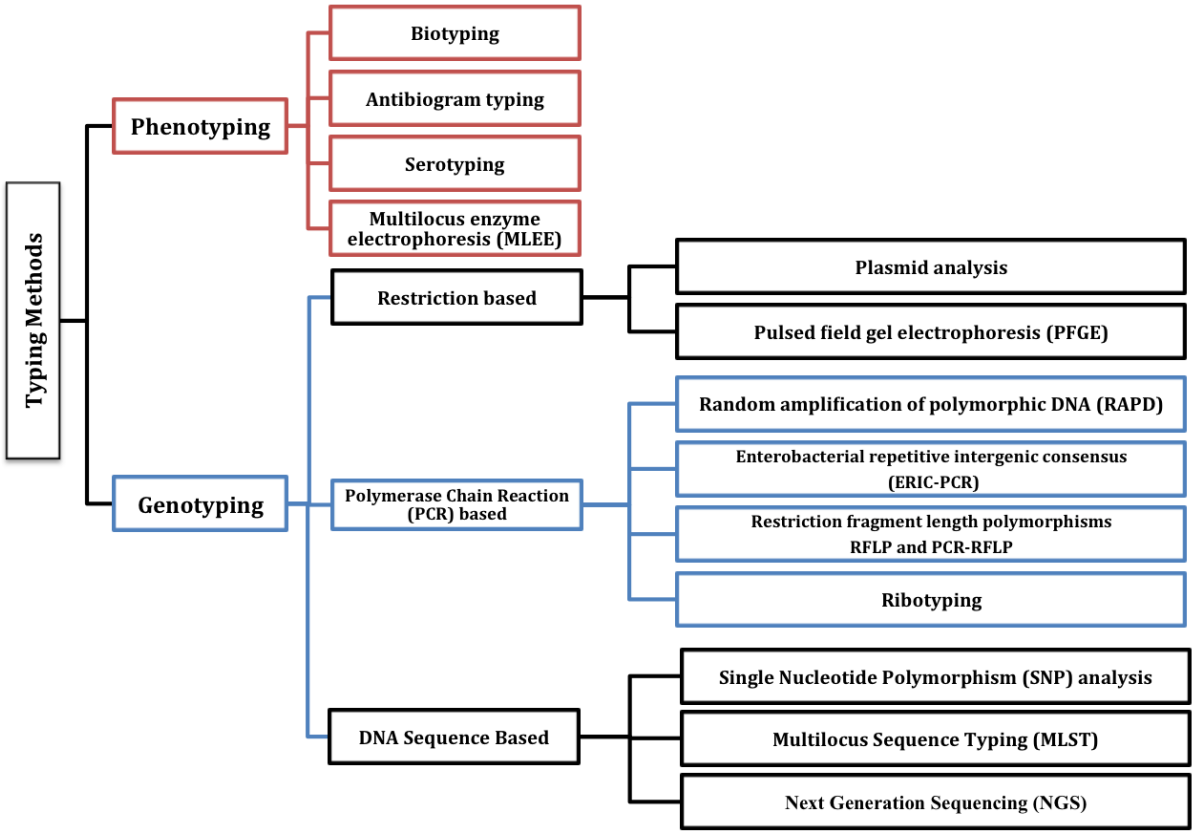

Figure 1.6 Flow diagram of typing methods used for epidemiological typing for pathogenic strains of *E. coli*.

1.7.1 Phenotyping methods

The phenotyping methods are divided into several types such as serotyping, biotyping, antibiotyping and multilocus enzyme electrophoresis. These methods characterise the products of gene expression to classify the strains into different subgroups. Most of these methods have been found to have limited discriminatory power (Van Belkum et al., 2001) and they are not now widely used.

Serotyping was historically considered to be one of most important epidemiological tools to describe the distribution of *E. coli* lineages. *E. coli* can be serotyped based on the determination of somatic (O), capsular (K) and flagellar (H) antigens (Kauffmann, 1947). The O antigen subunits can be used to distinguish between *E. coli* strains based on the structure of the O antigen gene clusters (DebRoy et al., 2011). The main method, and the most comprehensive for serotyping, is based on agglutination reactions, which is a simple method for testing O antigen groups (Orskov et al., 1977). *E. coli* serotypes can be used as epidemiological markers for specific lineages of *E. coli* that are equipped with specific virulence traits (Manges et al., 2001; Ziebell et al., 2008), for example the well known O157 lineage associated with production of verotoxin and development of haemolytic uremic syndrome in humans (Karmali et al., 2010). After the development of molecular technologies, the O antigen cluster genes have been sequenced for many O antigen groups (Liu et al., 2010, 2007; Wang et al., 2010). Subsequently, PCR assays have been developed for the 'molecular serotyping' of many pathogenic *E. coli* strains (Beutin et al., 2005; Wang and Reeves, 1998; Wang et al., 2010) and multiplex PCR assays targeting the most clinically relevant serotypes associated with urinary tract, bloodstream or gastrointestinal infections are very efficient means of serotyping strains (Clermont et al., 2007; Li et al., 2010; Sánchez et al., 2015).

Serotypes have been used to differentiate specific population as well as pathogenic from commensal *E. coli* (Johnson et al., 1994). Serotyping became an important tool for the classification of UPEC where specific O antigens have been described as being highly associated with UPEC (Li et al., 2010). Consequently, the major lineages of UPEC have been associated with specific O types (Table 1.2).

Table 1.2 Distribution of Serotypes among the major MLST lineages of UPEC

ST	Serotype	Reference
ST69	O11, O15. O17, O73, O77 and O86	(Lau et al., 2008a; Tartof et al., 2005)
ST73	O6, O25, O18	(Lau et al., 2008a; Suzuki et al., 2009)
ST95	O1, O2 and O45	(Lau et al., 2008a; Mora et al., 2013, 2009; Peigne et al., 2009; Vincent et al., 2010)
ST127	O6	(Harada et al., 2012; Johnson et al., 2008; Lau et al., 2008a)
ST131	O25 and O16	(Harada et al., 2012; Johnson et al., 2014; Lau et al., 2008a)

O antigen biosynthesis is an important virulence determinant that represents the main mechanism for serum resistance of UPEC (Phan et al., 2013). Recent reports have revealed that members of the globally disseminated ST131 multidrug resistant clonal group belong to serotypes O25 and O16 (Chang et al., 2014; Ho et al., 2015; Johnson et al., 2014). Members of ST69 and ST393 multidrug resistant clonal groups have been reported as belonging to O15 (Blanco et al., 2011).

1.7.2 Genotyping methods

There are several genotyping methods that vary depending on whether they are used to detect a certain gene or plasmid, or to screen for pathogenicity islands or a certain phylogenetic group (Figure 1.6).

The principle of genotypic methods basically relies on direct investigation and analysis of the genetic structure of the examined strains. Genotyping microbial organisms is used to study the relatedness of specific organisms that share certain traits, such as virulence factors, biochemical traits and genomic characteristics (Olive and Bean, 1999). Molecular epidemiological studies have revealed the diversity of species isolated from regions or locations at different times or from different sources. The individual methods applied are used to differentiate and classify microorganisms into genotypes or even to subtypes based on their respective levels of discriminatory power (Dias et al., 2010; Weissman et al., 2012). In the past decade, genotypic characterisation has become an essential factor in epidemiological investigation of UPEC strains. Historically, several studies have shown that genotyping is a reliable way to identify certain pathogenic strains that are usually recognised to cause outbreaks, such as *E. coli* O157:H7 (Manges et al., 2001; Tenover et al., 1997). However, a problem arises when it comes to characterising pathogenic strains that are not often found to cause outbreaks.

Community-acquired UTIs caused by UPEC were originally thought to not represent outbreaks, but in 2001 a report by Manges and colleagues identified clonal group A (CgA) strains, which were circulating in the community causing widespread outbreaks (Manges et al., 2001).

Manges and colleagues were concerned to find a reliable, relevant and consistent genotypic method to identify the distribution of UPEC strains (Manges et al., 2008). Some genotypic methods suffer from inter-laboratory variability such as pulsed field gel electrophoresis (PFGE). This technique is commonly used to identify food-borne outbreaks of *E. coli* O157:H7; however, it is labour-intensive and technically demanding. In contrast, typing methods that are based on DNA sequencing are more reliable and consistent, especially after the recent developments in DNA sequencing, which have allowed sequence analysis to become an important tool for epidemiological studies. Multilocus sequence typing (MLST) is the current method used to investigate the genetic differences between isolates of UPEC (Croxall et al., 2011; Gibreel et al., 2012b; Jaureguy et al., 2008; Maiden, 2006). This method has proved to be a useful tool for investigating local or sporadic outbreaks with no consideration of any genetic variation of the tested isolates. This tool has become established as one of the most important epidemiological methods used to identify UPEC, as well as other important pathogenic strains (Tartof et al., 2005). This method is based on direct detection of genetic variation of certain (housekeeping) genes (Figure 1.7).

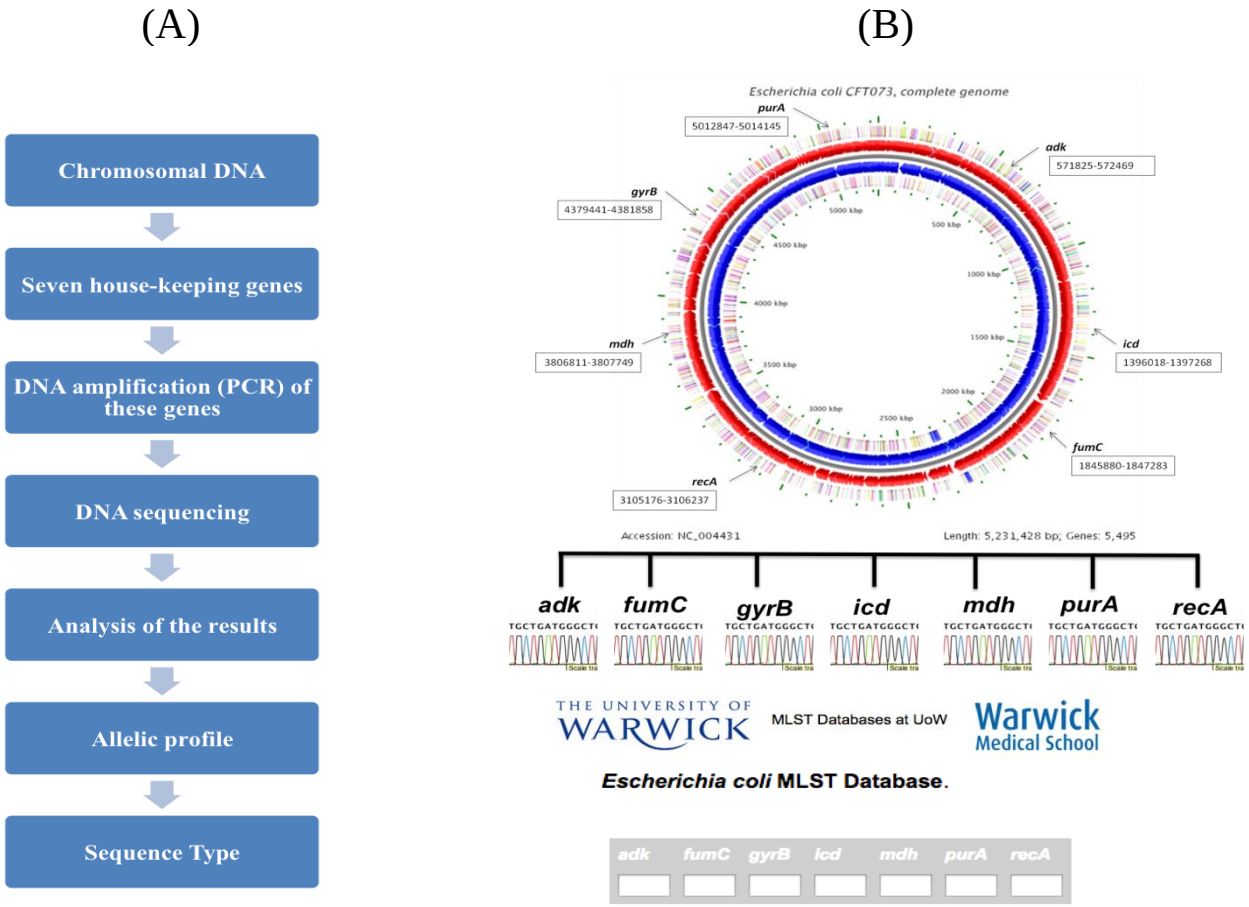

Figure 1.7 (A) Overview of the protocol used for MLST. (B) The complete genome of *E. coli* strain CFT073 showing the position of the seven housekeeping genes used in the Achtman MLST database, hosted at the University of Warwick (Wirth et al., 2006).

For each of the selected housekeeping genes, each allele has a certain number, so the combination of alleles at each of the seven loci provides an allelic profile. Thus, the allelic profile defines a sequence type (ST) that is assigned to the examined isolate. These genes are present at different loci around the genome to reduce the chance of co-transfer by mobile genetic elements. The candidate loci (usually seven housekeeping genes) are not exposed to selective forces; they are under neutral selection. The length of the amplified DNA fragments is selected to be around 450-500 base pairs, reflecting the sequence read lengths generally achievable when MLST was established. This ensures that the sequence data for these fragments are highly reproducible, which makes the method reliable for comparison of studies between different laboratories; the data are often described as being 'portable'. In addition, web-databases for MLST schemes have been developed as useful digital repositories for a wide variety of pathogenic and non-pathogenic micro-organisms (www.pubmlst.org).

The databases are easy to access globally and information can be transferred for analysis and comparisons carried out using the global epidemiological web-database. There are two well-recognised MLST schemes for *E. coli* hosted at the University of Warwick (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and the Pasteur Institute (http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html).

Due to the clonal distribution and high diversity of UPEC stains, there are many studies that illustrate the importance of high resolution typing methods (Banerjee et al., 2013b; Dias et al., 2010). The vast majority of *E. coli* strains encode type 1 fimbrial adhesin (*fimH*), which is a target that has recently been used in a sub-typing approach to obtain superior intra-clonal discrimination power.

This typing approach was developed by pairing analysis of the *fimH* fragment with one of the housekeeping loci, the *fumC* fragment, which gives optimal discrimination (Weissman et al., 2012). The results of this approach have been used to show the sub-clonal diversity among the worldwide pandemic clonal group ST131. A study by Adams-Sapper and others described typing of 246 *E. coli* bacteraemia isolates by MLST and *fimH*-subtyping methods, demonstrating that the three most frequent *fimH* types accounted for 96% of ST131 isolates (Adams-Sapper et al., 2013).

In addition, another study explored the sub-clonality of 352 isolates of ST131 (Johnson et al., 2013). The results showed that fluoroquinolone-resistant *E. coli* clinical isolates are significantly associated with the ST131 sub-clone *fimH*30. Despite the fact that MLST is the preferred method for clonal typing of UPEC it is clear that analysis of other targets for sub-clonal typing of STs can be useful to divide closely related strains into distinct sub-clones.

Molecular epidemiological research has been transformed over the last decade with the introduction of next generation sequencing (NGS). This has led to developments in the study of bacterial pathogens in terms of dissemination of antimicrobial resistant strains, understanding pathogenicity and investigation of complex microbial communities (Sherry et al., 2013). NGS technology has been significantly developed to offer dramatic increases in cost-effectiveness, with high quality sequencing platforms (Hodkinson and Grice, 2014).

Use of NGS has revolutionized molecular diagnostics at clinical laboratories where rapid diagnosis can be performed even for complex bacterial infections by targeting the 16S ribosomal RNA (rRNA) gene (Klindworth et al., 2013; Salipante et al., 2013) or for detailed investigation of individual strains (Köser et al., 2012; Mellmann et al., 2011). NGS based determination of bacterial genome sequences has become very routine and has been used for epidemiological investigations to determine and identify single-nucleotide polymorphisms (SNPs), which distinguish one lineage from another with the ultimate level of discriminatory power (Harris et al., 2013).

NGS has been used to determine the molecular epidemiology in outbreak settings (Arena et al., 2014; Harris et al., 2013; Köser et al., 2012; Sherry et al., 2013). More recently, and of direct relevance to the present project, several epidemiological studies have been performed by investigating the genome sequences of UPEC on a large-scale (Petty et al., 2014; Price et al., 2013; Salipante et al., 2015). These epidemiological studies have provided in-depth information about UPEC, which can be utilized to investigate the pathogenicity of strains and to track the dissemination of (antimicrobial resistant) strains.

1.8 Aims of this Study

The global dissemination and the emergence of antimicrobial resistant UPEC strains has demonstrated the importance of, and need for, further epidemiological studies to determine the role of key UPEC clones and identify the virulence factors they carry. Examination of clones known to cause infection in different body sites may increase our understanding of the role of specific virulence factors in development of a chosen infection. In addition, molecular typing of UPEC from geographically diverse locations will enhance our understanding of the epidemiological context in which key UPEC clones are disseminated.

The aim of this study is to generate a better understanding of the epidemiology and virulence of UPEC by studying a variety of UPEC populations. This includes UPEC populations collected from different infection sites and different geographic locations.

The fist work package will involve study of the population structure of UPEC causing UTI that results in urosepsis and compare these organisms to *E. coli* isolates that cause sepsis through a route other than the urinary tract; the biliary tract. The virulence potential of each of these groups of organisms will also be examined using PCR based screening methods.

The work has the following objectives -

- To generate a clearer understanding of the epidemiology and virulence of urosepsis and biliary sepsis isolates using molecular typing methods
- To identify key genetic markers of virulence in the tested isolates
- To determine the antimicrobial resistance and metabolic potential of the examined isolates

The second aspect of this research project is to investigate the virulence factors of the well-recognised major lineages of UPEC (ST69, ST73, ST95, ST127 and ST131) using an *in vivo* infection model.

The work has the following objectives -

- To evaluate the potential for using *Galleria mellonella* larvae as an alternative infection model to study UPEC pathogenesis
- To investigate the genetic mechanisms underlying any differences that may be observed between clonal groups with respect to virulence in the larvae

The proposed work includes a third major aim, which is to use molecular phylotyping and MLST methods to define the population biology of UPEC from Saudi Arabia. This will allow, for the first time, comparison with other parts of the world, as the population structure of UPEC has not been studied in detail in the Middle East.

The work has the following objectives -

- To collect representative UPEC isolates from Saudi Arabia
- To identify the population structure of UPEC in the Kingdom using MLST, phylogenetic grouping and molecular serotyping
- To identify key genetic markers of virulence in the tested isolates
- To determine the antimicrobial susceptibility of isolates and characterize antibiotic resistance related genes

Chapter Two

2 Molecular Epidemiological and Phylogenetic analysis of *Escherichia coli* Strains from Patients with Urosepsis and Biliary Sepsis

2.1 Abstract

Escherichia coli can invade the bloodstream, resulting in one of the most serious manifestations of *E. coli* infection. UPEC have been the focus of numerous recent epidemiological studies, but relatively little is known about the population biology of *E. coli* causing sepsis. In the current study, a total of 127 isolates of *E. coli* were recovered in the Northwest region of England from different infections sites (urine, blood and biliary isolates). The isolates included 100 from the blood and urine of 50 patients presenting with urosepsis and 27 isolates from cases of biliary sepsis blood culture. Molecular typing of the examined isolates was performed using phylogenetic grouping, multilocus sequence typing (MLST) and virulence gene profiling. In addition, antimicrobial susceptibility and metabolic profile were determined to generate a clearer understanding of the epidemiology of isolates that cause sepsis following UTI. Urine and blood paired isolates from each patient showed identical molecular types, virulence gene content and antimicrobial resistance profiles. However, several pairs of isolates showed different metabolic activity profiles, revealing the potential ecological impact of different niches on metabolic gene regulation or expression in some of the examined isolates.

The most frequently detected lineages among the urine and blood isolates were ST73, ST95, ST69 and ST131, which represented 30%, 12%, 10% and 8%, respectively. Members of ST131 were significantly associated with resistance to most of the tested antibiotics. In addition, ST131 showed a relatively high score of metabolic activities but the lowest virulence score, compared to other STs. Notably, one pair of isolates of ST167 showed the highest level of resistance, which warrants further attention. The MLST results of the biliary sepsis isolates revealed the most frequently detected isolates were from ST69, ST131 (detected at an equal level of 14.8%) and ST73 (7.4%). Members of phylogenetic group B2 and D were the most frequently detected clones, but with a low prevalence of antimicrobial resistant profiles. However, members of phylogenic group A (ST410 and S617) showed the highest antimicrobial resistance score compared to others STs. The most notable difference between the collections was the comparatively high incidence of ST131 isolates in the biliary sepsis collection, though numbers were too low to assign significance to this difference.

Further investigations will help to understand the distribution of antimicrobial resistance and virulence traits between UPEC and urosepsis isolates, in contrast to isolates that access the bloodstream via other sites. This may facilitate development of rapid prognostic and diagnostic assays for clinical patient management and surveillance.

2.2 Introduction

Urinary tract infections are among the most serious infections and account for millions of visits to physicians' offices annually, including the majority of the patients diagnosed with UTI infections, which requires immediate medical attention (Griebling, 2005; Tartof et al., 2005). UTI has considerable impact on health care-associated infections in patients at intensive care units, which leads to high mortality, an increased length of hospital stay and a significant burden on healthcare resources (Stone, 2009). In the United States alone, community-acquired UTIs were significantly associated with high annual costs that exceeded an estimated $1.6 billion (Foxman, 2003). *E. coli* is the most common cause of UTI and is responsible for 85-95% of uncomplicated cystitis in premenopausal women and around 90% of uncomplicated pyelonephritis (Foxman, 2003; Russo and Johnson, 2003). Moreover, *E. coli* is responsible for about 90% of all the UTIs which are noted among ambulatory patients and around 50% of all nosocomial UTIs (Tartof et al., 2005). It is also found to be a major cause of bloodstream infection with a high mortality rate. Epidemiological studies have shown a rapid global increase of bloodstream infections caused by *E. coli* during the past two decades (de Kraker et al., 2011; Martin et al., 2003; Stewardson et al., 2013).

According to recent antimicrobial resistance surveillance program conducted by Public Health England (PHE) in 2014, in the United Kingdom, *E. coli* bloodstream infections increased by 12% between 2010 and 2013 from 47.0 to 52.6 cases per 100,000 population (ESPAUR, 2014) (Figure 2.1).

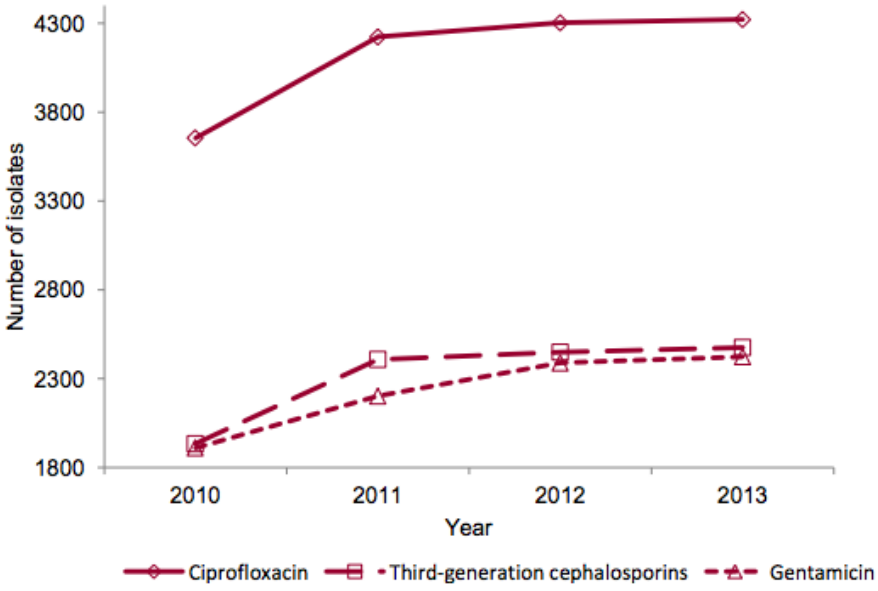

Figure 2.1 Figure adapted from ESPAUR annual report 2014 by PHE which showes an increase number of *E. coli* isolates non-susceptible to ciprofloxacin, third-generation cephalosporins and gentamicin between 2010 and 2013 (ESPAUR, 2014).

Previous studies showed the successful translocation of *E. coli* from the urinary tract to the bloodstream proceeded in an ascending manner, starting with bacterial colonization of the bladder. Subsequently, bacterial cells can cross the epithelial cells through the proximal tubule and then the endothelial cells of the capillary and gain access to the bloodstream (Bower et al., 2005; Duncan et al., 2004; Smith et al., 2010). This usually occurs when untreated UPEC with specific traits proceed in an ascending manner through the urinary tract and enter the bloodstream to cause systemic disease (Smith et al., 2010). The type 1 fimbrial adhesin FimH is a major virulence factor that facilitates bacterial colonisation and invasion of human bladder cells (Martinez et al., 2000; Mulvey et al., 1998). A study by Floyd and others demonstrated that UPEC, but not avian pathogenic *E. coli* strains or FimH mutant UPEC, were able to impair contractility in an intact rat ureter model (Floyd et al., 2012).

Despite the extensive studies into the population genetics of UPEC, little is known about the role of key UPEC clones in progression of uncomplicated UTI to urosepsis, in terms of the molecular basis and virulence factors involved. There are many virulence traits of *E. coli* that contribute to colonisation of the urinary tract and these include fimbrial adhesins, secreted toxins, the polysaccharide capsule, ferric iron acquisition systems (siderophores) and specific metabolic pathways (Alteri et al., 2009; Gibreel et al., 2012a; Johnson, 1991; Smith et al., 2010). The majority of UPEC encode adhesive structures, which are important virulence factors, including type 1, P, and S pili along with Dr adhesins. These promote bacterial attachment to, and invasion of, host tissues within the urinary tract and persistence in this environment (Johnson and Stell, 2000; Johnson, 1991; Martinez et al., 2000; Mokady et al., 2005; Mulvey, 2002).

The overall purpose of the work described in this chapter is to study the population structure of UPEC causing UTI that results in urosepsis, compared with *E. coli* isolates that cause sepsis through a route other than the urinary tract and to investigate the virulence of each of these groups of organisms.

The project aims to generate a better understanding of the epidemiology of UPEC that cause bloodstream infections by comparison to those that can cause sepsis, but do so via a different route. This may allow development of rapid diagnostic and prognostic assays for use in surveillance and patient management, respectively, and may identify targets that can be exploited in development of therapeutic interventions or preventative measures to reduce UTI and sepsis caused by UPEC.

2.3 Materials and methods

2.3.1 Bacterial isolates and DNA extraction

E. coli isolates were obtained from the blood and urine of each of 50 patients who had both bloodstream and urinary tract inactions. The isolates were recovered from two different hospitals in the UK; the Central Manchester Foundation Trust (CMFT), Manchester and the Mid Yorkshire Hospitals NHS Trust, Wakefield between July 2011 and March 2013. In addition, a total of 27 *E. coli* isolates were obtained from patients with biliary sepsis at Stepping Hill Hospital, Stockport, South Manchester, UK between September 2011 and February 2013.

All isolates were received in pure culture; recovery from clinical specimens was carried out by staff in respective laboratories. Bacterial identification was carried out at the clinical microbiology laboratories at CMFT using the VITEK 2.0 compact Automated Expert System (AES) (bioMérieux). The *E. coli* strains were routinely cultured on Columbia agar (CA; Oxoid) at 37^{o}C for 18 hours and were stored using Microbank beads (Pro-Lab Diagnostics) at -80^{o}C. DNA extraction was carried out using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, USA), following manufacturer instructions. DNA was stored at -80^{o}C.

2.3.2 Antibiotic susceptibility and metabolic activities

Bacterial identification and antibiotic susceptibility testing was performed at the clinical microbiology laboratories at the CMFT. The VITEK 2.0 AES (bioMérieux) was used for identification using the Gram-negative VITEK 2.0 ID-GNB identification card (Biomérieux). Bacterial identification occurs on the basis of metabolic activity in 47 biochemical tests to measure carbon source utilization and enzymatic activity (Table 2.1).

Table 2.1 VITEK 2.0 ASE Metabolic Tests and abbreviations

Test	Abb.	Test	Abb.	Test	Abb.
Adonitol	ADO	D-Mannitol	dMAN	Lipase	LIP
Alpha-Galactosidase	AGAL	D-Mannose	dMNE	Malonate	MNT
Glutamyl Arylamidse pNA	AGLTp	D-sorbitol	dSOR	Beta-N-Acetyl-Glucosaminidase	NAGA
Alpha-Glucosidase	AGLU	D-Tagatose	dTAG	O/129 Resistance	O129R
Ala-Phe-Pro-Arylamidase	APPA	D-Trehalose	dTRE	Ornithine Decarboxylse	ODC
Beta-Alanine Arylamidase pNA	BAIap	Ellman	ELLM	Fermentation/Glucose	OFF
Beta-Galactosidase	BGAL	Glu-Gly-Arg-Arylamidase	GGAA	Phosphatase	PHOS
Beta-Glucosidase	BGLU	Gamma-Glutamyl-Transferase	GGT	Palatinose	PLE
Beta-Glucoronidase	BGUR	Glycine Arylamidase	GlyA	L-Proline Arylamidase	ProA
Beta-N-Acetyl-Galactosaminidase	BNAG	H2S production	H2S	L-Pyrrolydonyl-Arylamidase	PyrA
Beta-Xylosidase	BXYL	L-Arabitol	IARL	Saccharose/Sucrose	SAC
Citrate	CIT	L-Histidine assimilation	IHISa	Succinate alkalinisation	SUCT
Courmarate	CMT	L-Lactate assimilation	ILATa	Tyrosine Arylamidase	TyrA
D-Cellobiose	dCEL	L-Lactate alkalinisation	ILATk	Urease	URE
D-Glucose	dGLU	L-Malate assimilation	IMLTa	5-Keto-D-Gluconate	5KG
D-Maltose	dMAL	Lysine Decarboxylase	LDC		

Antibiotic susceptibility testing was carried out using the Gram-negative Susceptibility Card, AST-GN206 based on kinetic analysis of the bacterial growth in the presence of selected antibiotics (21 antibiotics). These antibiotics representing several antibiotic families were as follows: penicillins (amoxicllin, ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam); cephalosporins (cefalotin, cefuroxime, cefuroxime axetil, cefoxitin, cefotaxime, ceftazidime, cefepime); carbapenems (ertapenem, meropenem); monobactams (aztreonam); aminoglycosides (amikacin, gentamicin, tobramycin); fluoroquinolones (ciprofloxacin, ofloxacin); tigecycline and trimethoprim.

2.3.3 Molecular typing

Multilocus sequence typing (MLST) was carried out to determine isolate sequence types (STs) by using the Achtman scheme for *E. coli* as specified at the University of Warwick *E. coli* MLST web site (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

This scheme is based on sequencing fragments of a selection of seven pre-specified housekeeping genes: *adk* (adenylate kinase); *fumC* (fumarate hydratase); *gryB* (DNA gyrase); *icd* (isocitrate dehydrogenase); *mdh* (malate dehydrogenase); *purA* (adenylosuccinate synthetase); and *recA* (ATP/GTP binding motif).

PCR amplification for the seven housekeeping genes was carried out by using pairs of primers (Table 2.2), as described previously (Wirth et al., 2006). Each PCR reaction was carried out in 50 µl reaction volumes by using a complete ready-to-use 2x reaction mix, BioMix™ Red (Bioline USA Inc.), which contains an ultra-stable Taq DNA polymerase. Each reaction contained 25 µl of 2x BioMix Red, 5 µl of each primer (10pmol/µl) and 1 µl of chromosomal DNA.

Table 2.2 PCR primers for the seven selected housekeeping genes used in MLST

Gene	Primer Sequence (5' – 3')	Annealing temp. (°C)	Product length (bp)
adk	*F*-ATTCTGCTTGGCGCTCCGGG *R*-CCGTCAACTTTCGCGTATTT	60	583
fumC	*F*-TCACAGGTCGCCAGCGCTTC *R*-GTACGCAGCGAAAAAGATTC	63	806
gyrB	*F*-TCGGCGACACGGATGACGGC *R*-ATCAGGCCTTCACGCGCATC	60	911
icd	*F*-ATGGAAAGTAAAGTAGTTGTTCCGGCACA *R*-GGACGCAGCAGGATCTGTT	53	878
mdh	*F*-ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG *R*-TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	53	932
purA	*F*-CGCGCTGATGAAAGAGATGA *R*-CATACGGTAAGCCACGCAGA	67	816
recA	*F*-CGCATTCGCTTTACCCTGACC *R*-TCGTCGAAATCTACGGACCGGA	60	780

PCR cycling parameters were initiated by an activation cycle at 94ºC for 2 min, followed by 35 cycles, each consisting of a denaturation step at 94ºC for 1 min, primer-annealing step at the temperature listed in Table 2.2 for 1 min and an extension step at 72ºC for 1 min, with a final extension at 72ºC for 5 min. The reactions were then held at 4ºC.

To eliminate unincorporated primers and master mix residues, ExoSAP-IT PCR Clean-up Kit (Affymetrix, Inc) was used as a single-step enzymatic *cleaner protocol.* This is simply a one-step enzymatic treatment of PCR products to remove unincorporated primers and dNTPs so that they cannot interfere with downstream sequencing reactions (Bell, 2008). The clean up reaction was carried out for 15 minutes at 37°C, followed by 15 minutes incubation at 80°C to completely inactivate both enzymes.

BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for sequencing of the seven housekeeping genes. Before the sequencing reaction, the template quality was determined by spectrophotometry using a NanoDrop spectrophotometer (Agilent; A260/A280 ratio should be 1.7 to 1.9). The template quantity was also measured. The following work for sequence determination was carried out by staff at the University of Manchester's Stopford Building DNA sequencing facility. As recommended by the manufacturer, the sequencing reactions were carried out in 20 µl volumes, each reaction containing 2 µl DNA template (approx. 250ng), 4 µl primer (4pmoles), 1 µl of BigDye Terminator Ready Reaction Mix v3.1 (a cocktail of sequencing Taq polymerase; dNTPs; fluorescently labelled ddNTPs; and buffers/salts), 3.5 µl of 5x Buffer (magnesium chloride and TrisHCl buffer) and 9.5 µl of molecular biology grade water.

The sequencing reactions were then performed under the following conditions: initiation cycle at 96°C for 2 min followed by 35 cycles; 96°C for 40 sec, 50°C for 15 sec, 60°C for 4 min, and finally the reaction was held by cooling at 4°C until ready to process. The completed reactions were then cleaned (to remove the unused fluorophores and dNTPS/salts) on a Biomek 3000 liquid handling robot utilising the Agencourt CleanSEQ magnetic bead separation method (SPRI: Solid Phase Reversible Immobilization). The sequences of the reaction products were then determined using the Applied Biosystems 3730 (48-capillary) Genetic Analyzer. The samples are generally electrophoresed for approximately 2 hours at 8.5 kV through 50cm array using POP7 ('performance optimized polymer 7') to generate approximately 750-850 base pairs of sequence data.

For data analysis and quality control, Analysis Package v5.3.1 software was used to base-call, trim, and display, edit and print data from the sequencing instrument. The CLCbio Multilocus Sequence Typing Module 1.0 (www.clcbio.com/mlst/) was used to eliminate much of the manual work required for assembly and analysis of MLST sequence data.

2.3.4 Phylogenetic grouping

Phylogenetic grouping was carried out based on previously described methods (Doumith et al., 2012). To determine the phylogenetic grouping of each isolate, a quadruplex PCR reaction was performed by using four sets of primers, as listed in Table 2.3, targeting four DNA markers (*gadA*, *chuA*, *yjaA* and TSPE4.C2). The PCR reactions were carried out in 50 µl volumes containing 25 µl of BioMix™ Red (Bioline USA Inc.), 1 µl of each primer (10pmol/µl) and 2 µl of 100 ng/µl chromosomal DNA.

Table 2.3 Primer sequences for the Phylogenetic grouping of *E. coli* (Doumith et al., 2012)

Marker	Primer Sequence (5' – 3')	Product length (bp)
gadA	F-GATGAAATGGCGTTGGCGCAAG R-GGCGGAAGTCCCAGACGATATCC	373
chuA	F-ATGATCATCGCGGCGTGCTG R-AAACGCGCTCGCGCCTAAT	281
yjaA	F-TGTTCGCGATCTTGAAAGCAAACGT R-ACCTGTGACAAACCGCCCTCA	216
TSPE4.C2	F-GCGGGTGAGACAGAAACGCG R-TTGTCGTGAGTTGCGAACCCG	152

PCR cycling parameters for the phylogenetic grouping were initiated by an activation cycle at 94ºC for 4 min, followed by 30 cycles, each consisting of a denaturation step at 94ºC for 5 sec, primer-annealing at 59ºC for 10 sec, an extension step at 72ºC for 10 sec, final extension at 72ºC for 5 min, and the reactions were held by cooling at 4ºC.

2.3.5 Analysis of virulence potential using multiplex PCR

Virulence factor (VF) profiles (Table 2.4) of urine, blood and biliary sepsis isolates were determined by using the primers listed in Table 2.5. This method of virulence factor screening was proposed by Johnson and Stell (Johnson and Stell, 2000) and is a widely adopted multiplex PCR assay.

Using this assay, all the isolates in the current study were screened for the presence of twenty-nine ExPEC associated VF genes encompassing five categories (adhesins, toxins, siderophores, capsule and 'miscellaneous'), as listed in Table 2.4.

PCR amplification was performed in 25 µl reaction volumes containing template (2 µl of 200 ng/µl chromosomal DNA), 4 mM MgCl2, 0.8 mM each of 4 dNTPs, 0.6 mM of each primer excluding *papC* allele, *sfaS*, *Afa/draBC*, *nfaE*, *cnfI* and *kpsMT-II*, which were used at a concentration of 0.3 mM, and 2.5 units AmpliTaq Gold in 1 × PCR buffer**.**

PCR cycling parameters were carried out using the following conditions: 12 min at 94ºC; followed by 25 cycles of 30 sec at 94ºC, 30 sec at 63ºC, 3 min at 68ºC, followed by 10 min at 72ºC, and finally the reactions were held at 4ºC. PCR products were then analysed by agarose gel electrophoresis passing the PCR product through a 2 % (w/v) agarose gel (Promega) in TBE at 120 V for 60 min and visualisation under UV transillumination (AlphaImager™ System 2200).

Table 2.4 Five categories of ExPEC virulence factors (Johnson & Stell 2000)

Virulence factors	Gene(s)	Remarks
Adhesins	*fimH*	Mannose specific adhesin of type I fimbriae
	papAH	P fimbriae elements
	papC	
	papEF	
	papG alleles I, II, III	
	sfaS	S fimbrial adhesin
	focG	The putative F1C fimbrial adhesin
	sfa/focDE	Central region of *sfaS* and *focG* operons
	afa/draBC	Dr antigen-specific adhesin operons
	bmaE	Blood group M-specific adhesion
	nfaE	Non-fimbrial adhesin
	gaf D	Glucosamine specific G fimbriae
Toxins	*cnf1*	Cytotoxic necrotizing factor
	cdtB	cytolethal distending toxin
	hlyA	*E. coli* hemolysin (encoded by the *hlyCABD* operon) is a prototypic repeat-in-toxin (RTX) molecule and an important model for acylation of virulence factors within the bacterial cytoplasm
Siderophores	*fyuA*	Yersiniabactin
	iutA	Aerobactin
Capsule	*kpsMT II* and *III*	
	kpsMT K1	
	kpsMT K5	
Miscellaneous	*cvaC*	Colicin V; multifunctional serum resistance-associated plasmids
	traT	Serum resistance associated
	ibeA	Invasion of brain endothelium
	PAI	Coding region of unknown significance of a sequenced PAI from archetypal ExPEC strain CFT073 was used as a generic marker for uropathogenic PAIs

Table 2.5 Primers used in multiplex PCR to detect different virulence factors (Johnson & Stell 2000)

Gene	Primer sequence (5'-3')	Product length (bp)
papAH	F-ATGGCAGTGGTGTCTTTTGGTG R-CGTCCCACCATACGTGCTCTTC	720
papC	F-GTGGCAGTATGAGTAATGACCGTTA R-ATATCCTTTCTGCAGGGATGCAATA	200
papEF	F-GCAACAGCAACGCTGGTTGCATCAT R-AGAGAGAGCCACTCTTATACGGACA	336
papG I, II,III ***papG II, III*** ***papG I***	F-CTGTAATTACGGAAGTGATTTCTG R-ACTATCCGGCTCCGGATAAACCAT R-TCCAGAAATAGCTCATGTAACCCG	 1070 1190
allele I	F-TCGTGCTCAGGTCCGGAATTT R-TGGCATCCCCCAACATTATCG	461
allele II	F-GGGATGAGCGGGCCTTTGAT R-CGGGCCCCCAAGTAACTCG	190
Allele III	F-GGCCTGCAATGGATTTACCTGG R-CCACCAAATGACCATGCCAGAC	258
Sfa/focDE	F-CTCCGGAGAACTGGGTGCATCTTAC R-CGGAGGAGTAATTACAAACCTGGCA	410
sfaS	F-GTGGATACGACGATTACTGTG R-CCGCCAGCATTCCCTGTATTC	240
focG	F-CAGCACAGGCAGTGGATACGA R-GAATGTCGCCTGCCCATTGCT	360
Afa/draBC	F-GGCAGAGGGCCGGCAACAGGC R- CCCGTAACGCGCCAGCATCTC	559
bmaE	F-ATGGCGCTAACTTGCCATGCTG R-AGGGGGACATATAGCCCCCTTC	507
gafD	F-TGTTGGACCGTCTCAGGGCTC R-CTCCCGGAACTCGCTGTTACT	952
nfaE	F-GCTTACTGATTCTGGGATGGA R-CGGTGGCCGAGTCATATGCCA	559
fimH	F-TGCAGAACGGATAAGCCGTGG R-GCAGTCACCTGCCCTCCGGTA	508
hlyA	F-AACAAGGATAAGCACTGTTCTGGCT R-ACCATATAAGCGGTCATTCCCGTCA	1177
cnfI	F-AAGATGGAGTTTCCTATGCAGGAG R-CATTCAGAGTCCTGCCCTCATTATT	498
cdtB	F-AAATCACCAAGAATCATCCAGTTA R-AAATCTCCTGCAATCATCCAGTTTA	430
fyuA	F-TGATTAACCCCGCGACGGGAA R-CGCAGTAGGCACGATGTTGTA	880
iutA	F-GGCTGGACATCATGGGAACTGG R-CGTCGGGAACGGGTAGAATCG	300
kpsMT-II	F-GCGCATTTGCTGATACTGTTG R-CATCCAGACGATAAGCATGAGCA	272
kpsMT-III	F-TCCTCTTGCTACTATTCCCCCT R-AGGCGTATCCATCCCTCCTAAC	392
kpsMT K1	F-TAGCAAACGTTCTATTGGTGC R-CATCCAGACGATAAGCATGAGCA	153
kpsMT K2	F-CAGTATCAGCAATCGTTCTGTA R-CATCCAGACGATAAGCATGAGCA	159
rfc	F-ATCCATCAGGAGGGGACTGGA R-AACCATACCAACCAATGCGAG	788
ibeA	F-AGGCAGGTGTGCGCCGCGTAC R-TGGTGCTCCGGCAAACCATGC	170
cvaC	F-CACACACAAACGGGAGCTGTT R-CTTCCCGCAGCATAGTTCCAT	680
traT	F-GGTGTGGTGCGATGAGCACAG R-CACGGTTCAGCCATCCCTGAG	290
PAI	F-GGACATCCTGTTACAGCGCGCA R-TCGCCACCAATCACAGCCGAAC	930

2.3.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 5.0d. The analyses were carried out to examine the relationship of isolates according to their STs, phylogenetic grouping and the distribution of virulence factors among different STs. The analysis was performed based on nonparametric tests using Mann-Whitney U test and Fisher's exact test, where the threshold for statistical significance was a P value of ≤ 0.05.

Aggregate scores for metabolic activity, antimicrobial resistance and virulence factors were calculated for each isolate. The resistance and metabolic scores were calculated for each isolate as the sum of all parameters for which the isolates tested positive divided by the total number of the tested parameters. Virulence score was calculated for each isolate as the sum of positive VF PCRs for each isolate.

2.4 Results

2.4.1 Molecular typing and clonal relationships of the STs

2.4.1.1 Urine and blood isolates

Phylogenetic grouping of the 50 pairs of isolates from blood and urine recovered from the same patients showed that most of the isolates belonged to B2 (n=28, 56% of the total) followed by D (n=15, 30%), A (n=5, 10%) and B1 (n=2, 4%). MLST analysis identified 20 unique STs and the most frequently detected one was ST73 (30%) followed by ST95 (12%), ST69 (10%) and ST131 (8%) (Table 2.6). To compare the level of genetic diversity in the unique STs, maximum likelihood phylogenies were constructed showing the genetic relatedness among the 20 STs.

Figure 2.2 shows the maximum likelihood phylogenetic tree segregating STs into three different clusters. Members of STs within cluster 1 belonged to phylogenetic groups A and B1. Clusters 2 and 3 and were observed to exclusively include STs belonging to phylogenetic groups D and B2, respectively.

Table 2.6 Frequency of detected sequence types (ST) and their allelic profiles, clonal complex (CC) and phylogenetic group of the urine and blood isolates

ST	CC	Grouping	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	Frequency %
ST73	ST-73	B2	36	24	9	13	17	11	25	30
ST95	ST-95	B2	37	38	19	37	17	11	26	12
ST69	ST-69	D	21	35	27	6	5	5	4	10
ST131	-	B2	53	40	47	13	36	28	29	8
ST48	ST-10	A	6	11	4	8	8	8	2	4
ST127	-	B2	13	14	19	36	23	11	10	4
ST38	ST-38	D	4	26	2	25	5	5	19	4
ST14	ST-14	D	14	14	10	14	17	7	10	4
ST167	ST-10	A	10	11	4	8	8	13	2	2
ST973	-	D	6	29	5	26	24	8	6	2
ST2102	-	B1	6	8	4	16	7	8	7	2
ST10	ST-10	A	10	11	4	8	8	8	2	2
ST12	ST-12	B2	13	13	9	13	16	10	9	2
ST23	ST-23	A	6	4	12	1	20	13	7	2
ST2459	-	D	21	35	27	6	5	2	4	2
ST372	-	D	88	103	19	36	23	44	26	2
ST393	ST-31	D	18	106	17	6	5	5	4	2
ST405	ST-405	D	35	37	29	25	4	5	73	2
ST56	ST-155	B1	6	4	4	18	24	5	14	2
ST59	ST-59	D	27	32	24	29	26	19	22	2
Total										100

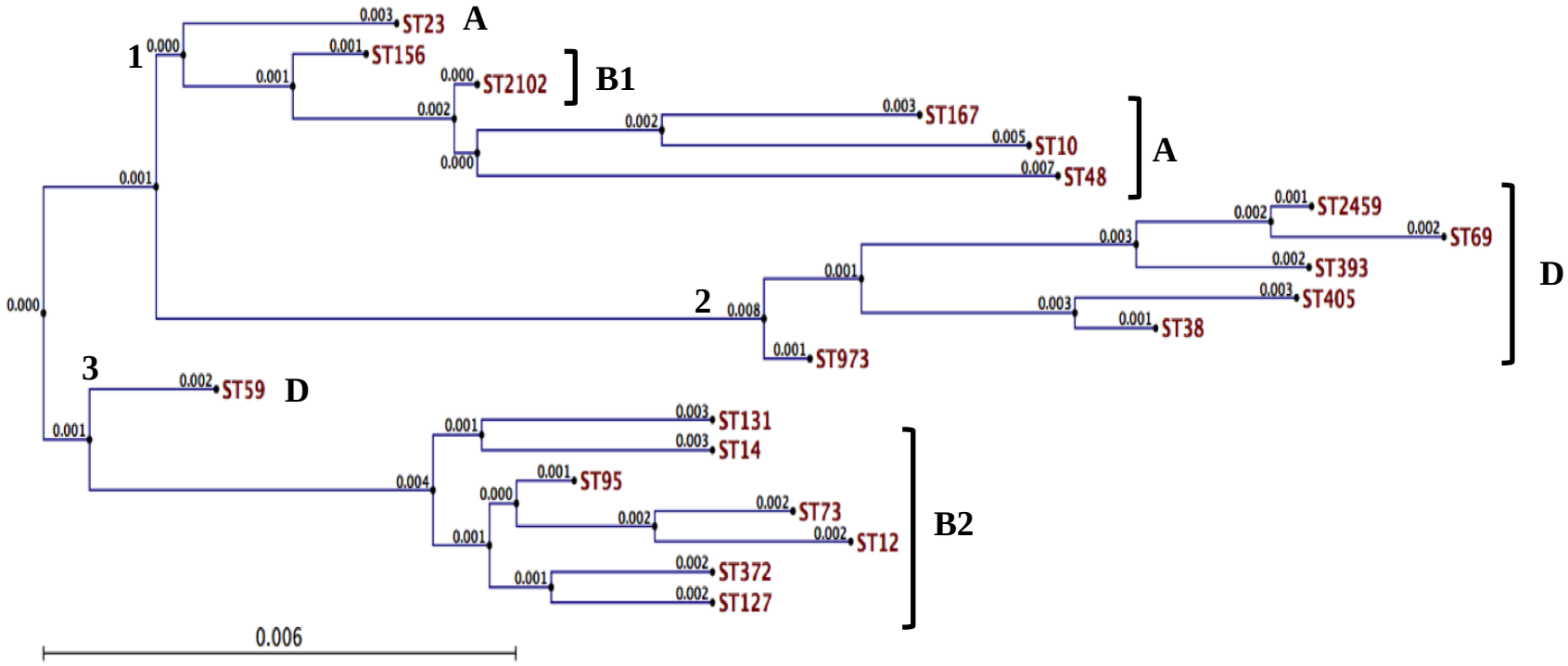

Figure 2.2 Maximum likelihood phylogeny tree of the urine and blood isolates belonging to 20 unique STs. The analysis was performed using concatenated nucleotide sequences of the seven MLST gene fragments in the order *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* using the Kimura two-parameter (K80) evolution model with the gamma distribution (4 substitution rate categories). A bootstrap value is attached to each node, this value is a measure of the confidence in the subtree rooted at the node. The sequence type number and phylogenetic grouping are indicated at all branches.

eBURST analysis was performed to indicate the genetic relatedness of STs based on MLST allelic profiles. Table 2.7 shows the estimated relationship between STs based on the highest numbers of SLVs and DLVs as a root node. The analysis revealed two CCs encompassing 5 STs representing 20 isolates with 80 isolates appearing as singletons. The most abundant SLVs were from CC10, which accounts for three isolates.

Table 2.7 eBURST analysis of 50 isolates showing the frequency of each sequence type and their corresponding SLVs and DLVs

No. isolates=100, NO. STs= 20			
Group 1: No. isolates = 8 | No. STs = 3 | Predicted Founder = ST10			
ST	FREQ	SLV	DLV
10	4	2	0
48	2	1	1
167	2	1	1
Group 2: No. isolates = 12 | No. STs = 2 | Predicted Founder = ST69			
ST	FREQ	SLV	DLV
69	10	1	0
2459	2	1	0
Singletons: No. isolates =80			
14, 23, 38, 56, 59, 73, 95, 127, 131, 156, 372, 393, 405, 973, 2102			

2.4.1.2 Biliary sepsis isolates

The phylogenetic grouping analysis of 27 biliary sepsis isolates segregated them into four groups where the most commonly detected was B2 (n=16, 59%) followed by D (n=7, 26%), A (n=3, 11%) and B1 (n=1, 4%). MLST analysis identified 20 unique STs and the most commonly detected STs were ST131 and ST69 (both with n=4, 15%), followed by ST73 (n=2, 7%) and 17 singletons of a variety of STs including well-recognised clones ST14, ST38, ST95 and ST410 (Table 2.8).

Table 2.8 Frequency of detected sequence types (ST) and their allelic profiles, clonal complex (CC) and phylogenetic group of the biliary sepsis isolates

ST	CC	Grouping	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	Frequency	%
ST69	**ST-69**	**D**	**21**	**35**	**27**	**6**	**5**	**5**	**4**	**4**	**14.8**
ST131	**-**	**B2**	**53**	**40**	**47**	**13**	**36**	**28**	**29**	**4**	**14.8**
ST73	**ST-73**	**B2**	**36**	**24**	**9**	**13**	**17**	**11**	**25**	**2**	**7.4**
ST617	**ST-10**	**A**	**10**	**11**	**4**	**8**	**8**	**13**	**37**	**1**	**3.7**
ST14	**ST-14**	**B2**	**14**	**14**	**10**	**14**	**17**	**7**	**10**	**1**	**3.7**
ST88	**ST-23**	**A**	**6**	**4**	**12**	**1**	**20**	**12**	**7**	**1**	**3.7**
ST410	**ST-23**	**A**	**6**	**4**	**12**	**1**	**20**	**18**	**7**	**1**	**3.7**
ST393	**ST-31**	**D**	**18**	**106**	**17**	**6**	**5**	**5**	**4**	**1**	**3.7**
ST38	**ST-38**	**D**	**4**	**26**	**2**	**25**	**5**	**5**	**19**	**1**	**3.7**
ST538	**ST-538**	**B2**	**13**	**40**	**19**	**13**	**36**	**28**	**30**	**1**	**3.7**
ST80	**ST-568**	**B2**	**13**	**24**	**19**	**14**	**23**	**1**	**10**	**1**	**3.7**
ST104	**ST-73**	**B2**	**13**	**24**	**9**	**13**	**17**	**11**	**25**	**1**	**3.7**
ST95	**ST-95**	**B2**	**37**	**38**	**19**	**37**	**17**	**11**	**26**	**1**	**3.7**
ST847	**-**	**B1**	**6**	**6**	**5**	**26**	**9**	**13**	**98**	**1**	**3.7**
ST91	**-**	**B2**	**13**	**40**	**34**	**13**	**23**	**28**	**30**	**1**	**3.7**
ST144	**-**	**B2**	**13**	**43**	**9**	**36**	**30**	**44**	**25**	**1**	**3.7**
ST1170	**-**	**B2**	**13**	**40**	**19**	**13**	**23**	**28**	**109**	**1**	**3.7**
ST429	**-**	**B2**	**97**	**40**	**93**	**13**	**23**	**28**	**66**	**1**	**3.7**
ST547	**-**	**B2**	**13**	**52**	**10**	**119**	**17**	**37**	**25**	**1**	**3.7**
ST174	**-**	**D**	**20**	**67**	**56**	**64**	**5**	**50**	**45**	**1**	**3.7**
Total										**27**	**100**

The maximum likelihood phylogenetic tree of the biliary sepsis isolates revealed 2 clusters showing the genetic relatedness among 20 STs (Figure 2.3). Members of STs belonging to phylogenetic group B2, representing 12 STs, were observed in cluster 1, which included one ST that belonged to phylogenetic group D. Cluster 2 was observed to include STs belonging to phylogenetic groups A, B and D representing 7 STs.

Table 2.9 shows the eBURST analysis, which indicates genetic relatedness between the STs of 27 biliary sepsis isolates. The analysis revealed two CCs encompassing 4 STs, representing 5 isolates with 22 isolates contained in 16 singleton STs.

Table 2.9 eBURST analysis of 27 biliary sepsis isolates showing the frequency of each sequence type and their corresponding SLVs and DLVs

No. isolates=27, NO. STs= 20			
Group 1: No. isolates = 3 | No. STs =2 | Predicted Founder = ST73			
ST	FREQ	SLV	DLV
73	2	1	0
104	1	1	0
Group 2: No. isolates = 2 | No. STs = 2 | Predicted Founder = ST23			
ST	FREQ	SLV	DLV
88	1	1	0
410	1	1	0
Singletons: No. isolates = 22			
14, 38, 69, 80, 91, 95, 131, 144, 174, 393, 429, 538, 547, 617, 847, 1170			

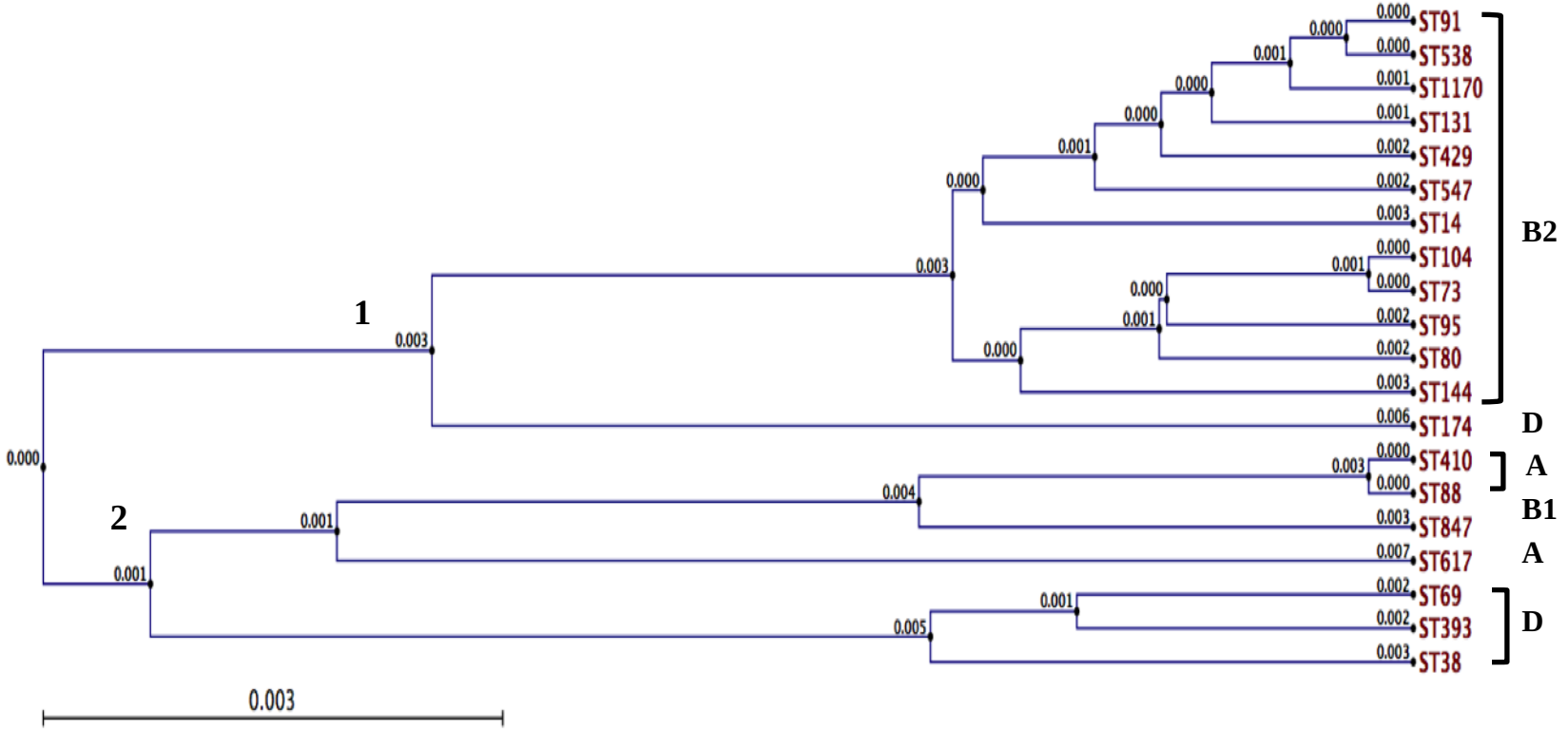

Figure 2.3 Maximum likelihood phylogenetic tree of the biliary sepsis isolates belonging to 20 unique STs. The analysis was performed using concatenated nucleotide sequences of the seven MLST gene fragments in the order *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* using the Kimura two-parameter (K80) evolution model with the gamma distribution (4 substitution rate categories). A bootstrap value is attached to each node, this value is a measure of the confidence in the subtree rooted at the node. The sequence type number and phylogenetic grouping are indicated at all branches.

2.4.2 Carriage of selected virulence factors by urosepsis and biliary sepsis UPEC isolates

2.4.2.1 Urine and blood isolates

Surveillance of 29 virulence genes was carried out to detect the prevalence of virulence factors within the urine and blood isolates. The virulence factor profiles among paired isolates recovered from different infection sites (blood and urine) within the same patients were identical, as determined by multiplex PCR for virulence genes. Among the urine and blood isolates, a total of 27 virulence genes were detected with a prevalence ranging from 2% (*bmaE* and *nfaE*) to 100% (*fimH*). Virulence factor *papG I* was not detected in the collection of isolates. The prevalence of VF genes was significantly associated with members of STs belonging to phylogenetic groups B2 and D, compared to phylogenetic groups A and B1 (Figure 2.4). The adhesin genes *papAH, papC* and *papGII,III* occurred in more than 50% of isolates and *papEF* and *allele-II* were found in more than 40% of the isolates, most of which were within phylogenetic groups B2 and D.

The toxin-associated genes had a high prevalence in phylogenetic group B2 isolates. The toxin VFs (*cnf* and *cvaC*) were detected in more than 20% and 12% of the isolates, respectively, whereas hemolysin A (*hlyA*) had a slightly higher prevalence (24%). Of the siderophores, ferric yersiniabactin (*fyaA*) was more prevalent (86%) than *iut* (70%). Capsule synthesis genes were detected with varying rates of 52% (*kpsM II*), 24%, (*kpsM III*), 16% (*K1*) and 34% (*K5*). The serum resistance gene (*traT*) occurred in 70% of the isolates. Finally the pathogenicity island (*PAI*) marker gene was present in 56% of the isolates (Table 2.10).

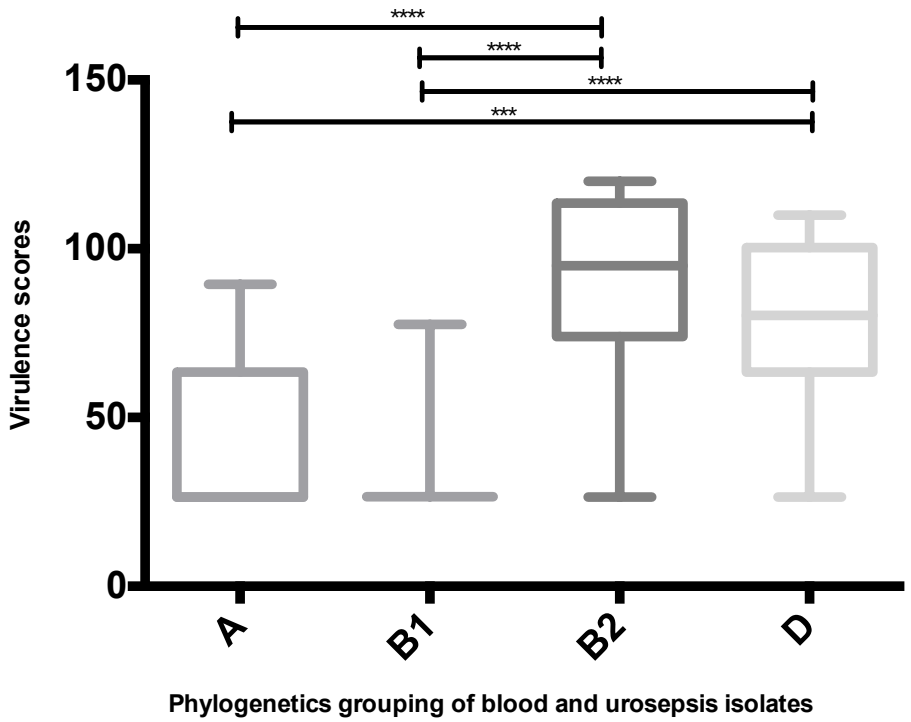

Figure 2.4 Statistical analysis of the urine and blood isolates to compare the prevalence of VF factor scores between the phylogenetic grouping A, B1, B2 and D. Phylogenetic group B2 and D show a significantly enhanced virulence capacity, compared to that of other groups.

STs varied considerably in VF content, from ST48 with the lowest VF score (mean 4.0) to ST127 with the highest VF score (mean 12.5). Such a high virulence score was notable, even given the relatively low number of ST127 isolates detected in this study. Table 2.11 shows the virulence profiles that had a significant positive or negative association with strains of the most commonly detected STs. The adhesion factors, especially the Pap elements, were significantly associated with ST69 and ST95 clonal groups. Members of ST73 were also significantly associated with *papAH* and with other adhesion elements including *sfa/focDE* and *focG*. Isolates from the ST131 clonal group showed moderate VF scores (mean=6.75), with significantly low prevalence for several factors including *papGII,III, kpsMT K5* and *traT*.

Table 2.10 Prevalence of various UPEC associated virulence factors of phylogenetic groups observed in the urine and blood collections. The data presented are for VFs showing a degree of association with the different phylogenetic groups. The urine and blood paired isolates of each patient show identical VFs profiles (not visible in Table)

Phylogenetic groups	Isolates No	Prevalence (%) of VF by phylogenetic grouping and STs (No. of isolates positive)																			VF score [mean, median (range)]
		Adhesion (%)							Toxins (%)			Siderophore (%)		Capsule (%)				Miscellaneous (%)				
		papAH	*papC*	*papEF*	*papG II,III*	*allele-II*	*sfa/focDE*	*focG*	*hlyA*	*cnfI*	*cdtB*	*fyuA*	*iutA*	*kpsMT II*	*kpsMT III*	*kpsMT k1*	*kpsMT K5*	*ibeA*	*cvaC*	*traT*	PAI	
		58	57	48	63	46	24	12	24	20	12	85	70	52	24	16	34	18	14	70	56	
A	10	0	0	2 (4)	0	0	0	0	0	2 (10)	0	8 (9)	2 (3)	0	4 (17)	0	0	0	2 (14)	8 (11)	0	4, 4 (2-6)
	p value	*0.0001*	*0.0001*	-	*0.0001*	*0.001*	-	-	-	-	-	-	*0.0009*	*0.0004*	-	-	*0.014*	-	-	-	*0.0001*	
B1	4	0	0	0	0	0	0	0	0	0	0	4 (5)	4 (6)	0	2 (8)	0	0	0	2 (14)	2 (3)	0	4.5, 4.5 (3-6)
	p value	*0.029*	*0.032*	-	*0.017*	-	-	-	-	-	-	-	-	*0.049*	-	-	-	-	-	-	*0.034*	
B2	56	36 (62)	38 (67)	28 (58)	39 (62)	30 (65)	20 (83)	8 (67)	22 (92)	16 (80)	12 (100)	48 (56)	34 (49)	32 (62)	6 (25)	14 (87)	18 (53)	12 (67)	4 (29)	34 (49)	46 (82)	10.2, 10 (2-17)
	p value	-	*0.016*	-	-	-	*0.0021*	-	*0.0001*	*0.022*	*0.001*	-	*0.028*	-	-	*0.0059*	-	-	*0.0402*	*0.028*	*0.0001*	
D	30	22 (38)	19 (33)	18 (38)	24 (38)	16 (35)	4 (17)	4 (33)	2 (8)	2 (10)	0	25 (29)	30 (43)	20 (38)	12 (50)	2 (13)	16 (47)	6 (33)	6 (43)	26 (37)	10 (18)	10.2, 10 (5-19)
	p value	0.049	-	-	*0.0247*	-	-	-	*0.009*	*0.031*	*0.016*	-	*0.0001*	-	*0.021*	-	*0.011*	-	-	*0.018*	*0.004*	

P values (by Fisher's exact test) where shown when $P \leq 0.05$; underlining indicates a negative association.

Table 2.11 Prevalence of various UPEC associated virulence factors of STs observed in the urine and blood collections. The data presented are for VFs showing a degree of association with the different phylogenetic groups. The urine and blood paired isolates of each patient show identical VFs profiles (not visible in Table)

Phylogenetic groups	Isolates No	Prevalence (%) of VF by phylogenetic grouping and STs (No. of isolates positive)																				VF score [mean, median (range)]
		Adhesion (%)							Toxins (%)			Siderophore (%)		Capsule (%)				Miscellaneous (%)				
		papAH	*papC*	*papEF*	*papG II,III*	*allele-II*	*sfa/focDE*	*focG*	*hlyA*	*cnfI*	*cdtB*	*fyuA*	*iutA*	*kpsMT II*	*kpsMT III*	*kpsMT k1*	*kpsMT K5*	*ibeA*	*cvaC*	*traT*	PAI	
		58	57	48	63	46	24	12	24	20	12	85	70	52	24	16	34	18	14	70	56	
ST73	30	22 (38)	20 (35)	16 (33)	21 (33)	18 (39)	18 (75)	8 (67)	18 (75)	12 (60)	10 (83)	24 (28)	18 (26)	16 (31)	2 (8)	4 (25)	14 (41)	8 (44)	0 (0)	18 (26)	28 (50)	11.2, 10 (6-17)
	p value	*0.0489*	-	-	-	-	*0.0001*	*0.0059*	*0.0001*	*0.0021*	*0.0001*	-	-	-	*0.0095*	-	-	-	*0.009*	-	*0.0001*	
ST95	12	6 (10)	12 (21)	6 (13)	12 (19)	10 (22)	0	0	0	0	2 (17)	12 (14)	10 (14)	10 (19)	0	10 (63)	0	0	4 (29)	10 (14)	8 (14)	10.7, 11 (8-14)
	p value	-	*0.001*	-	*0.0032*	*0.0108*	-	-	-	-	-	-	-	*0.0013*	-	*0.0001*	*0.0073*	-	-	-	-	
ST69	10	10 (17)	9 (16)	10 (21)	10 (16)	10 (22)	0	0	0	0	0	9 (11)	10 (14)	6 (12)	2 (8)	0 (0)	6 (18)	0	0	10 (14)	0	10.3, 10.5 (9-12)
	p value	-	*0.0402*	*0.0004*	*0.0122*	*0.0002*	-	-	-	-	-	-	*0.0302*	-	-	-	-	-	-	*0.0302*	*0.0001*	
ST131	8	4 (7)	2 (4)	2 (4)	2 (3)	2 (4)	0	0	4 (17)	4 (20)	0 (0)	8 (9)	6 (9)	2 (4)	2 (8)	0	0	0		2 (3)	6 (11)	6.75, 5.5 (3-13)
	p value	-	-	-	*0.0489*	-	-	-	-	*0.0487*	-	-	-	-	-	-	*0.0484*	-	-	*0.0085*	-	
ST48	4	0	0	2 (4)	0	0	0	0	0	2 (10)	0 (0)	2 (2)	2 (3)	0	2 (8)	0	0	0	0	2 (3)	0	4, 4 (2-6)
	p value	*0.0285*	*0.0315*	-	*0.0168*	-	-	-	-	-	-	-	-	*0.0496*	-	-	-	-	-	-	*0.0346*	
ST127	4	4 (7)	4 (7)	4 (8)	4 (6)	0	0	0	0	0	0	4 (5)	0	4 (8)	2 (8)	0	4 (12)	4 (22)	0	4 (6)	4 (7)	12.5, 12.5 (12-13)
	p value	-	-	*0.0496*	-	-	-	-	-	-	-	-	*0.007*	-	-	-	*0.0118*	*0.0008*	-	-	-	
ST38	4	2 (3)	2 (4)	0	2 (3)	0	0	0	0	0	0	4 (5)	4 (6)	2 (4)	2 (8)	0	2 (6)	0	0	4 (6)	0	7.5, 7.5 (7-8)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*0.0346*	
ST14	4	4 (7)	2 (4)	2 (4)	4 (6)	2 (4)	2 (8)	2 (17)	0	0	0	4 (5)	4 (6)	2 (4)	2 (8)	0	2 (6)	2 (11)	0	2 (3)	4 (7)	11, 11 (10-12)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*0.0346*	

P values (by Fisher's exact test) where shown when $P \leq 0.05$; underlining indicates a negative association.

2.4.2.2 Biliary sepsis isolates

The prevalence of VF genes was determined among the 27 biliary sepsis isolates. A total of 23 VF genes were detected with a prevalence ranging from 4% (*afa/draBC*) to 96% (*fimH*). Virulence factors *allele-I, nfaE, rfc, cvaC, papG I sfaS,* were not detected in the collection of isolates. The prevalence was significantly higher among STs belonging to phylogenetic groups B2 and D (Figure 2.5). Members of STs belonging to phylogenetic group A and B1 were detected infrequently in the examined isolates and low counts for VF gene carriage were observed for these STs/phylogenetic groups. The adhesion genes (*papAH, papC* and *papEF*) occurred in around 30% of the isolates, while *papGII,III* occurred only in 19% of the isolates (Table 2.12). The toxin-associated genes (*hlyA, cnf1* and *cdtB)* were detected in relatively few isolates; 15%, 7% and 7%, respectively. Of the siderophores, *fyuA* (yersiniabactin) was observed in 85% of the isolates and *iutA* was slightly lower (63% of the isolates). Capsule synthesis genes had varying prevalence rates of 70% (*kpsM II*), 15%, (*kpsM III*), 7% (K1) and 41% (*K5*). The serum resistance gene (*traT*) occurred in 37% of the isolates while the pathogenicity island (*PAI*) marker gene was present in 67% of the isolates (Table 2.12). STs varied considerably in VF content, from ST38 with the lowest VF score (mean 3.0) to ST73 with the highest VF score (mean 10.5) (Table 2.12). The adhesion factor *papEF* was significantly associated with ST69 isolates. Due to the low number of biliary sepsis isolates, no other significant results were observed.

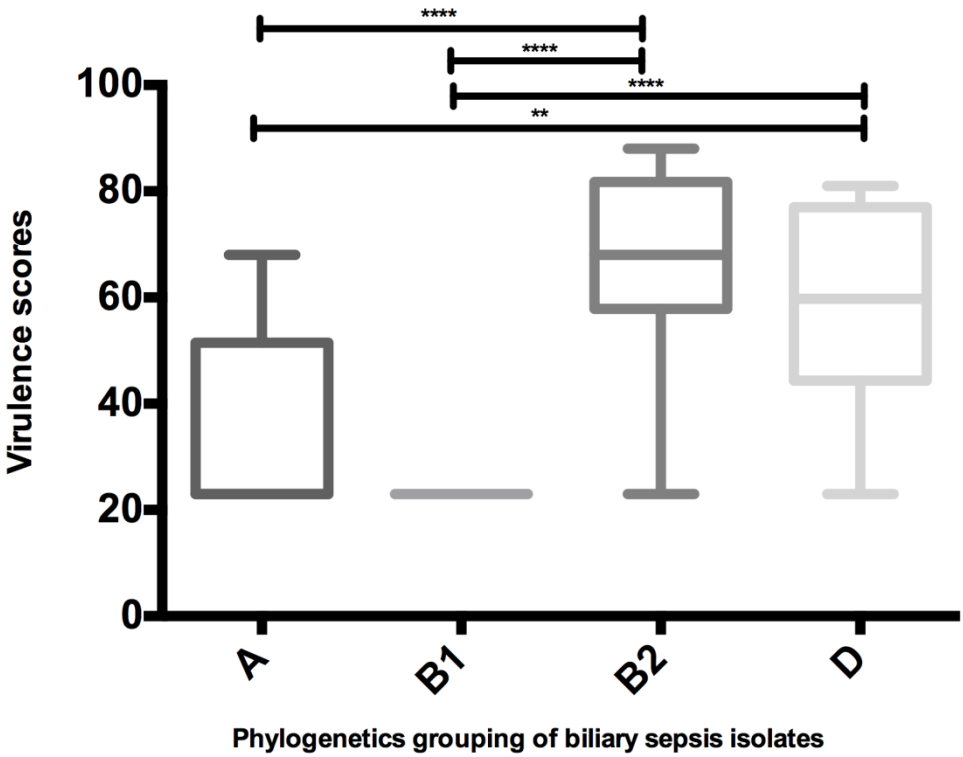

Figure 2.5 Statistical analysis of the biliary sepsis isolates to compare the prevalence of VF scores between the phylogenetic groupings A, B1, B2 and D. Phylogenetic group B2 shows a significantly enhanced virulence capacity compared to that of other groups.

Table 2.12 Prevalence of various UPEC associated virulence factors observed in biliary sepsis isolates

Phylogenetic groups	Isolates No	Prevalence (%) of VF by phylogenetic grouping and STs (No. of isolates positive)																				VF score [mean, median (range)]
		Adhesion (%)									Toxins (%)			Siderophore (%)		Capsule (%)				Miscellaneous (%)		
		papAH	*papC*	*papEF*	*papG II,III*	*allele-II*	*allele-III*	*sfa/focDE*	*afa/draBC*	*focG*	*hlyA*	*cnfI*	*cdtB*	*fyuA*	*iutA*	*kpsMT II*	*kpsMT III*	*kpsMT k1*	*kpsMT K5*	*ibeA*	*traT*	PAI	
		8	8	7	5	7	3	2	1	3	4	2	2	23	17	19	4	2	11	9	10	18	
A	3	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (13)	2 (12)	0 (0)	0 (0)	0 (0)	0 (0)	1 (11)	1 (10)	2 (11)	4, 4 (3-5)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*0.0191*	-	-	-	-	-	-	
B1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1, 1 (1-1)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
B2	16	4 (50)	4 (50)	3 (43)	1 (20)	4 (57)	3 (100)	2 (100)	1 (100)	2 (67)	4 (100)	1 (50)	1 (50)	14 (61)	9 (53)	14 (74)	3 (75)	2 (100)	7 (64)	7 (78)	6 (60)	13 (72)	8.1, 7 (4-14)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*0.0332*	-	-	-	-	-	-	
D	7	4 (50)	4 (50)	4 (57)	3 (60)	3 (43)	0	0	0	0	0	1 (50)	1 (50)	5 (22)	5 (29)	5 (26)	1 (25)	0	4 (36)	0	3 (30)	2 (11)	7.6, 9 (3-11)
	p value	-	-	*0.0496*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*0.0235*	
ST																							
ST69	4	3 (38)	3 (38)	3 (43)	2 (40)	2 (29)	0	0	0	0	0	0	1 (50)	4 (17)	4 (24)	3 (16)	1 (25)	0	2 (18)	0 (0)	3 (30)	1 (6)	9.3, 9.5 (7-11)
	p value	-	-	*0.0419*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST131	4	0	0	0	0	0	0	0	0	1 (33)	0	0	0	4 (17)	4 (24)	3 (16)	1 (25)	0	2 (18)	1 (11)	2 (20)	4 (22)	6.5, 6 (6-8)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST73	2	1 (13)	1 (13)	1 (14)	0	1 (14)	1 (33)	0	0	0	1 (25)	1 (50)	0	2 (9)	2 (12)	2 (11)	1 (25)	0	1 (9)	1 (11)	1 (10)	1 (6)	10.5, 10.5 (7-14)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST38	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (5)	0	0	1 (9)	0	0	0	3, 3 (3-3)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST88	1	0	0	0	0	0	0	0	0	0	0	0	0	1 (4)	(1) 6	0	0	0	0	(1) 11	0	1 (6)	5, 5 (5-5)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST410	1	0	0	0	0	0	0	0	0	0	0	0	0	1 (4)	1 (6)	0	0	0	0	0	0	0	3, 3 (3-3)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST617	1	0	0	0	1 (20)	0	0	0	0	0	0	0	0	1 (4)	0	0	0	0	0	0	1 (10)	1 (6)	4, 4 (4-4)
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

P values (by Fisher's exact test) where shown when $P \leq 0.05$; underlining indicates a negative association.

2.4.3 Antibiotic resistance profiles of urosepsis and biliary sepsis isolates

2.4.3.1 Urine and blood isolates

In the present study, with respect to antibiotic resistance, paired isolates from blood and urine exhibited identical profiles (data not shown). Overall, 25% of the total urine and blood isolates were susceptible to all tested antibiotics, while the rest of the isolates demonstrated a wide range of susceptibility, from resistance to just one antibiotic (6%) to resistance to 15 out of 21 tested antibiotics (< 2%) (Table 2.13). A high level of resistance was observed towards a number of commonly used antibiotics: amoxicillin (75%); ampicillin (64%); and trimethoprim (48%), as shown in Figure 2.6.

The antibiotic profiles of paired isolates shows different mechanisms of resistance are predicted for the major antibiotic classes (Table 2.13). According to the VITEK 2 AES, 8% of the total paired isolates were identified as ESBL producers including members of STs belonging to phylogenetic groups A and B2. This is perhaps higher than may be expected in UPEC selected from uncomplicated UTI. Two patterns of aminoglycoside resistance mechanisms were seen, based on enzymatic modification by N-Acetytransferase (AAC) and O-Adenyltransferase (ANT), which were collectively acquired by 6% of the isolates.

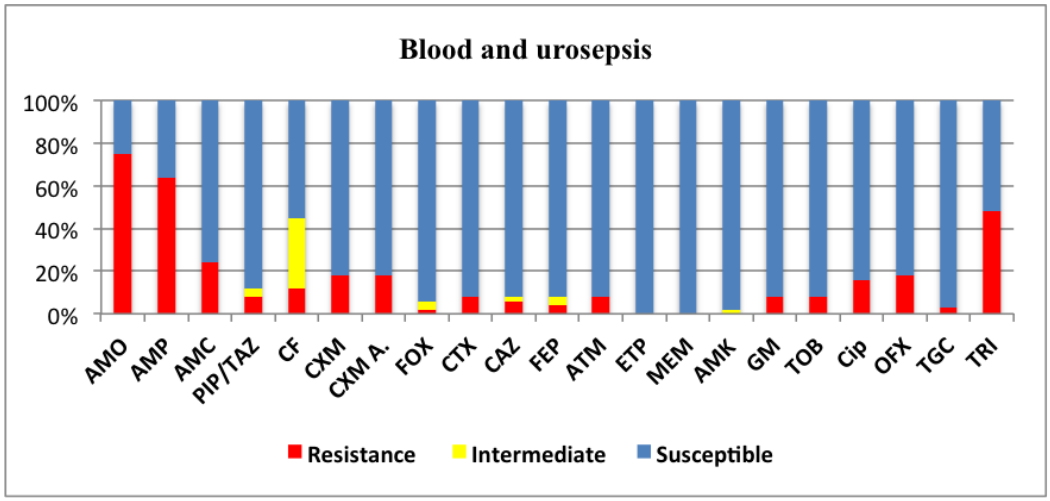

Figure 2.6 Distribution of antibiotic susceptibility of urine and blood isolates. AMO: amoxicllin; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; PIP/TAZ: piperacillin/tazobactam; CF cefalotin; CXM: cefuroxime; CXM A: cefuroxime axetil; FOX: cefoxitin; CTX: cefotaxime; CAZ: ceftazidime; FEP: cefepime; ETP: ertapenem; MEM: meropenem; ATM: Aztreonam; CIP: Ciprofloxacin; OFX: Ofloxacin; AMK: Amikacin; GM: Gentamicin; TOB: Tobramycin; TGC: Tigecycline; TRI: Trimethoprim

Table 2.13 Association of urine and blood isolates of various phylogenetic groups and STs with resistance to different antibiotic agents

Phylogenetic group	Isolates No	β-Lactams (%)												ESBL (%)	Aminoglycosides (%)		Quinolones (%)		Trimethoprim (%)	Resistance-score
		AMO	AMP	AMC	PIP	CF	CXM	CXM.A	FOX	CTX	CAZ	FEP	ATM		AAC	AAC(3) ANT(2)	CIP	OFX		
		73	62	24	8	12	18	18	2	8	6	4	8	8	2	4	16	18	46	[mean, median (range)]
A	10	8 (11)	7 (11)	2 (8)	5 (63)	4 (33)	5 (28)	5 (28)	2 (100)	4 (50)	2 (33)	0	4 (50)	4 (50)	0	2 (50)	2 (13)	2 (11)	6 (13)	0.4, 0.61 (0.04-1.38)
P value		-	-	-	0.016	0.017	0.016	0.016	0.009	0.003	-	-	0.003	0.003	-	0.049	-	-	-	
B1	4	4 (5)	4 (6)	2 (8)	0	0	0	0	0	0	0	0	0	0	0	0	2 (13)	2 (11)	2 (4)	0.4, 0.4 (0.28-0.52)
P value		-	-	-	-	-	-	-	-	-	-	-	-	-			-	-	-	
B2	56	35 (48)	30 (48)	11 (46)	8 (100)	8 (67)	8 (44)	8 (44)	0 (0)	4 (50)	4 (67)	4 (100)	4 (50)	4 (50)	2 (100)	0	6 (38)	8 (44)	20 (43)	0.19, 0.39 (0-1.4)
P value		0.012	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.026	
D	30	26 (36)	21 (34)	9 (38)	5 (63)	0	8 (44)	8 (44)	0	0	0	0	0	0	0	2 (50)	6 (38)	6 (33)	18 (39)	0.33, 0.37 (0-0.95)
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
STs	**Isolates**																			
ST73	30	17 (23)	13 (21)	5 (21)	2 (25)	2 (17)	2 (11)	2 (11)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (9)	0.2, 0.19 (0-0.67)
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST95	12	12 (16)	3 (5)	0	0	0	0	0	0	0	0	0	0	0	0	0	2 (13)	2 (11)	4 (9)	0.12, 0.05 (0-0.48)
P value		0.033	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST69	10	10 (14)	6 (10)	4 (17)	3 (38)	0 (0)	3 (17)	3 (17)	0	0	0	0	0	0	0	0	0	0	5 (11)	0.35, 0.33 (0-0.71)
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST131	8	8 (11)	8 (13)	4 (17)	6 (75)	6 (50)	6 (33)	6 (33)	0	4 (50)	4 (67)	4 (100)	4 (50)	4 (50)	2 (100)	0	4 (25)	6 (33)	6 (13)	0.92, 0.88 (0.43-1.48)
P value		-	0.023	-	0.0003	0.0001	0.0003	0.0003	0.0011	0.001	0.0003	0.0001	0.001	0.001	0.006	-	0.021	0.0003	0.0003	
ST48	4	4 (5)	4 (6)	0	3 (38)	2 (17)	3 (17)	3 (17)	0	2 (25)	2 (33)	0	2 (25)	2 (25)	0	2 (50)	0	0	2 (4)	0.71, 0.79 (0.24-1.05
P value		-	-	-	0.018	-	0.018	0.018	0.031	0.031	0.017	-	0.031	0.031	-	0.0172	-	-	-	
ST127	4	4 (5)	3 (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (4)	0.26, 0.24 (0.19-0.38)
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST38	4	4 (5)	2 (3)	0 (0)	0	0	0	0	0	0	0	0	0	0	0	0	2 (13)	2 (11)	4 (9)	0.52, 0.52 (0.33-0.71)
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0416	
ST14	4	2 (3)	2 (3)	2 (8)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2 (4)	0.19, 0.19 (0-0.38)
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST167	2	2 (3)	2 (3)	2 (8)	2 (25)	2 (17)	2 (11)	2 (11)	2 (100)	2 (25)	2 (33)	2 (50)	2 (25)	2 (25)	0	0	2 (13)	2 (11)	2 (4)	1.38, 1.38 (1.38-1.38)
P value		-	-	-	0.031	0.013	0.031	0.031	0.0002	0.006	0.003	0.006	0.006	0.006	-	-	0.003	0.003	-	

P values (by Fisher's exact test) where shown when $P \leq 0.05$ and these relate to differences found when susceptibility profiles for isolates of each Phylogenetic group or ST were compared with those of all others combined

Table 2.13 shows the association of the phylogenetic groups and STs with antibiotic resistance profiles. Members of ST131 (50% of ST131) were significantly associated with production of ESBL enzymes (P <0.001) and resistance to ciprofloxacin (P <0.02) and ofloxacin (P <0.0003). Almost all ST131 (75% of ST131) were associated with resistance to trimethoprim (P <0.0003). ST48 (n=4) and ST167 (n=2) also showed a high prevalence of resistance profile, but members of these STs were detected in small numbers (Table 2.13).

2.4.3.2 Biliary sepsis isolates

The antimicrobial resistance profile of the biliary sepsis isolates shows that 26% of the isolates were susceptible to all tested antibiotics, while the rest of the isolates demonstrated a wide range of susceptibility, from resistance to just one antibiotic (7%) to resistance to 16 out of 21 tested antibiotics (< 4%). A high level of resistance was observed to a number of commonly used antibiotics: amoxicillin and ampicillin (59%), followed by amoxicillin/clavulanic acid (44%), cefuroxime (33%), ciprofloxacin (26%) and ofloxacin (26%), as shown in Figure 2.7.

According to the VITEK 2 AES, two isolates (8% of total) were identified as ESBL producers (Table 2.14). These isolates belonged to phylogenetic group A (ST410 and ST617). One pattern of aminoglycoside resistance mechanism (ANT) was seen, which was acquired by 7% of the isolates belonging different STs (ST131, ST410 and ST617).

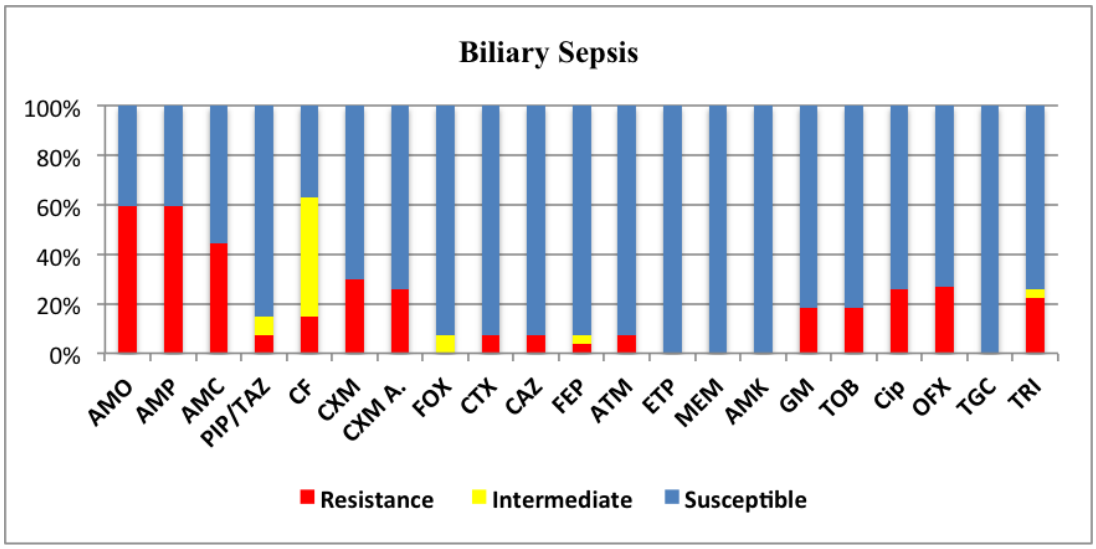

Figure 2.7 Distribution of antibiotics susceptibility of biliary sepsis isolates. AMO: amoxicllin; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; PIP/TAZ: piperacillin/tazobactam; CF cefalotin; CXM: cefuroxime; CXM A: cefuroxime axetil; FOX: cefoxitin; CTX: cefotaxime; CAZ: ceftazidime; FEP: cefepime; ETP: ertapenem; MEM: meropenem; ATM: Aztreonam; CIP: Ciprofloxacin; OFX: Ofloxacin; AMK: Amikacin; GM: Gentamicin; TOB: Tobramycin; TGC: Tigecycline; TRI: Trimethoprim

Table 2.14 Association of biliary sepsis isolates of various phylogenetic groups and STs with resistance to different antibiotic agents

Phylogenetic group	Isolates No	β-Lactams (%)											ESBL (%)	Aminoglycosides (%)	Quinolones (%)		Trimethoprim (%)	Resistance-score
		AMO	AMP	AMC	PIP	CF	CXM	CXM.A	CTX	CAZ	FEP	ATM		AAC(3), ANT(2)	CIP	OFX		
		16	16	12	2	4	8	7	2	2	1	2	2	7	7	7	6	[mean, median (range)]
A	3	3 (19)	3 (19)	3 (25)	1 (50)	2 (50)	3 (38)	3 (43)	2 (100)	2 (100)	1 (100)	2 (100)	2 (100)	2 (29)	2 (29)	2 (29)	1 (17)	1.38, 1.13 (0.52-1.48)
P value		-	-	-	-	0.0485	0.0191	0.012	0.0085	0.0085	-	0.0085	0.0085	-	-	-	-	
B1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
B2	16	6 (38)	6 (38)	4 (33)	1 (50)	2 (50)	3 (38)	2 (29)	0	0	0	0	0	2 (29)	3 (43)	3 (43)	4 (67)	0.24, 0.26 (0-0.95)
P value		0.0076	0.0076	0.022	-	-	-	-	-	-	-	-	-	-	-	-	-	
D	7	7 (44)	7 (44)	5 (42)	0	0	2 (25)	2 (29)	0	0	0	0	0	1 (14)	2 (29)	2 (29)	1 (17)	0.52, 0.44 (9-0.57)
P value		0.0216	0.0216	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
STs	Isolates																	
ST69	4	4 (25)	4 (25)	4 (33)	0	0	1 (13)	1 (14)	0	0	0	0	0	1 (14)	1 (14)	1 (14)	0	0.52, 0.49 (0.33-0.57)
P value		-	-	0.0282	-	-	-	-	-	-	-	-	-	-	-	-	-	
131	4	3 (19)	3 (19)	2 (17)	1 (50)	1 (25)	1 (13)	1 (14)	0	0	0	0	0	2 (29)	3 (43)	3 (43)	2 (33)	0.64, 0.62 (0.24-0.95)
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	0.0419	0.0419	-	
ST73	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.17, 0.17 (0-0.33)
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST38	1	1 (6)	1 (6)	1 (8)	0	0	1 (13)	1 (14)	0	0	0	0	0	0	0	0	0	0.57
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST88	1	1 (6)	1 (6)	1 (8)	0 (0)	0 (0)	1 (13)	1 (14)	0	0	0	0	0	0	0	0	0	0.52
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST410	1	1 (6)	1 (6)	1 (8)	0 (0)	1 (25)	1 (13)	1 (14)	1 (50)	1 (50)	1 (100)	1 (50)	1 (50)	1 (14)	1 (14)	1 (14)	0	1.38
P value		-	-	-	-	-	-	-	-	-	0.037	-	-	-	-	-	-	
ST617	1	1 (6)	1 (6)	1 (8)	1 (50)	1 (25)	1 (13)	1 (14)	1 (50)	1 (50)	0	1 (50)	1 (50)	1 (14)	1 (14)	1 (14)	1 (17)	1.48
P value		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

P values (by Fisher's exact test) where shown when $P \leq 0.05$ and these relate to differences found when susceptibility profiles for isolates of each phylogenetic group ST were compared with those of all others combined

2.4.4 Metabolic activity profiles of urosepsis and biliary sepsis isolates

2.4.4.1 Urine and blood isolates

Metabolic profiles of the urine and blood isolates were determined using the VITEK 2.0 ID-GNB card (Biomérieux). A total of 47 fluorescence based biochemical tests was performed to measure carbon source utilization and enzymatic activity of the examined isolates.

Table 2.15 shows the statistical analysis of individual metabolic activity with the phylogenetic groups and common STs using Fisher's exact test. Of the 47 biochemical tests, phylogenetic groups were significantly associated with six tests and the common STs were associated with eight tests. Assimilation of saccharose/sucrose (SAC) appears to be a key characteristic in some common STs including ST14, ST38, ST69 and ST131, though this was negatively associated with ST95 and ST73. Despite the low number of ST38 isolates (4% of the total), they showed the highest bio-score (mean= 0.4) compared to other STs.

Table 2.15 Statistical analysis of association between phylogenetic groups, STs and metabolic activity of urine and blood isolates

Phylogenetic groups	(No)	Biochemical tests												Bio-score
		ADO* (2)	GGT (3)	OFF (98)	SAC (45)	dTAG (10)	5KG (59)	ILATk (84)	SUCT (94)	PHOS (2)	GlyA (8)	ODC (72)	IMLTa (5)	[mean, median (range)]
A	(10)	0.0091	-	-	-	-	-	-	-	-	-	**0.0005**	-	0.36, 0.36 (0.3-0.38)
B1	(4)	-	-	-	0.038	-	**0.0258**	-	-	-	-	-	-	0.37, 0.37 (0.36-0.38)
B2	(56)	-	-	-	0.0001	-	-	-	-	-	**0.0201**	0.0001	-	0.37, 0.36 (0.32-0.43)
D	(30)	-	0.0251	-	0.0001	-	0.0073	-	-	-	-	**0.0001**	-	0.38, 0.38 (0.3-0.43)
STs														
ST73	(30)	-	-	-	**0.0001**	-	-	-	-	-	-	0.0015	0.0019	0.37, 0.36 (0.34-0.43)
ST95	(12)	-	-	-	**0.0004**	-	-	**0.0035**	-	-	-	0.0181	-	0.35, 0.36 (0.32-0.38)
ST69	(10)	-	-	-	0.0002	-	-	-	-	-	-	**0.0001**	-	0.38, 0.38 (0.36-0.42)
ST131	(8)	-	-	-	0.0012	-	-	-	-	-	-	-	-	0.39, 0.39 (0.36-0.4)
ST14	(4)	-	-	**0.0012**	0.038	0.0488		-	**0.0172**	0.0012	-	-	-	0.36, 0.36 (0.3-0.43)
ST38	(4)	-	-	-	0.038	-	-	-	-	-	-	-	-	0.4, 0.4 (0.4-0.4)
ST48	(4)	-	-	-	-	-	-	-	-	-	-	**0.0052**	-	0.33, 0.34 (0.3-0.34)
ST127	(4)	-	-	-	-	-	-	**0.0125**	-	-	-	-	-	0.38, 0.38 (0.36-0.4)

P values (by Fisher's exact test) were calculated for the phylogenetic groups and for the most detected STs compared to the rest of the examined isolates shown only where P≤0.05. Bold P values are for negative associations.

* Two isolates belong to ST10 where the only positive to ADO

The metabolic profiles were identical for 32 pairs of isolates and 18 pairs showed discrepant profiles for a total of 12 biochemical tests. These isolates are members of different STs including ST73 (n=6, meaning 3 pairs of isolates), ST95 (n=8), ST69 (n=6), ST127 (n=4), ST48 (n=2) and ST2459 (n=2). The majority of these paired isolates showed a positive result in urine and were negative in blood isolates for each of the 12 biochemical tests. In four biochemical tests, the differences were in the opposite direction (Table 2.16).

The test most likely to have discrepant results was ILATk (eight pairs of isolates). Four pairs of isolates belonging to ST73 (n=2) and ST95 (n=2) differed in the O/129 utilization test, with three of the pairs showing positive activity in the blood isolates and negativity in the urine. The ELLM and SUCT tests showed different results for two pairs of isolate, where one of them was positive in the blood isolate and negative in the urine. The BGUR test was non-identical in only one pair of isolates, which belonged to ST73 (positive in the blood isolate). A large number of ST73 paired isolates showed non-identical blood and urine biochemical activities, but more notable was the fact that the two ST127 pairs observed in the collection both had differential results, one pair being discrepant in four tests (Table 2.16).

Biochemical activities of urosepsis isolates were compared to previously reported data for UPEC and the general *E. coli* population. The data of Gibreel and colleagues are most relevant as the isolates being studied here and in their work were collected in the same geographical location and within 5 years of each other. The results show similar biochemical profiles of the different populations with some exceptions (Table 2.17). Several biochemical activities were observed to be different in the urine and blood isolates compared to the UPEC population, as shown in Figure 208. A significant difference was observed considering the ELLM test, where 14% of the urosepsis isolates were positive, compared to 96% reported by previously (Gibreel, 2011) ($P < 0.0001$). In addition, other biochemical tests were observed with a notable difference including alpha-galactosidase, l-lactate alkalinisation, succinate alkalinisation and tyrosine arylamidase.

Table 2.16 Results of biochemical tests that was non-identical in the urine and blood isolates

NO	Sample	ST	GROUPING	Biochemical Tests											
				GGT	ProA	TyrA	SAC	5KG	ILATk	SUCT	GlyA	BGUR	O129R	IMLTa	ELLM	Bio-score
46	Urine	48	A			+			+							16
46	Blood	48	A			-			-							14
17	Urine	69	D	+	+						+					20
17	Blood	69	D	-	-						-					17
48	Urine	69	D								+					19
48	Blood	69	D								-					18
69	Urine	69	D						+							18
69	Blood	69	D						-							17
7	Urine	73	B2						+	-					+	18
7	Blood	73	B2						-	+					-	17
15	Urine	73	B2		+									+		18
15	Blood	73	B2		-									-		17
20	Urine	73	B2					+					-			17
20	Blood	73	B2					-					+			17
21	Urine	73	B2											+		19
21	Blood	73	B2											-		18
22	Urine	73	B2		+							-		+		19
22	Blood	73	B2		-							+		-		18
23	Urine	73	B2											+		18
23	Blood	73	B2											-		17
25	Urine	73	B2						+				+			18
25	Blood	73	B2						-				-			16
2	Urine	95	B2										-			17
2	Blood	95	B2										+			18
9	Urine	95	B2			+			+							17
9	Blood	95	B2			-			-							15
28	Urine	95	B2						+							17
28	Blood	95	B2						-							16
66	Urine	95	B2										-			16
66	Blood	95	B2										+			17
18	Urine	127	B2			+	+				+					18
18	Blood	127	B2			-	-				-					15
41	Urine	127	B2			+			+	+					-	19
41	Blood	127	B2			-			-	-					+	17
16	Urine	2459	D						+		+					18
16	Blood	2459	D						-		-					16

Table 2.17 Biochemical activity profiles of the biliary sepsis and urosepsis isolates studied here, compared to those previously reported by other groups (bold face type indicates major difference)

Test	Abb.	% of Biochemical activities				
		Biliary sepsis	Urine and blood	UPEC as reported by	*E. coli* as reported by	
				(Gibreel, 2011)	(Ewing, 1986)	(Farmer *et al.* 1985)
		(n=27)	(n=100)	(n=300)	(n=1.231)	(n=ND)
Adonitol	ADO	0	2	0.7	5.6	5
Alpha-Galactosidase	**AGAL**	**81**	**96**	**69**	**ND**	**99**
Glutamyl Arylamidse pNA	AGLTp	0	0	0	ND	ND
Alpha-Glucosidase	AGLU	0	0	0	0	0
Ala-Phe-Pro-Arylamidase	APPA	0	0	0	ND	ND
Beta-Alanine Arylamidase pNA	BAIap	0	0	0	ND	ND
Beta-Galactosidase	BGAL	100	100	98.7	ND	95
Beta-Glucosidase	BGLU	0	0	0	ND	ND
Beta-Glucoronidase	BGUR	96	95	94	ND	ND
Beta-N-Acetyl-Galactosaminidase	BNAG	0	0	0	ND	ND
Beta-Xylosidase	BXYL	0	0	0	ND	ND
Citrate	CIT	0	0	0	ND	1
Courmarate	CMT	100	100	100	ND	ND
D-Cellobiose	dCEL	0	0	0	2.4	2
D-Glucose	dGLU	100	100	100	100	100
D-Maltose	dMAL	100	100	97.4	89.9	95
D-Mannitol	dMAN	100	100	100	96.8	98
D-Mannose	dMNE	100	100	100	ND	98
D-sorbitol	dSOR	100	100	92.1	93.4	94
D-Tagatose	dTAG	**33**	**10**	**7.3**	**ND**	**ND**
D-Trehalose	dTRE	100	100	99.3	98.8	98
Ellman	ELLM	**15**	**14**	**96.7**	**ND**	**ND**
Glu-Gly-Arg-Arylamidase	GGAA	0	0	0	ND	ND
Gamma-Glutamyl-Transferase	GGT	7	3	0.7	ND	ND
Glycine Arylamidase	GlyA	4	8	0	ND	ND
H2S production	H2S	0	0	0	ND	1
L-Arabitol	IARL	0	0	0	ND	5
L-Histidine assimilation	IHISa	0	0	0	ND	ND
L-Lactate assimilation	ILATa	0	2	0	ND	ND
L-Lactate alkalinisation	ILATk	**67**	**84**	**23.8**	**ND**	**ND**
L-Malate assimilation	IMLTa	4	5	2	ND	ND
Lysine Decarboxylase	LDC	93	96	95.4	88.7	90
Lipase	LIP	0	0	0	0	0
Malonate	MNT	0	0	0.7	0	0
Beta-N-Acetyl-Glucosaminidase	NAGA	0	0	0	ND	ND
O/129 Resistance	O129R	59	72	60.9	ND	ND
Ornithine Decarboxylse	ODC	74	72	78.8	64.2	65
Fermentation/Glucose	OFF	100	98	96.7	ND	100
Phosphatase	PHOS	0	2	6	ND	ND
Palatinose	PLE	0	0	0	ND	ND
L-Proline Arylamidase	ProA	7	9	9.3	ND	ND
L-Pyrrolydonyl-Arylamidase	PyrA	0	0	0	ND	ND
Saccharose/Sucrose	**SAC**	**70**	**45**	**49.7**	**48.9**	**50**
Succinate alkalinisation	**SUCT**	**85**	**94**	**33.8**	**ND**	**ND**
Tyrosine Arylamidase	**TyrA**	**63**	**88**	**36.4**	**ND**	**ND**
Urease	URE	0	0	0.7	0	1
5-Keto-D-Gluconate	5KG	52	59	49	ND	ND

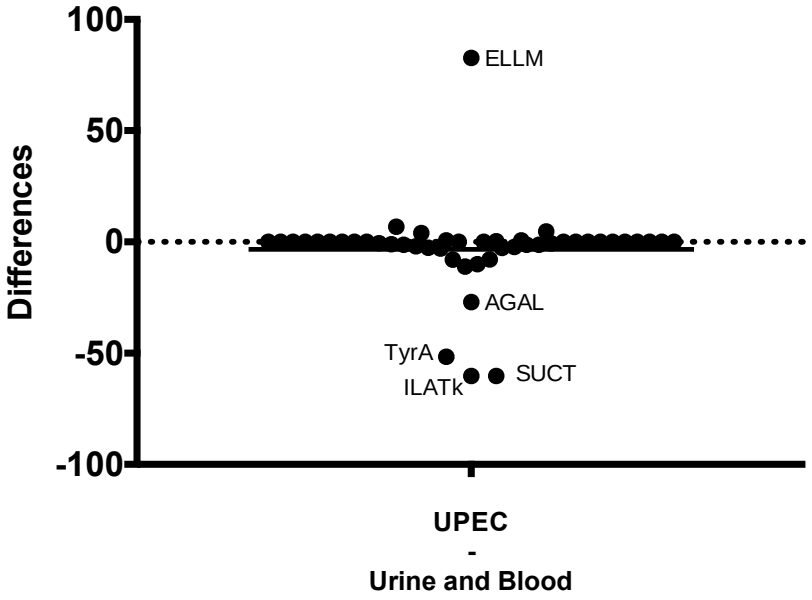

Figure 2.8 Comparative analysis of metabolic activities of urine and blood isolates studied here, with UPEC previously reported previously (Gibreel, 2011).

2.4.4.2 Biliary sepsis isolates

The metabolic profiles of the biliary sepsis isolates were similar to the urine and blood isolates, with some exceptions. Three biochemical tests showed notable differences in results compared with the urine and blood isolates including assimilation of sucrose, production of D-tagatose and tyrosine arylamidase (Table 2.17). In addition, a clear difference was observed between the metabolic profiles of the biliary sepsis isolates compared to UPEC and the general *E. coli* populations previously reported by other groups (Table 2.17)(Edwards and Ewing, 1986; Farmer et al., 1985; Gibreel, 2011). Table 2.18 shows the association between the clonal groups of biliary sepsis isolates and specific metabolic activities. Phylogenetic group B2 was significantly associated with three biochemical tests; d-tagatose, l-lactate alkalinisation and ornithine decarboxylase.

Table 2.18 Statistical analysis of association between the clonal groups of biliary sepsis isolates and selected metabolic activity assays

		Biochemical tests					**Bio-score**
Phylogenetic groups	(No)	dTAG (9)	5KG (14)	ILATk (18)	ODC (20)	IMLTa (1)	**[mean, median (range)]**
A	(3)	-	-	-	-	-	0.38, 0.38 (0.34-0.42)
B1	(1)	-	-	-	-	-	0.38
B2	(16)	0.0417	-	0.0417	0.0087	-	0.36, 0.36 (0.32-0.4)
D	(7)	-	-	-	**0.0047**	-	0.37, 0.36 (0.34-0.45)
STs							
ST69	(4)	-	-	-	**0.0419**	-	0.37, 0.37 (0.36-0.38)
ST131	(4)	-	**0.0407**	-	-	-	0.37, 0.37 (0.32-0.4)
ST73	(2)	-	-	-	-	-	0.36, 0.36 (0.34-0.38)
ST38	(1)	-	-	-	-	-	0.45
ST88	(1)	-	-	-	-	-	0.38
ST410	(1)	-	-	-	-	0.037	0.43
ST617	(1)	-	-	-	-	-	0.34

P values (by Fisher's exact test) were calculated for the phylogenetic groups and for the most frequently detected STs compared to the rest of the examined isolates, shown only where $P \leq 0.05$. Bold *P* values are for negative associations.

2.5 Discussion

UTI is a frequent source of bacteraemia caused by *E. coli* strains (Hounsom et al., 2011). These strains need to overcome and adapt to a new host environment by acquiring, or expressing, niche specific traits including virulence determinants or metabolic capabilities (Johnson et al., 1987). Walters and colleagues tried to understand the population dynamics during bacterial movement between sites (Walters et al., 2012). Their study tracked the movement of UPEC during UTI and the passage of invading UPEC into the bloodstream *in vivo* using a murine model. The results showed that some UPEC reached he bloodstream in less than four hours after inoculation of the bladder.

The current study aims to understand the population structure, virulence factor carriage, metabolic potential and antibiotic susceptibility of extraintestinal *E. coli* isolates selected for study on the basis of known bloodstream infection originating from urine or the biliary duct.

2.5.1 Multilocus sequence typing and phylogenetic analysis of UPEC and biliary sepsis strains

2.5.1.1 Urine and Blood isolates

MLST was carried out to understand the population structure of *E. coli* strains recovered from blood and urine cultures from single patients (paired isolates). Previous studies have described the population structure of UPEC by using MLST (Gibreel et al., 2012b; Lau et al., 2008b; Tartof et al., 2005). MLST is a trusted molecular epidemiological typing method that has been used to define the population structure of many pathogenic bacteria (Maiden et al., 1998).

The MLST scheme used in the current study was constructed by Achtman and colleagues (Wirth et al., 2006) and is the most widely adopted of the two schemes for *E. coli*. The targeted genes have been selected on the basis that they encode proteins that are under stabilizing selection for conservation of metabolic function (Maiden 2006), rather than rapidly evolving genes, which are subject to positive selection. This allows sufficient discriminatory power to differentiate alleles within the population.

The use of MLST in the current study indicated that the different pathogenic *E. coli* strains from the same patients were identical. This was not surprising because complicated UTI primarily occurs in an ascending manner, where the bacteria ascend through the bladder to the kidneys and potentially cause bacteraemia. A recent study by Walters and colleagues aimed to examine the population dynamics of UPEC and track certain lineages in a murine model (Walters et al., 2012). The study revealed that UPEC strains that are able to express certain factors, such as the ability to adhere to host cells, will support bacterial migration between the kidney and the bladder and resist the action of urine flow (Floyd et al., 2012). In contrast, movement of virulent UPEC strains from kidneys to the bloodstream appears to be a restriction point, which required certain characteristics to allow strains to successfully invade the bloodstream. These characteristics within strains with specific virulence traits promote bacterial invasion and colonization enhanced by adhesion factors such as the type 1 fimbrial FimH adhesin (Bien et al., 2012; Korhonen et al., 1986; Mulvey, 2002).

In addition, these strains should have efficient serum resistant mechanisms. O-antigens and K capsules are known to be important mechanisms for serum resistance (Phan et al., 2013). In chapter 3, it is demonstrated that O-antigen biosynthesis is an important virulence determinant that plays a significant role in the virulence of ST127 UPEC strains, which rapidly kill *Galleria mellonella* wax moth larvae (Alghoribi et al., 2014). Other factors have been reported to contribute to serum resistance, such as the major outer membrane protein OmpA (Weiser and Gotschlich, 1991), plasmid-encoded proteins like TraT (Moll et al., 1980) and the phage membrane protein Bor (Barondess and Beckwith, 1995).

MLST analysis of the examined populations (urine and blood strains) revealed a profile of STs that consisted of ST73 (30%) followed by ST95 (12%), ST69 (10%), ST131 (8%) and ST127 (4%). Previous epidemiological studies have determined these STs to be major lineages of UPEC, which have been reported globally as a cause of UTI and bloodstream infection associated with both community-onset and healthcare-associated infections (Doumith et al., 2015; Lau et al., 2008b; Riley, 2014). International UPEC population structure analyses have revealed the predominance of sequence types ST69, ST73, ST95, and ST131 among large collections of ExPEC from human infections (Adams-Sapper et al., 2013; Banerjee et al., 2013a; Gibreel et al., 2012b).

Members of the ST73 and ST127 lineages are epidemiologically associated with extraintestinal infection syndromes such as pyelonephritis (Johnson *et al*. 2008). It should be noted, however, that there has been little description of the properties and virulence of ST127 isolates, though this may be an emerging lineage of importance (Gibreel et al., 2012b).

As mentioned above, in chapter 3, it is demonstrated that ST127 strains have pronounced virulence in the *G. mellonella* model (Alghoribi et al., 2014), which may indicate a potential for aggressive infection in humans (Brennan et al., 2002; Champion et al., 2009; Mukherjee et al., 2010; Wand et al., 2013). The other STs observed are associated with important emerging antimicrobial resistance profiles, such as ST69 with trimethoprim/sulfamethoxazole resistance and ST131 with fluoroquinolone resistance and ESBL production (Weissman et al., 2012). Recent studies using whole genome sequencing of ST131 strains collected from different countries have demonstrated the high prevalence of ESBL production among ST131 strains (Petty et al., 2014; Price et al., 2013).

These and other studies have concluded that ST131 fluroquinolone resistant (FQ-R) strains carrying the *fimH30* allele, acquired the CTX-M-15 ESBL enzyme giving rise to the emergence of the *fimH30*-Rx, or Clade C, clone, which is globally disseminated and the cause of significant morbidity and mortality (Banerjee and Johnson, 2014; Petty et al., 2014; Price et al., 2013). Although FQ-R, CTX-M-15 positive ST131 isolates were seen in the strains examined here, the *fimH* locus was not sequenced. However, it is highly likely that these strains are of the Clade C lineage.

In addition, MLST allowed identification of three clones, namely ST10, ST167 and ST48, which are important *E. coli* lineages belonging to CC10 and are related to phylogenetic group A. Members of ST10 were reported previously as the most frequent isolates in the MLST database. These isolates can carry most of the ESBL types including CTX-M-14 and CTX-M-15 (Oteo et al., 2009).

2.5.2 Biliary sepsis isolates

Phylogenetic analysis of *E. coli* biliary sepsis strains has not been previously reported. MLST analysis revealed eight STs for the 27 biliary sepsis isolates examined in the current study. The predominant STs identified are of clones previously associated with UTI (Lau et al., 2008b); ST131 and ST69 were equally prevalent in the population (14.8%) followed by ST73 (7.4%).

ST131 clonal group was found to be a successful pathogen, able to invade host tissues and harbouring virulence genes leading to extraintestinal diseases (Rogers et al., 2011; Van Der Bij et al., 2011). Although *E. coli* ST131 isolates have been previously reported to be recovered mostly from urine and blood cultures (Johnson et al., 2010), four isolates have been recovered from bile cultures in the current study, representing the first report of this important pathogen in such samples. In addition, most of the STs that have been observed from bile cultures in this study are also been reported previously to cause UTI and bacteraemia. Only one recent study has reported ST847 causing infection in humans, but this was in a cystic fibrosis patient and was not associated with UTI or bloodstream infection (Barillova et al., 2014).

Other studies listed on the MLST database for the scheme used in this thesis (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) have reported ST847 to cause infection in animals including pigs, camels and birds (Guenther et al., 2012) suggesting ST847 strains may represent zoonotic pathogens. ST91 has been reported previously to cause bloody diarrhoea (Lu et al., 2006), which has relevance here, given the proximity of the bile duct to the gastrointestinal tract. No other studies have reported this ST to cause either UTI or bloodstream infections.

2.5.3 Virulence profiling

The virulence factor profiles of isolates from different STs illustrate the impact of one or several properties or virulence factors on the ability of *E. coli* strains to cause different clinical syndromes. All four recognised phylogenetic groups (A, B1, B2 and D) were detected among the examined isolates. Most of the virulence factors were associated with isolates from both B2 and D phylogenetic groups. Carriage of virulence factors was significantly more frequent in these phylogenetic groups, compared to groups A and B1 (Figure 2.4 and Figure 2.5). This is in line with numerous previous reports (Basu et al., 2013; Gibreel et al., 2012b; Johnson and Stell, 2000; Picard et al., 1999).

The virulence factor profiles of *E. coli* isolates recovered from urine and blood cultures of the same patient were identical, as may be expected. A study by Bergsten and others supports the notion that gene expression is down regulated in the bladder, which allows accumulation of mutations during long-term carriage and loss of virulence genes (Bergsten et al., 2005). The correlation of virulence gene carriage in the urine and blood paired isolates was not particularly surprising, given the immediacy of recovery, but longer-term studies may be warranted.

The mechanisms that enable *E. coli* strains to colonize and persist in the urinary tract and then progress to cause bloodstream infection are poorly understood. It would be interesting and of relevance to study the expression of virulence genes in the urine and sepsis isolates from individual patients to examine possible differences in expression of genes that may be up- or down-regulated in response to niche-specific environmental cues.

In addition, whole genome sequencing and examination of single nucleotide polymorphisms (SNPs) may be expected to reveal differences that would affect gene expression. However, similar work has previously demonstrated very little to no variation at the SNP level in a small collection of paired urosepsis isolates (McNally et al., 2013).

The siderophores ferric yersiniabactin (*fyaA*) and *iutA* were frequently observed among the *E. coli* isolates. These factors enable the bacterium to persist and multiply in environments with a limited concentration of free iron (Lee et al., 2010). A study by Johnson and Stell (2000) reported a high prevalence of *fyuA* among urosepsis *E. coli*, which is confirmed by the present study.

In addition, the presence of the *fyuA* gene (efficient iron uptake) in UPEC isolates is considered to be an essential factor for the bacterium to facilitate entry into the bloodstream, resulting in bacteraemia (Ananias and Yano, 2008).

Successful *E. coli* strains that cause bacteraemia need to possess effective virulence factors to mediate avoidance or disruption of the host immune defence mechanisms. Such virulence genes, including the capsular gene *kpsMTII* (52%) and serum resistance associated gene *traT* (70%), were frequently observed among the examined isolates.

As part of the pathogenic process, it is important for the bacteria to bind to and invade host cells. Invasive *E. coli* strains require virulence genes that can facilitate attachment to host cells and penetration of bladder epithelial cells to allow formation of intracellular biofilm-like communities (Rosen et al., 2007). Adhesion genes are commonly found among *E. coli* strains that cause UTI and are significantly associated with pyelonephritis (Johnson 1991).

The prevalence rate of adhesion genes among the examined isolates including *fimH* (100%), P fimbriae elements *papAH, papC, papEF* and *papG* II, III (over 50%) and allele II (46%) were considerably high, as would be expected for these key factors for successful invasive UPEC strains. These results were compared to previous studies that reported similar prevalence rates of different *pap* genes, which ranged from 50% up to 79% (Johnson et al., 2001; Leflon-Guibout et al., 2008). Host cell exfoliation has an impact on bacterial persistence, as the process disseminates bacteria within and outside the urinary tract. Exfoliation of mucosal uroepithelial cells provides an effective defence system to clear adherent and intracellular bacteria by producing focal adhesion-associated components like paxillin. A recent study by Dhakal and Mulvey illustrates that pore-forming toxin α-hemolysin (*hlyA*) produced by UPEC strains can play an important role in disruption of focal adhesion-associated components like paxillin. Such toxins not only affect the function of epithelial cells but also disable macrophages and suppress the inflammatory responses (Dhakal and Mulvey, 2012).

In the current study, the virulence factor profiles of the examined biliary sepsis isolates revealed a lower prevalence rate than was seen in urine and blood isolates, though the number of biliary isolates examined was limited and this may have reduced the power of any comparisons made. However, several individual virulence genes showed considerable differences, such as the *papG II,III* factor, which was detected in 63% of the urine and blood isolates but only observed in 19% of the biliary sepsis isolates.

A previous study by Wang and others highlighted the importance of the *papGII* gene, which may play a more central role in the development of bacteraemia in patients with upper UTI, than in those with bile duct infection (Wang et al., 2002). Adhesion-receptors for P fimbriae have been identified in humans that mediate binding to receptors presented on the uroepithelium (Leffler and Edén, 1980; Wilson, 2002). Taken together with the findings presented here, the differences in *pap* gene carriage may be an example of niche specialisation within strains of the same genetic background (i.e. ST).

The *traT* genes were found to be highly prevalent in the urine and blood isolates (70%) compared to those from biliary sepsis (37%). It is not yet clear why serum resistance (encoded by *traT*) would be more significant for UPEC than biliary sepsis pathogens, but the difference between the populations of bacteria was less marked for this gene than for other virulence determinants.

Further studies are required to compare VFs and investigate the expression of a variety of virulence genes in urine and blood models for strains from different STs. Quantitative monitoring of gene expression should be carried out immediately following collection of isolates from different niches. The advent of next generation sequencing technologies has created opportunities to rapidly and accurately quantify gene expression. Studying bacterial transcriptomes using RNA-seq has proven to be highly accurate and informative, which will refine our understanding of bacterial gene regulation (Croucher and Thomson, 2010). This may reveal the differential significance of genes in the various environments to which the organisms are exposed during the infectious process.

2.5.4 Antibiogram profiling

Analysis of the antimicrobial susceptibility of the examined populations showed a high prevalence of resistance to penicillin and trimethoprim antibiotics. However, ESBL producing isolates were observed among the urine, blood (8% n=8 isolates) and biliary sepsis (7% n=2 isolates), which all belonged to phylogenetic groups A or B2. A low prevalence of antibiotic resistance was observed within the most frequently detected STs, including ST73, ST95 and ST69. These findings are in line with those reported from a recent study carried out in the same geographic region (Gibreel et al., 2012b) and are as would be expected for the UK.

Members of ST95 (n=12 of the total isolates) were observed to have the lowest antibiotic resistance score (mean = 0.12) followed by ST14 (0.19), ST73 (0.2), ST127 (2.6) and ST69 (3.5) (Table 2.14). These STs were the most commonly detected in this study and accounted for more than 60% of the total urine and blood isolates examined. However, one pair of isolates belonging to ST167 had the highest resistance score (mean = 1.38). Thesis isolates were significantly associated with an ESBL phenotype and resistance to most of the tested antibiotics (Table 2.13).

ST167 has been reported in previous studies with broad resistance and was found to be more prevalent among ESBL-producing *E. coli* isolates recovered from animals (Guenther et al., 2012; Schink et al., 2013). A study by Zhang and others reported the first instance of $bla_{\text{NDM-1}}$ carriage in *E. coli* ST167 isolates and demonstrated coexistence of $bla_{\text{NDM-1}}$ and $bla_{\text{CMY-42}}$ in same isolate (Zhang et al., 2013).

These previous reports, combined with the broad resistance observed in the pair of isolates studied here indicate that ST167 may be an emerging clonal group, which warrants close monitoring and further study.

ST131 has been reported previously as the most globally disseminated multidrug resistant clonal group (Nicolas-Chanoine et al., 2008; Petty et al., 2014). In this study, ST131 was detected as a relatively in low proportion of the collection, but isolates had a significant association with multidrug resistance, so the isolates examined here do not appear to differ significantly in this respect from the reported literature. ST48 was also detected with low frequency, but with a high resistance score. ST48 is a member of CC10, the largest clonal complex of closely related ST in the MLST database.

The antimicrobial resistance profile of the biliary sepsis isolates in this study showed no significant difference compared to the urine and blood isolates (Figure 2.9). However, ST131 was the most commonly detected clone (14%) among the biliary sepsis collection. These biliary ST131 strains were generally susceptible to β-lactam antibiotics, but were significantly associated with fluoroquinolone resistance ($P \geq 0.04$) and a lack of ESBL production. The latter point is intriguing as fluoroquinolone resistant ST131 strains are commonly ESBL positive (Gibreel et al., 2012b; Petty et al., 2014). Interestingly, only two isolates exhibited an ESBL phenotype, and these belonged to phylogenetic group A (ST410 and ST617). Among the biliary sepsis isolates these strains were associated with the highest resistance score, compared to isolates of other STs in the same collection (Table 2.15).

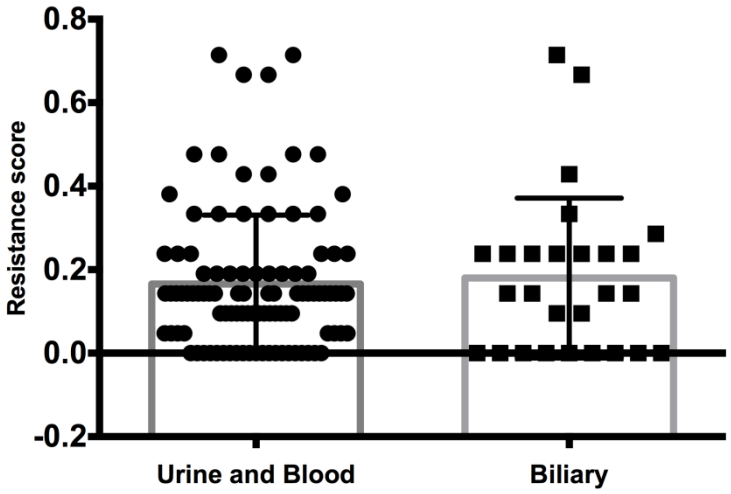

Figure 2.9 Comparison of the antimicrobial resistance score of the urine-blood isolates and biliary sepsis isolates. The analysis showed no significant difference between the groups.

ST410 is a member of CC23 and is classified in phylogroup A. It has been reported globally with various resistance traits and has been recovered from human, meat, water and animal samples (Egea et al., 2012; Ewers et al., 2012; Mavroidi et al., 2012a; Peirano et al., 2014a; Poirel et al., 2012). A study by Correia and others reported, for the first time, that ST410 isolates carried the *bla*CTX-M-14 gene (Correia et al., 2012).

However, NDM-1 producing ST410 was recovered from a urine sample of a critically ill patient and this was the first incidence of NDM-positive CPE in Poland (Fiett et al., 2014). ST410 was previously reported to cause both urinary tract and bloodstream infections that were associated with different antibiotic resistance patterns (Mushtaq et al., 2011; Park et al., 2014; Shin et al., 2011).

ST617 is also a member of largest complex, CC10. This clonal group has been reported in Nigeria as the predominant CTX-M-15-producing UPEC (Aibinu et al., 2012). ST617 has been reported to cause both urinary tract and bloodstream infections and is associated with fluoroquinolone and multidrug resistance (Giufrè et al., 2012).

The antimicrobial resistance profile of the biliary sepsis isolates showed a low prevalence of resistance among the examined isolates. Despite the low number of isolates in this collection, it was surprising to find the highest resistance score was among members of phylogroup A. Previous studies have described phylogroup A as a globally distributed clonal group, which infects urinary tract and non-urinary tract sites and highly is associated with trimethoprim-sulfamethoxazole resistance (Johnson et al., 2005).

A recent study reported the prevalence of phylogroup A *E. coli* causing non-urine, extraintestinal infections in a northern European country (Skjøt-Rasmussen et al., 2013). The study included a collection of blood and paired urine isolates from patients with bacteraemia of urinary tract origin. The finding of this study showed that 15% of the blood isolates belonged to phylogroup A.

2.5.5 Metabolic profiling

It has been suggested that ST131 UPEC in Northwest England are more metabolically diverse than other strains from the same area (Gibreel et al., 2012a). This hypothesis was contested in a study of UPEC from a different region of the UK, but some of the justification for the counter claims was based on analysis of a very small number of strains selected to represent each ST, which undermines the validity of these claims (Alqasim et al., 2014).

In current study, we have examined activity of the biliary sepsis and paired urosepsis isolates in 47 metabolic reactions. These reactions are used conventionally in the VITEK 2.0 system for the classification of bacteria into families and species. Biochemical reactions have long been used as typing methods within species, based on the variability and reproducibility of these reactions (Katouli et al., 1990).

In 1985, biochemical profiling was used to epidemiologically characterise *E. coli* isolates and it was reported that the kinetics of 24 biochemical reactions could be used to create a distinctive fingerprint for *E. coli* strains (Kühn, 1985). Recent advancements in technology have facilitated development of automated biochemical testing systems. The VITEK 2.0 compact system has been used in several studies to determine the biochemical activity profiles, generating reliable and reproducible results (Funke et al., 1998; Gavin et al., 2002; Gibreel et al., 2012a).

On the whole, urine, blood and biliary sepsis isolates in this study showed similar biochemical profiles to those previously reported for UPEC and the general *E. coli* population (Farmer et al., 1985; Gibreel, 2011).

However, comparison between these collections showed some notable differences for several biochemical tests, as shown in Table 2.17. Low incidence of the ELLM test was observed in urine, blood (14%) and biliary sepsis (15%) compared 96.7% of the UPEC population reported by (Gibreel, 2011). A previous study has used various common isolation media to evaluate the automated VITEK 2.0 identification GN card, which revealed extreme variability in ELLM reactions obtained from different media (Lowe et al., 2006) and this may explain difference between studies.

Other metabolic reactions were observed to be high in urine, blood and biliary sepsis populations compared to other UPEC collections, such as alkalinization of lactate and succinate (Table 2.17). The elevated incidence of alkalinization of lactate and succinate might occur in bacteria to relieve acid stress exerted by amino acid metabolism (Gibreel et al., 2012a).

Less pronounced differences in biochemical activity were also observed for alpha-galactosidase for the urine, blood (96%) and biliary sepsis (81%) isolates compared to other UPEC (69%) populations. However, alpha-galactosidase activities in urine and blood (96%) were almost identical to those for the general *E. coli* population (99%). The reason of these different activities in the α-galactosidase assay among the examined populations is not clear. The metabolic activity analysis of the urine and blood isolates revealed that nine pairs of isolates (18% of the total isolates) showed non-identical metabolic profiles, as described in Table 2.16. The non-identical metabolic profiles of these paired isolates indicate that the UPEC strains are modulating biochemical activity to potentially suit different niches (Berg et al., 2002).

The significance of these discrepancies cannot be determined at this point, but this is a particularly intriguing finding that justified further study of the expression of these and other genes in model systems, perhaps with analysis of gene expression using RNAseq based approaches.

In conclusion, the work described in this study aims to reveal the relative importance of each *E. coli* from different STs recovered from various infection sites (UTI, bloodstream infection and biliary sepsis) and expose the virulence potential that enables these pathogenic bacteria to persist and cause infection.

Comparison of the results for the two collections of isolates points towards importance of certain adhesins, P fimbrae for example, in determining the ability to cause bloodstream infection via the UTI route rather than following biliary infection. It should be remembered though, that differences in metabolic capacity might influence survival in the GIT that have an impact on progression to UTI or colonization and infection in the biliary tract.

Obtaining a clear picture of the relative pathogenicity of isolates of key STs will be important for ongoing surveillance of these important clones and allow early identification of emerging clones. Examining the virulence traits of the different organisms may allow development of rapid prognostic assays that can be used in clinical management of patients to improve recovery rates for these serious, life-threatening infections.

Chapter Three

Published as -

Alghoribi MF, Gibreel TM, Dodgson AR, Beatson SA, Upton M. *Galleria mellonella* infection model demonstrates high lethality of ST69 and ST127 uropathogenic *E. coli*. *PLoS One* 2014; 9: e101547.

3 *Galleria mellonella* infection model demonstrates high lethality of ST69 and ST127 uropathogenic *E. coli*

3.1 Abstract

Galleria mellonella larvae are an alternative *in vivo* model for investigating bacterial pathogenicity. Here, we examined the pathogenicity of 71 isolates from five leading uropathogenic *E. coli* (UPEC) lineages using *G. mellonella* larvae. Larvae were challenged with a range of inoculum doses to determine the 50% lethal dose (LD_{50}) and for analysis of survival outcome using Kaplan-Meier plots. Virulence was correlated with carriage of a panel of 29 virulence factors (VF). Larvae inoculated with ST69 and ST127 isolates (10^4 colony-forming units/larvae) showed significantly higher mortality rates than those infected with ST73, ST95 and ST131 isolates, killing 50% of the larvae within 24 hours. Interestingly, ST131 isolates were the least virulent. We observed that ST127 isolates are significantly associated with a higher VF-score than isolates of all other STs tested ($P \leq 0.0001$), including ST69 ($P<0.02$), but one ST127 isolate (strain EC18) was avirulent. Comparative genomic analyses with virulent ST127 strains revealed an IS1 mediated deletion in the O-antigen cluster in strain EC18, which is likely to explain the lack of virulence in the larvae infection model. Virulence in the larvae was not correlated with serotype or phylogenetic group.

This study illustrates that *G. mellonella* are an excellent tool for investigation of the virulence of UPEC strains. The findings also support our suggestion that the incidence of ST127 strains should be monitored, as these isolates have not yet been widely reported, but they clearly have a pathogenic potential greater than that of more widely recognised clones, including ST73, ST95 or ST131.

3.2 Introduction

Escherichia coli is the major cause of extraintestinal infections including urinary tract infection (UTI), Gram-negative bacteraemia and neonatal meningitis. Uropathogenic *E. coli* (UPEC) are the most frequent cause of UTI, being responsible for up to 85% of community acquired UTI and 40% of nosocomial UTI (Berry et al., 2009; Ronald, 2003; Tartof et al., 2005).

Multilocus sequence typing (MLST) is the current method used to investigate the genetic differences between isolates of UPEC. This method has been used to good effect to identify UPEC as well as other important pathogenic *E. coli* (Bengtsson et al., 2012; Gibreel et al., 2012b; Lau et al., 2008b; Tartof et al., 2005). Our own work and that of others has highlighted the importance of several leading lineages of UPEC (e.g. Sequence Type 69 (ST69), ST73, ST95, ST127 and ST131) and we have recently suggested that ST127 is a newly evolved clone, with particularly high virulence potential (Gibreel et al., 2012b). Numerous other recent studies have highlighted the virulence and antimicrobial resistance of members of these clones (Banerjee et al., 2013a; Bengtsson et al., 2012; Croxall et al., 2011; Johnson et al., 2008; Nicolas-Chanoine et al., 2008).

Based on PCR surveillance of virulence factors, UPEC have been shown to possess multiple virulence-associated determinants that include diverse adhesins, toxins, siderophores, capsule variants and other miscellaneous traits (Johnson and Stell, 2000; Johnson, 1991; Kaper et al., 2004; Lloyd et al., 2007).

Although a great deal of research effort has been devoted to understanding UPEC virulence mechanisms, much remains for further investigation and animal models of UTI are resource intensive.

The larvae of the wax moth *Galleria mellonella* have been used as an infection model to describe and evaluate microbial pathogenicity for a number of bacterial pathogens, including enteropathogenic *E. coli* (EPEC) (Leuko and Raivio, 2012; Mukherjee et al., 2010; Mylonakis et al., 2005; Olsen et al.; Wand et al., 2013). The virulence mechanisms of many pathogens in *G. mellonella* show a high degree of similarity to mammals, including humans (Champion et al., 2009; Wand et al., 2011). Previous studies have shown a strong and positive correlation of virulence of different pathogens between mouse infection systems and *G. mellonella* (Brennan et al., 2002; Cotter et al., 2000; Jander et al., 2000). In this study, *G. mellonella* larvae were used as an *in vivo* model to investigate the virulence of the major lineages of UPEC.

3.3 Material And Methods

3.3.1 Bacterial strains

A total of 71 non-duplicate isolates of *E. coli* from patients with UTI were examined in this study. The patients included those presenting in the community and nosocomial infections. All isolates were recovered at clinical bacteriology laboratories at Central Manchester Foundation Trust, Preston Royal Hospital and the Mid Yorkshire Hospital Trust, Wakefield, between 2007 and 2011. The MLST and virulence typing of 57 of these isolates has been previously described (Gibreel et al., 2012b).

The isolates were selected on the basis of assignment to the major lineages of UPEC, as determined by using the Achtmann MLST scheme, and were from ST69 (n=11), ST73 (n=20), ST95 (n=10), ST127 (n=10) and ST131 (n=20), using previous methods (Lau et al., 2008b).

PCR based detection of 29 uropathogen associated virulence factors (previously defined by Johnson et al., 2000 (Johnson and Stell, 2000)) was carried out for each of the examined isolates.

3.3.2 Phylogenetic grouping

Phylogenetic grouping was determined by triplex PCR reaction targeting three DNA markers (*chuA, yjaA* and TSPE4.C2), as described previously by Clermont and colleagues (Clermont et al., 2000).

3.3.3 Serotyping

Molecular serotyping was performed on all the isolates using a multiplex PCR method to detect 14 *Escherichia coli* serogroups associated with UTI (O1, O2, O4, O6, O7, O8, O15, O16, O18, O21, O22, O25, O75 and O83), as described previously (Li et al., 2010). Isolates that were not able to be typed using this method (i.e. gave negative results with all primer pairs) were classified as nontypable (nt).

3.3.4 Identification of LD_{50} in *G. mellonella* larvae infection

Larvae of the Greater Wax Moth, *G. mellonella* (GM) were obtained from Live Foods Ltd (Rooks Bridge, UK). Larvae were stored in the dark and used within 10 days of receipt. Larvae were selected to be 15-25mm in length, having a cream colour with minimal speckling and no grey markings. To prepare UPEC inoculum, strains were grown in nutrient broth overnight at 37^{o}C and collected by centrifugation at 13,000 × *g* for two minutes.

The cell suspensions were normalised using optical density (OD_{600}) and the colony forming units (cfu/ml) were confirmed by viable count assay.

A minimum of three biological replicates of 10 larvae were injected per serial dilution of UPEC (10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 cfu/10µl) using a Hamilton syringe (26S gauge, 50 µl capacity). Larvae were then incubated at 37°C in the dark and the dilution that killed 50% of the larvae (LD_{50}) for each replicate was determined after 24 hours. Larvae were monitored for an additional 120 hours and survival outcome was determined; larvae were considered dead when no response was observed following touch.

In addition, 10 larvae were injected with non-pathogenic *E. coli* DH5α to evaluate whether *G. mellonella* larvae were killed by non-infection related reactions and a control inoculation (n=10) was performed with 10µl of PBS to measure any lethal effects due to physical injury. An additional control group (n=10) had no manipulation.

Survival analysis and statistical significance were determined using the log-rank test and the Kaplan–Meier survival curves were plotted using SPSS v.20. The LD_{50} was calculated using probit regression model implemented in SPSS v.20 at a significance level of P=0.05.

3.3.5 Assessment of virulence of UPEC strains *in vivo*

Having identified the LD_{50} for each isolate, we investigated the virulence of each strain over 120hrs to assess the utility of the model for comparison of virulence of the UPEC strains. Larvae were inoculated with a dose corresponding to the LD_{50} and survival followed over 120hrs, as described above.

3.3.6 Correlation analyses

In order to investigate correlations between the LD_{50} and virulence profiles of the isolates from the five STs, data were examined by using the Kruskal-Wallis test, followed by pairwise analysis of differences performing Mann–Whitney U-tests in Prism v.6 (www.graphpad.com/).

The LD_{50} was taken to be a continuous variable and the values for all isolates were used to divide them into three groups; low LD_{50} (~10^2-10^3 cfu/10µl, the volume initially used to inoculate the larvae), medium LD_{50} (~10^4-10^5 cfu/10µl) and high LD_{50} (~10^5-10^7 cfu/10µl).

Virulence factors were correlated with LD_{50} using Prism v.6 by fitting Pearson's correlation coefficients between the three LD_{50} groups and virulence factors. The correlation was used to describe the virulence factors that are associated with each group.

3.3.7 Genome sequencing and annotation of *E. coli* EC18 and EC41

Genomic DNA of ST127 strains EC18 and EC41 (virulent and virulent in GM larvae, respectively) was sequenced using Illumina MiSeq by the Centre for Genomic Research, University of Liverpool. Velvet 1.2.10 software (Zerbino and Birney, 2008) was used to assemble sequence reads of both genomes into contigs. For each strain (EC18 and EC41), a total of 6,666,026 and 4,165,821 sequence reads were assembled into 149 and 178 contigs greater than 200 bp in length with and average depth of coverage of 196.71 and 114.92, respectively. Contigs were ordered according to the complete genome of UPEC ST127 strain 536 **(**Accession ref |NC_008253; (Hochhut et al., 2006)) and annotated using Prokka 1.7 (*Prokka: Prokaryotic Genome Annotation System - http://vicbioinformatics.com/*)**.** The Illumina sequence reads are available under the Bioproject PRJEB6308/ERP005824 (Accession Numbers: EC18, ERS497039; EC41, ERS497040). Comparison and visualization of UPEC genomes were carried out using BLAST (Altschul et al., 1990), Artemis comparison tool (ACT) (Carver et al., 2005), BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011), Easyfig (Sullivan et al., 2011) and Tablet (Milne et al., 2013).

3.3.8 Gap Region Amplification and Sequencing of the O-antigen deletion region in EC18

Primers were design to target the gap of the O-antigen deletion region in EC18 strains (gap-F 5′-TCA AGC ACC GAA TAA CCT -3′) and (gap-R 5′-TAC CTG AAG TAC GTA GCC-3′). The primers were designed based on the sequence of the two contigs adjacent to the O-antigen deletion region, for which no direct linkage information was available from the genome sequence.

As the size of the expected product was not immediately clear from the genome assembly alone, long-range PCR was performed using Q5® High-Fidelity 2X Master Mix (New England Biolabs). PCR was performed as follows; 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 52°C for 30 sec and 72°C for 30 sec, and a final extension at 72°C for 2 min. Electrophoresis in a 1% agarose gel was used to determine the size of the PCR product by running with Quick-Load® 1 kb Extend DNA Ladder (New England Biolabs). The DNA sequence of the amplified product was determined by Sanger sequencing at the Plymouth University Systems Biology Centre using an Applied Biosystems 3130 Genetic Analyzer. Sequence data were assembled using CLC Main Workbench v.6.9.1.

3.3.9 Phylogenetic grouping

Phylogenetic analysis of the 71 strains examined indicated that they belonged to groups B2 (51%), D (48%) and B1 (1%). All ST69 strains belonged to phylogenetic group D, ST73 to both group D (13 strains) and B2 (7 strains), ST95 to both group D (4 strains) and B2 (6 strains), ST127 belonged to groups D (4 strains) and B2 (6 strains), and ST131 belonged to groups D (2 strains), B2 (17 strains) and B1 (1 strain) (Table 3.1).

3.3.10 Serotyping

Serotyping of the 71 strains indicated that they belonged to several serogroups with O6 being the most prevalent (32%) followed by O25 (25%), O15 (13%), O2 (7%), O16 (4%), O18 (3%), O22 (1%); 14% were classified as nt (Table 3.1). Serogroup O6 was seen for all of the ST127 strains, except EC18, which was nt. ST73 (9 strains) and ST95 (5 strains) were also O6. Fifteen strains of ST131 belonged to O25, with the remainder (3 strains) belonging to O16. O25 was also observed for ST73 (2 strains) and ST69 (1 strain). Most of the ST69 strains (n=6) and 3 ST95 strains were O15. Nontypable strains were observed for all STs (Table 3.1).

UPEC from ST69 and ST127 are significantly more pathogenic towards *G. mellonella* than those from other STs

The larvae were injected with a range of inoculum doses to determine the mortality rates of each isolate. An example of the Kaplan-Meier survival analysis for 2.87×10^4 cfu/10µl of a strain from each ST investigated is shown in Figure 3.12. The survival outcome of the tested UPEC clones varied, where ST69 and ST127 showed high mortality rates compared to ST73 ($P\leq 0.248$), ST95 ($P\leq 0.303$) and ST131 ($P\leq 0.054$). Isolates of ST131 were observed to be the least virulent in this model. Larvae injected with non-pathogenic *E. coli* DH5α, showed no sign of stress, with an insignificant level of larval death recorded, indicating that larval death is related to overt pathogenic mechanisms in the UPEC strains. However, an interesting result to emerge from the data was the observation that one of the ST127 isolates (strain EC18) did not show any lethal effects, even for high inoculum doses (up to 2.33×10^7 cfu/10µl).

Table 3.1 Serotyping, Phylo-grouping, median lethal value LD50 and virulence factor of each strain* examined from the five UPEC lineages

ST69					ST73					ST95				
Isolate no	**Serotyping**	**Phylo-grouping**	**LD_{50}**	**VF Score**	**Isolate no**	**Serotyping**	**Phylo-grouping**	**LD_{50}**	**VF Score**	**Isolate no**	**Serotyping**	**Phylo-grouping**	**LD_{50}**	**VF Score**
73	nt	D	2.47E+03	6	21U	O6	D	1.90E+04	17	28U	nt	D	1.60E+04	12
23	nt	D	2.78E+03	10	217	O2	B2	2.39E+04	12	64	O15	B2	4.15E+04	13
48U	nt	D	6.80E+03	9	B54	O6	D	2.47E+04	11	9U	nt	B2	4.18E+04	14
83	O15	D	7.97E+03	9	12	O2	B2	3.40E+04	12	37	O15	D	7.46E+04	8
110	O15	D	9.62E+03	9	B84	O2	D	4.12E+04	12	159	O6	B2	8.68E+04	11
135	O15	D	1.59E+04	11	20U	O6	D	4.22E+04	17	33	O15	D	1.21E+05	11
20	O15	D	1.75E+04	9	B60	O25	D	4.71E+04	13	3	O6	B2	1.52E+05	13
119	O15	D	2.53E+04	5	9	O6	B2	5.46E+04	16	55	O6	B2	1.88E+05	12
84	O15	D	2.87E+04	11	B51	O2	D	5.64E+04	16	22	O6	B2	2.04E+05	10
17U	nt	D	1.96E+05	12	45U	O6	B2	6.48E+04	15	50	O6	D	2.29E+05	9
94	O25	D	2.08E+07	6	44	O2	D	7.53E+04	13					
					T15	O6	B2	8.11E+04	8					
					26U	O6	D	8.48E+04	6					
					23U	O6	D	9.80E+04	13					
					24	nt	D	1.01E+05	10					
					43U	O6	B2	1.30E+05	15					
					B47	O25	D	1.88E+05	9					
					25U	O22	D	2.97E+05	10					
					207	O18	B2	2.87E+06	6					
					15U	O18	D	1.80E+07	7					
Average			**1.92E+06**	**8.82**	**Average**			**1.12E+06**	**11.90**	**Average**			**1.15E+05**	**11.30**
Median			**1.59E+04**	**9**	**Median**			**7.01E+04**	**12**	**Median**			**1.04E+05**	**11.50**

* - Isolates have been arranged in rank order based on LD50 value

Table 3.1 Serotyping, Phylo-grouping, median lethal value LD50 and virulence factor of each strain* examined from the five UPEC lineages (continued)

ST127					ST131				
Isolate no	**Serotyping**	**Phylo-grouping**	**LD_{50}**	**VF Score**	**Isolate no**	**Serotyping**	**Phylo-grouping**	**LD_{50}**	**VF Score**
18	nt	B2	Not lethal	13	241	O25	B2	1.48E+04	8
112	O6	B2	8.24E+02	14	14	O16	B2	2.70E+04	12
B53	O6	D	2.56E+03	16	208	O25	B2	2.85E+04	8
B24	O6	D	8.44E+03	17	19	nt	B2	3.47E+04	6
72	O6	B2	9.71E+03	12	25	O25	B2	7.91E+04	7
B67	O6	D	1.17E+04	9	164	O25	B2	8.14E+04	5
224	O6	B2	1.27E+04	17	52	O16	B2	8.93E+04	6
B72	O6	D	1.78E+04	11	160	O25	B2	9.33E+04	5
41	O6	B2	6.50E+04	12	75	O25	B2	1.30E+05	6
21	O6	B2	4.61E+05	14	183	O25	B2	1.36E+05	6
					56	O25	B1	1.41E+05	5
					186	O25	D	1.56E+05	6
					184	O25	B2	1.87E+05	7
					230	O25	B2	2.05E+05	6
					2	O25	D	8.55E+05	7
					53	O25	B2	1.38E+06	8
					240	O25	B2	1.49E+06	9
					124	O25	B2	2.64E+06	5
					205	O16	B2	2.96E+06	4
					163	nt	B2	2.26E+07	6
Average			**6.55E+04**	**13.50**	**Average**			**1.67E+06**	**6.6**
Median			**1.17E+04**	**13.50**	**Median**			**1.39E+05**	**6**

* - Isolates have been arranged in rank order based on LD50 value

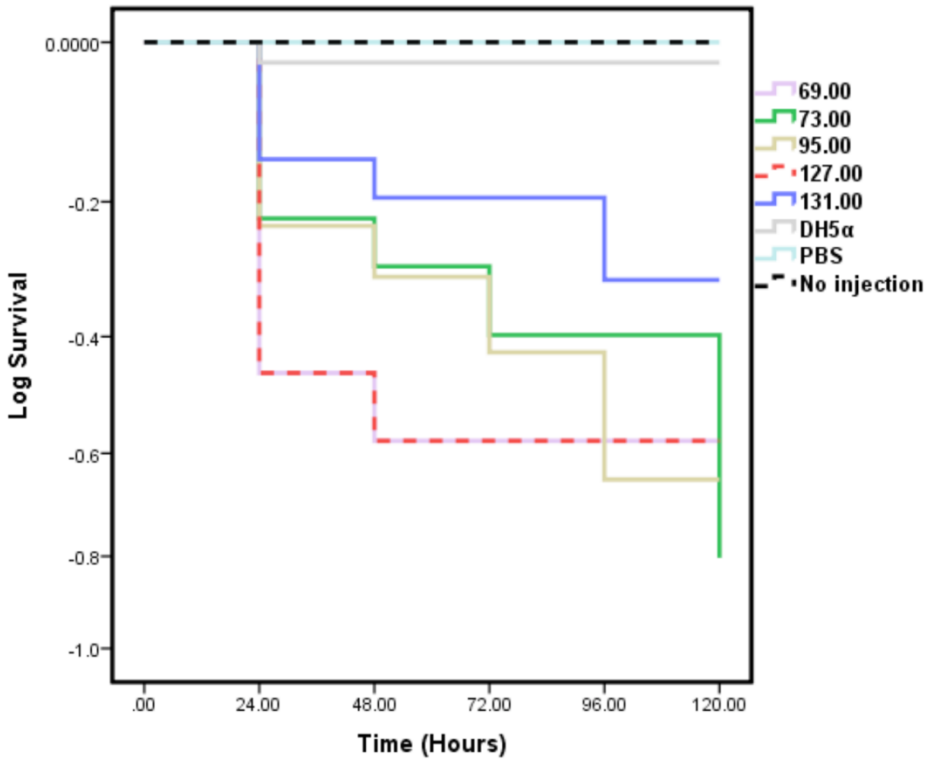

Figure 3.1 Kaplan-Meier survival analysis of *G. mellonella* larvae following injection of UPEC cells (2.87×10^6 cfu/ml) of different sequence types (ST). Data presented are the mean of three independent assays with each UPEC isolate. Low larvae mortality was recorded following injection of DH5α. Non-injected larvae and PBS injected larvae showed no mortality. Survival outcome of larvae injected with ST69 and ST127 isolates recorded the highest mortality compared to ST73 ($P\leq 0.248$), ST95 ($P\leq 0.303$) and ST131 ($P\leq 0.054$).

Lethality tests conducted to investigate LD_{50} confirmed that isolates of ST69 and ST127 were significantly more virulent than those of the other STs tested (Table 3.2). The median LD_{50} for ST69 and ST127 isolates was 1.59×10^4 cfu/10µl ($P\leq 0.0021$) and 1.17×10^4 cfu/10µl ($P\leq 0.047$), respectively (Figure 3.2 A). Isolate EC18 was not included in statistical calculations, as it had no recordable LD_{50} value.

Table 3.2 Statistical analysis indicates the significant difference in carriage of virulence factors (based on a PCR survey) and lethal effects (Low LD_{50}) with larvae inoculated with ST69 and ST127 strains

STs	ST69		ST127	
	VF (*P* value)	LD_{50} (*P* value)	VF (*P* value)	LD_{50} (*P* value)
ST69	-	-	0.02	0.401
ST73	0.0124	0.0023	0.6985	0.0024
ST95	0.0185	0.0079	0.3653	0.0051
ST127	0.02	0.401	-	-
ST131	0.0115	0.0021	0.0001	0.0004

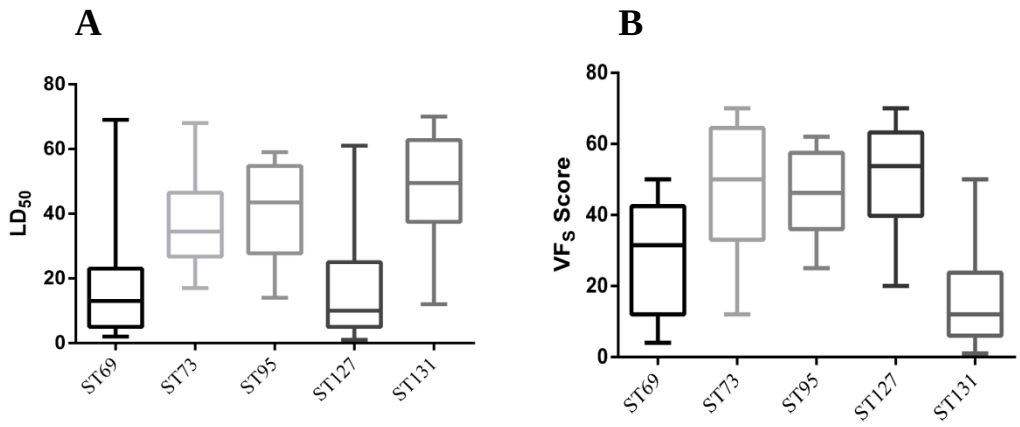

Figure 3.2 Correlation analysis between the five UPEC sequences types: (A) Low LD_{50} shows significant lethal effects with larvae inoculated with ST69 and ST127 strains. (B) ST127 shows high virulence capacity compared to that of other STs and ST131 shows relatively low virulence capacity.

3.3.11 Deletion of O-antigen gene cluster in UPEC strain EC18 results in avirulence in *G. mellonella*

Comparative genomic analysis was carried between strains EC18, EC41 and 536, the only completely sequenced ST127 strain available in the public databases (Hochhut et al., 2006). This analysis indicated an insertion-sequence (IS1) mediated deletion (from *glaE* to *wcaA*) within the O-antigen gene cluster in strain EC18. UPEC 536 is a model UPEC strain used for studies on ExPEC pathogenesis and belongs to serogroup O6 (Johnson et al., 2008). The O-antigen gene cluster, which is involved in the synthesis of the O-antigen, is encoded by the constituent genes *galF* to *gnd* (DebRoy et al., 2011; Reyes et al., 2012; Samuel and Reeves, 2003). In EC18, there is a deletion of the majority of the O-antigen gene cluster, which results in a contig break in the assembly and sequence evidence of an insertion sequence (IS1) at the position where the deletion occurs (Figure 3.3). In contrast the O-antigen region was completely assembled into a single contig in the EC41 assembly (Figure 3.3). To ensure that the missing EC18 O-antigen genes were not encoded elsewhere in the EC18 assembly we carried out a whole genome comparison between the 536 complete genome and the EC18 and EC41 draft genomes (data not shown). Furthermore, BLASTn and BLASTp comparisons with 95 O-antigen sequences, as described by DebRoy and colleagues (DebRoy et al., 2011), showed that none of the known O-antigen regions were present in the EC18 assembly.

Due to the short insert size of Illumina paired-end sequence data it was not immediately clear from the draft genome assembly of EC18 if there was a single IS1 sequence, or if two or more IS1 along with intervening sequence were arranged in tandem within the O-antigen region. PCR and subsequent Sanger end-sequencing of the product confirmed the presence of a single IS1 sequence replacing the O-antigen region in EC18 from *galE* to the 3' end of *wzc*, inclusive (Figure 3.3).

The IS1 sequence in EC18 is 100% identical to an IS1 annotated in *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. T000240 (dbj|AP011957). The closest match in the ISfinder database is 96% nucleotide identity to IS1X2 from *E. vulneris* ATCC29943 (gb|Z11605).

Comparison of the virulence genes carried by ST127 strains, using a PCR survey, indicated no other missing gene targets that could clearly explain the difference in virulence between EC18 and other strains (Figure 3.3).

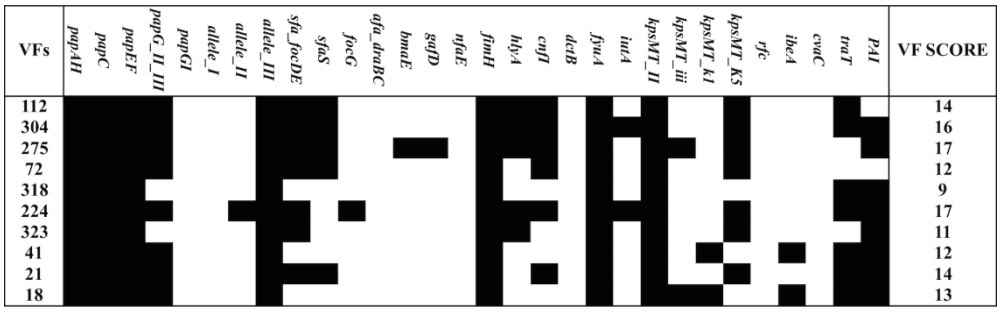

Figure 3.3 Virulence profile of ST127 strains based on PCR detection of 29 uropathogen associated virulence factors. EC18 (avirulent strain) shows a similar profile compared to other ST127 virulent strains. Black blocks represent positive PCR results and strain numbers are in the left hand column.

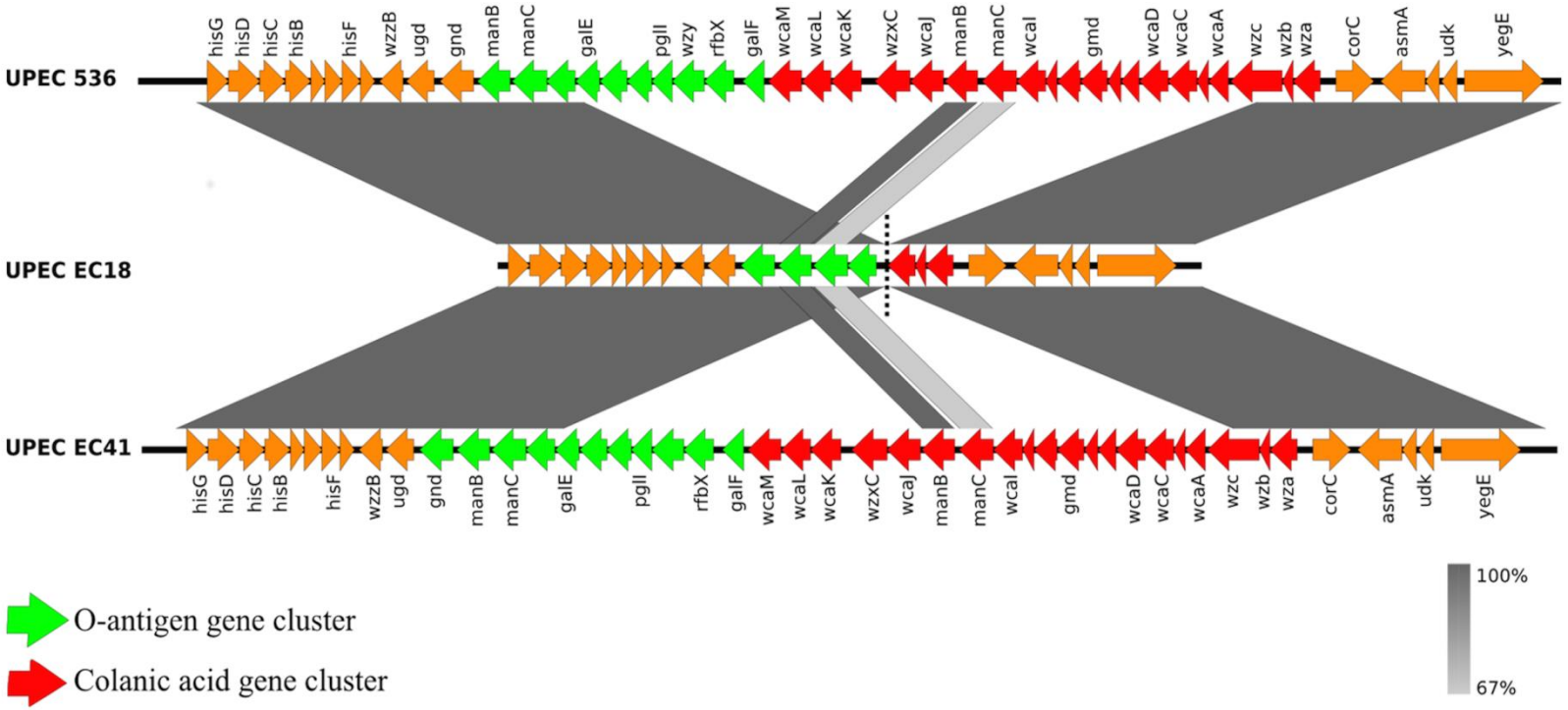

Figure 3.4 BLASTn comparison of O-antigen gene cluster (green) and colanic acid gene cluster (red) from UPEC ST127 strains 536, EC18 and EC41. The gray shading indicates high nucleotide identity between the sequences (99–100%). In EC18, there is an insertion-sequence (IS1) mediated deletion of most of the O-antigen gene cluster and the colanic acid gene cluster (vertical dotted line denotes contig boundaries in EC18). Figure was prepared using Easyfig (Sullivan et al., 2011).

Table 3.3 Prevalence of various UPEC associated virulence factors in the strains studied

Category	Specific VF	Prevalence (%) of VF by sequence type					
		Total no. 71	ST69 (n=11)	ST73 (n=20)	ST95 (n=10)	ST127 (n=10)	ST131 (n=20)
Adhesion	*papA*	41 (58)	8 (73)	13 (65)	9 (90)	10 (100)	1 (5)
	papC	41 (58)	7 (64)	14 (70)	10 (100)	10 (100)	0
	papEF	40 (56)	9 (82)	13 (65)	7 (70)	10 (100)	1 (5)
	papGII,III	42 (59)	7 (64)	16 (80)	10 (100)	8 (80)	1 (5)
	allele-II	27 (38)	6 (55)	11 (55)	9 (90)	1 (10)	0
	allele-III	15 (21)	0	4 (20)	0	10 (100)	1 (5)
	sfa/foc DE	20 (28)	0	13 (65)	0	7 (70)	0
	afa/draBC	10 (14)	0	0	0	0	10 (50)
	sfaS	7 (10)	0	2 (10)	0	5 (50)	0
	focG	12 (17)	0	10 (50)	1 (10)	1 (10)	0
	bmaE	2 (3)	1 (9)	0	0	1 (10)	0
	gafD	2 (3)	1 (9)	0	0	1 (10)	0
Toxins	*hlyA*	21 (30)	0	15 (75)	0	5 (50)	1 (5)
	cnf1	19 (27)	0	12 (60)	0	6 (60)	1 (5)
	cdtB	5 (7)	0	4 (20)	1 (10)	0	0
Siderophore	*fyuA*	69 (97)	10 (91)	19 (95)	10 (100)	10 (100)	20 (100)
	iutA	49 (69)	8 (73)	16 (80)	5 (50)	2 (20)	18 (90)
Capsule	*kpsM II*	49 (69)	6 (55)	12 (60)	10 (100)	10 (100)	11 (55)
	kpsM III	6 (8)	3 (27)	1 (5)	0	2 (20)	0
	K1	16 (25)	2 (18)	5 (25)	8 (80)	1 (10)	0
	K5	31 (44)	4 (36)	6 (30)	2 (20)	8 (80)	11 (55)
Miscellaneous	*cvaC*	6 (8)	1 (9)	0	5 (50)	0	0
	ibeA	7 (10)	0	2 (10)	0	2 (20)	3 (15)
	traT	54 (76)	11 (100)	12 (60)	9 (90)	7 (70)	15 (75)
	PAI	53 (75)	2 (18)	18 (90)	7 (70)	8 (80)	18 (90)

In order to assess the correlation between the LD_{50} and carriage of specific virulence factors, the continuous variable of LD_{50} was divided into three groups: low, medium and high. The medium and the high LD_{50} groups varied, in terms of constituent STs, but the low LD_{50} (i.e. high virulence) group contained only isolates belonging to ST69 and ST127 (Table 3.1). It should be stated that not all members of these STs exhibited the same LD_{50} values: in ST69 there were isolates of high (n=1), medium (n=5) and low (n=5) LD_{50}; in ST127 these values were 2 (including EC18), 5 and 3, respectively.

Importantly, a low LD_{50} was seen to have a significant positive association with several virulence factors; *papAH, papC, papEF, sfaS, bmaE, gafD* and *kpsMTIII* (Table 3.4). In contrast, the pathogenicity island marker gene (*PAI*) was negatively associated with a low LD_{50}.

Table 3.4 Association of LD_{50} with selected virulence factor

LD_{50} category	Number of isolates	Number of isolates carrying selected virulence factors							
		papAH	*papC*	*papEF*	*sfaS*	*bmaE*	*gafD*	*kpsMTIII*	*PAI*
Low LD_{50}	8	7	7	7	3	2	2	3	3
P value		0.0436	0.0436	0.0355	0.0001	<0.0001	<0.0001	0.0036	0.0019
Medium LD_{50}	51	32	32	30	3	0	0	2	40
P value		—	—	—	0.0101	0.0269	0.0269	0.0379	
High LD_{50}	12	2	2	3	1	0	0	1	10
P value		0.0013	0.0013	0.016	—	—	—	—	—

Underlining indicates a negative association.

Isolates of ST69 were not significantly associated with carriage of a high number of VFs (Figure 3.2 B). The mechanism underlying the pronounced virulence of ST69 isolates in this model has not yet been determined. Previous work has correlated growth rate with lethality in *G. mellonella,* where mortality was associated with proliferation of *Burkholderia species, Klebsiella pneumoniae, Staphylococcus aureus* or *Streptococcus pneumoniae* within the larvae (Desbois and Coote, 2012; Evans and Rozen, 2012; Wand et al., 2013, 2011). However, we investigated growth rate in LB medium and the ST69 isolates we have examined here were in fact seen to have the slowest growth rate, or longest doubling time (data not shown).

3.4 DISCUSSION

UPEC are a major cause of UTI and the severity of the infection is due to the contribution of many virulence factors including adhesins, toxins, siderophores and capsule. The diversity of the virulence factors enable UPEC to escape host immune responses and persist to cause infection (Kaper et al., 2004; Lloyd et al., 2007). In the current study, *G. mellonella* larvae were used as an *in vivo* model to investigate the virulence of UPEC from the leading lineages known to cause UTI. Previous studies indicate that the *G. mellonella* model is a powerful tool to investigate the virulence of a range of bacterial and fungal pathogens (Desbois and Coote, 2012; Kavanagh and Reeves, 2004; Peleg et al., 2009). Of most relevance to the current study is the work of Leuko and colleagues, who demonstrated that pathogenicity of EPEC could be dissected using *G. mellonella* larvae, and that *E. coli* K12 was non-pathogenic (Leuko and Raivio, 2012). The innate immune systems of insects such as *G. mellonella* display a high degree of similarity to the mammalian immune systems, which make *G. mellonella* an attractive alternative to animal models for investigation of pathogenicity (Kavanagh and Reeves, 2004; Ratcliffe, 1985; Salzet, 2001; Wand et al., 2013). Plasmatocytes and granulocytes have been identified in *G. mellonella* as types of haemocytes that are involved in phagocytosis, encapsulation and nodule formation, which are important elements in the defence against pathogenic bacteria (Walters and Ratcliffe, 1983), as suggested for EPEC (Leuko and Raivio, 2012). In addition, larvae of *G. mellonella* are large enough to allow easy handling, inexpensive to purchase and, being invertebrates, investigations do not require ethical permission.

The findings presented here illustrate different levels of virulence among isolates from the leading UPEC lineages and reveal that these phenotypes are largely conserved within the tested clones. We observed a significant association between lethality and carriage of specific virulence factors, but not with growth rate *in vitro*.

Based on virulence factor surveillance, ST127 has the highest virulence potential, which is consistent with our previous findings (Gibreel et al., 2012b). The median LD_{50} of virulent ST127 strains was 1.17×10^4 cfu, almost one log higher than that recently reported for a single strain of EPEC (Leuko and Raivio, 2012). A previous study by Johnson and colleagues showed that ST127 causes extraintestinal infections in humans, dogs, and cats (Johnson et al., 2008). The clonal group ST127 includes the reference strain 536, which is a model organism of extraintestinal *E. coli* infections and the first ST127 complete genome to be reported (Hochhut et al., 2006; Johnson et al., 2008). Other than strain 536, members of ST127 have not been widely reported, presumably because it is a recently evolved clone. Due to its pathogenic potential, ST127 may represent a significant health problem in the future, especially if strains were to acquire extensive antimicrobial resistance.

Comparative genomic analyses were carried out by Hochhut and colleagues between 536 and another reference strain, CFT073, which revealed at least five pathogenicity islands (PAI I-V_{536}) specific to strain 536 (Dobrindt et al., 2002; Hochhut et al., 2006; Schneider et al., 2004). Strain 536 (O6:K15:H31) is well-characterized and it has been demonstrated that it produces various types of fimbrial adhesins, such as S fimbriae (*sfa*) and type 1 and P-related fimbriae (Blum et al., 1994).

The P-related fimbriae genes and S fimbrial adhesins are located on PAI I_{536} and PAI II_{536}, respectively and deletion mutants in these regions show decreased potency *in vivo* (Blum et al., 1994; Hacker et al., 1990).

These observations are supported by our correlation analysis between LD_{50} and virulence profiles, which showed that ST127 isolates with significant lethal effects in *G. mellonella* are associated with the fimbrial adhesins *bmaE* (M fimbriae), *gafD* (G fimbriae) *papAH, papC, papEF* (P fimbriae) and *sfaS* (S fimbriae).

It has been demonstrated that EPEC with a defective type III secretion system (T3SS) have reduced virulence in *G. mellonella* (Leuko and Raivio, 2012). The same paper describes how activation of the Cpx envelope stress response pathway, removing all significant cell envelope associated virulence factors, including T3SS and the bundle forming pillus, will render EPEC avirulent. The data we present here regarding the VFs selected for analysis by PCR, indicate that additional virulence factors contribute to pathogenicity of UPEC in the *G. mellonella* model, but support suggestions that this insect is a valid tool for investigation of the pathogenicity of *E. coli*. We also suggest that, as the ST127 strain 536 is a recognized human pathogen, our observations support the use of *G. mellonella* as a model to indicate potential for causing disease in mammals. The discovery of a single avirulent ST127 strain, EC18, has allowed a deeper investigation of the mechanisms contributing to survival, a prerequisite for virulence, of *E. coli* in the larvae. All other strains examined had some degree of lethality and the observation of a single avirulent strain in ST127 was rather striking.

Comparative genomic analysis between EC18, EC41 and strain 536 revealed a deletion of the O-antigen and the colanic acid gene cluster in EC18. The O-antigen, part of lipopolysaccharide (LPS) present in the outer membrane of Gram-negative bacteria, is a major virulence factor of UPEC. Previous studies have demonstrated that bacterial LPS is important for virulence in the nematode model of infection (Aballay et al., 2003; Bender et al., 2013; Browning et al., 2013).

In one study, it was demonstrated that *Salmonella* Typhimurium required an intact LPS to resist the immune response, persist and multiply within *G. mellonella* (Bender et al., 2013). A recent study by Browning and others (2013) showed the essential nature of O-antigen production as a key virulence determinant mediating killing of *Caenorhabditis elegans* worms by *E. coli* (Browning et al., 2013). Browning demonstrated that regeneration of the O-antigen biosynthesis cluster renders *E. coli* K-12 strain MG1655 pathogenic in *C. elegans*. In this study we demonstrate the importance of the O-antigen gene cluster in the ability of UPEC strain EC18 to kill *G. mellonella.* Given that the VF score for the avirulent ST127 strain EC18 was similar to that for the virulent ST127 strains (Figure 3.3), it is clear that loss of O-antigen can supersede the virulence potential of UPEC in *G. mellonella.*

The normal bactericidal effects of the innate immune system of higher animals, including *G. mellonella*, play a crucial protective role during bacterial infection (Palusińska-Szysz et al., 2012; Phipps et al., 1994; Tichaczek-Goska et al., 2012). Antimicrobial peptides in the *G. mellonella* hemolymph are key factors in the humoral immune response against invading microorganisms (Cytryńska et al., 2007; Noh et al., 2014).

Several antimicrobial peptides that are effective against Gram-negative bacteria, including apolipophorin III (apoLp-III), lysozyme and anionic peptide 2, have been identified in the *G. mellonella* hemolymph (Brown et al., 2009; Zdybicka-Barabas et al., 2013). It has been suggested that O-antigens and colanic acid provide an effective protective barrier against desiccation, phagocytosis and serum complement-mediated killing, including the action of antimicrobial peptides (Miajlovic et al., 2014; Ortega et al., 2009; Oztug et al., 2012; Ramamoorthy et al., 2006; Vincent et al., 2000).

A recent study by Phan and others demonstrated the importance of the O antigen and colanic acid to serum resistance in ST131 UPEC strain EC958 (Phan et al., 2013). The study identified 56 serum resistance genes, of which the majority encode membrane proteins or factors involved in LPS biosynthesis. In addition, another study by Sarkar and colleagues showed the important role of O-antigen in the virulence of UPEC were it was demonstrated that the O6 antigen has a major impact on the colonization of the mouse urinary tract (Sarkar et al., 2014). In our study, serotype O6 was the most prevalent (32%) among the 71 examined isolates, which were distributed across different STs in strains that demonstrated different levels of lethality. This indicates that, although the O6 antigen may be important for bacterial survival during UTI, it is not correlated with virulence in the *G. mellonella* hemolymph.

Our results suggest that the absence of a functional O antigen and colanic acid gene cluster in EC18 renders the bacteria sensitive to the activity of *G. mellonella* hemolymph. However, the mechanisms that lead to the pronounced lethality of some strains from the ST127 lineage are yet to be deciphered.

The genome sequence data we have generated, in combination with publically available sequences for ST69 strains will allow us to begin to explore this in greater depth. We have also demonstrated a correlation between carriage of certain virulence factors with low LD_{50}, which warrants further experiments with mutant strains, and their complemented derivatives, to investigate the role of individual virulence factors in pathogenicity in the *G. mellonella* model.

The ST69 lineage is part of phylogenetic group D and is also described as clonal group A (CGA), which has been identified as an important cause of UTI, with CGA-D-ST69 strains being responsible for up to 50% of infections caused by trimethoprim-sulfamethoxazole-resistant isolates (Bert et al., 2010; Blanco et al., 2011; Johnson et al., 2002a). Analysis of the virulence profile of CGA strains has indicated similarity to O15:K52:H1 isolates, which were found to be more virulent than other *E. coli* (Johnson et al., 2002b; Prats et al., 2000). The O15:K52:H1 clonal group is considered to be a widely disseminated and important UPEC lineage (Johnson et al., 2009). In the current study, ST69 isolates were highly lethal in *G. mellonella.* This may be associated with specific adhesins in this clone, including the *pap* alleles. Interrogation of the VF data recorded during this study did not reveal any obvious similarity in VF profile between the low LD_{50} isolates from ST69 and ST127.

In conclusion, this is the first study to investigate the virulence of UPEC using *G. mellonella* as an *in vivo* model. The findings demonstrate that ST69 and ST127 isolates are, with one exception, highly virulent. We have demonstrated that the O-antigen cluster is essential for resistance to the action of the innate immune response in *G. mellonella.*

Given previous studies with ST69 (CGA) and ST127 (strain 536) UPEC, and our demonstration of the correlation between lethality and specific virulence factors, we suggest that the *G. mellonella* model is a good model to study virulence of UPEC strains, and is a useful tool for discovery of candidate vaccine targets. The high virulence potential and lethality of ST127 isolates emphasises the need to perform a comprehensive analysis of the genetics underlying the virulence of members of this clonal group and suggests that increased surveillance for the clone is justified.

Chapter Four

Published as -

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. dkv188–.

4 Molecular epidemiology of Uropathogenic *E. coli* isolates from Riyadh Saudi Arabia reveals the importance of antibiotic resistant ST131, ST38 and ST405 strains.

4.1 Abstract

The emergence of antimicrobial resistant uropathogeneic *E. coli* (UPEC) is a major issue worldwide. We investigated the molecular epidemiology of UPEC from a tertiary care hospital in Riyadh, Saudi Arabia revealing, for the first time, the population structure of UPEC in the region

A total of 202 UPEC isolates were recovered from hospital and community patients with urinary tract infection (UTI) in December 2012 and January 2013. Strains were not selected on the basis of any resistance profile. They were characterized by multi-locus sequence typing, antibiotic susceptibility determination, phylo-grouping and virulence gene detection.

The most common lineages were ST131 (17.3%), ST73 (11.4%), ST38 (7.4%), ST69 (7.4%), ST10 (6.4%), ST127 (5.9%), ST95 (5.4%), ST12 (3.5%), ST998 (3.5%) and ST405 (3%). ST131 and ST405 isolates were significantly associated with high levels of antibiotic resistance (60% of ST131 carried CTX-M-14 or CTX-M-15 and 66% of ST405 isolates carried CTX-M-15). ST131, CTX-M-15 positive isolates were predominantly of the *fimH*30/Clade C group, resistant to fluoroquinolones and more likely to carry a high number of genes encoding selected virulence determinants. In contrast to many previous studies, ST38 isolates were detected in a high proportion of samples and 40% of these carried CTX-M-15. Four out of the 15 ST38 isolates harbour *aggR*.

Our findings highlight the successful spread of multidrug resistant, CTX-M positive ST38, ST131 and ST405 UPEC in Saudi Arabia. The high proportion of community-acquired isolates with CTX-M is a particular concern. We suggest that ST38 UPEC warrant further study.

4.2 Introduction

Urinary tract infection (UTI) is considered to be amongst the most common infections affecting humans with up to 85% of community-acquired UTI and up to 40% of hospital-acquired UTIs being caused by uropathogenic *Escherichia coli* (UPEC) (Berry et al., 2009; Ejrnæs, 2011; Tartof et al., 2005). Over the last two decades, there has been a proliferation of clinical and molecular epidemiology studies clarifying the population structure, virulence potential and antibiotic susceptibility of UPEC (Arthur et al., 1989; Gibreel et al., 2012b; Johnson and Russo, 2005; Johnson et al., 2002b). Molecular epidemiology has provided numerous important insights into the phylogenetic background, emergence of antimicrobial resistance, pathogenicity and transmission pathways of UPEC (Johnson and Russo, 2005). Strain-typing methods are essential for surveillance to identify the population structure and determine the epidemiological distribution of UPEC and multilocus sequence typing (MLST) has been used to good effect in this respect to describe the emergence and dissemination of leading UPEC clonal groups (Lau et al., 2008b; Oteo et al., 2009; Tartof et al., 2005).

Epidemiological investigation of Extra-intestinal pathogenic *E. coli* (ExPEC) has revealed that UPEC exhibit specific characteristics such as virulence associated traits, distinctive O antigens and degrees of multidrug resistance (Bidet et al., 2007; Gibreel et al., 2012b; Zhang et al., 2000). Carriage by UPEC of a variety of virulence-associated traits is an essential factor to assist in colonization and to facilitate infection in the urinary tract. These virulence determinants include factors that assist UPEC to resist host immune defenses, adhesins, toxins, iron acquisition systems, or means to paralyze ureteric peristalsis (Johnson, 1991).

In previous studies, it has been suggested that some UPEC strains carry virulence properties of enteroaggregative *E. coli* (EAEC), which might indicate that at least some faecal EAEC strains have become uropathogens (Abe et al., 2008; Wallace-Gadsden et al., 2007). Several cases have been reported recently where patients with diarrheagenic EAEC infection have developed UTI and blood stream infections caused by the diarrheagenic EAEC strains (Ahmed et al., 2014; Herzog et al., 2014) and recent evidence indicates that members of the EAEC ST38 clone are evolving to cause UTI infection. ST38 have been reported recently as an evolving clonal group, which may carry the EAEC transport regulator gene (*aggR*) located on the EAEC plasmid (Chattaway et al., 2014a).

Recently, we have witnessed the global dissemination of multidrug-resistant O25b:H4-ST131 *E. coli* (ST131), which is responsible for a high proportion of community- and hospital-acquired urinary tract and bloodstream infections (Petty et al., 2014; Phan et al., 2013; Price et al., 2013; Totsika et al., 2011). The ST131 lineage is mostly associated with the widespread dissemination of extended spectrum β-lactamase (ESBL) enzymes, with representative isolates commonly producing CTX-M-15 and often displaying co-resistance to fluoroquinolones (Coque et al., 2008; Johnson et al., 2010, 2009; Nicolas-Chanoine et al., 2008).

The most prevalent ST131 sub-clone that accounts for both fluoroquinolone (FQ) resistance and CTX-M-15 ESBL production belongs to the *H30*-Rx group, which contains the H30 variant of the type 1 fimbrial adhesin gene *fimH* (Banerjee and Johnson, 2014; Petty et al., 2014) and has also been described as clade C (Petty et al., 2014).

However, the ST131 clonal group, which is the most problematic multidrug resistant clone currently in circulation shows moderate virulence factor (VF) profiles compared to other successful sequence types (STs) including ST69, ST73, ST95 and ST127 (Alghoribi et al., 2014; Nicolas-Chanoine et al., 2008). Other factors might play an important role to enhance the fitness of ST131 and contribute to pathogenesis, including increased bacterial metabolic capability (Gibreel et al., 2012a; Le Bouguénec and Schouler, 2011).

Although the global epidemiology of UPEC has been extensively studied (Banerjee et al., 2013a; Dahbi et al., 2014; Petty et al., 2014; Price et al., 2013), to our knowledge, there have been no robust epidemiological studies examining the population structure of UPEC in Saudi Arabia. Saudi Arabia is key member state of the Middle East and the Gulf Cooperation Council (GCC) states. A recent systematic review shows a growing problem of antibiotic resistance in this region in Gram-negative bacteria that are resistant to commonly used antibiotics. Due to a paucity of studies on the molecular epidemiology and antimicrobial resistance mechanisms in the region, the roots of the problem have never been fully investigated (Zowawi et al., 2013).

For this reason, we have used molecular epidemiology techniques to identify the population structure of UPEC at King Abdulaziz Medical City, a tertiary care hospital in Riyadh, Saudi Arabia. This is an essential and urgently needed study, which will help to inform infection control and prevent the spread of multidrug resistant strains from the region.

4.3 Materials and methods

4.3.1 Bacterial isolates and DNA extraction

A total of 202 non-duplicate consecutive clinical isolates of *E. coli* from patients with UTI were obtained from the King Abdulaziz Medical City (KAMC) in Riyadh, Saudi Arabia. This 1000 bed tertiary healthcare centre serves a population of 500,000 of the Saudi National Guard soldiers and their dependants. The isolates under investigation were collected prospectively from both inpatients and outpatients from December 2012 through January 2013 in collaboration with the microbiology diagnostic laboratory. The identification as *E. coli* was performed using the VITEK II XL (BioMerieux, France) automated system in the microbiology laboratory at KAMC using the Gram Negative Susceptibility Card, AST-GN26. Isolates were inoculated on Sheep Blood Agar (SBA) (Saudi Prepared Media Laboratory) and Transwabs® in Amies transport medium (Medical Wire and Equipment, England) were used to transport the isolates to the UK for analysis, following guidelines for transport of infectious substances (UN 2814).

Prior to DNA extraction, strains were cultured on Columbia agar (Oxoid) at 37^{o}C for 18 hrs and pure single colonies were used for extraction. DNA extraction was performed according to the manufacturer's instructions using a PrepMan Ultra sample preparation kit (PrepManTM Ultra, Applied Biosystems, USA).

4.3.2 Detection of clonal groups

MLST was carried out to determine the strain sequence type (ST) by using the Achtman scheme for *E. coli*, as specified at the University of Warwick *E. coli* MLST web site (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). PCR amplification and sequencing of seven housekeeping gene fragments (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*) was performed following the protocols described previously (Lau et al., 2008b). Additional typing method, *fimH* typing, was performed on members of the major clonal groups to investigate possible sub-typing within STs. Amplification and sequencing of *fimH* gene was performed as described previously by Weissman and others (Weissman et al., 2012).

4.3.3 Phylogenetic grouping

Phylogenetic grouping of isolates was determined using the recently published quadruplex PCR method, which targets four DNA markers (*gadA*, *chuA*, *yjaA* and TSPE4.C2), as described by Doumith and colleagues (Doumith et al., 2012). More details of phylogenetic grouping have been described in chapter 2 section 2.3.4.

4.3.4 Serotyping

Molecular serotyping was performed on the major clonal groups using a multiplex PCR method to detect 14 *E. coli* serogroups associated with UTI (O1, O2, O4, O6, O7, O8, O15, O16, O18, O21, O22, O25, O75 and O83) (Li et al., 2010). Isolates that could not be typed using this method (i.e. gave negative results with all primer pairs) were classified as nontypable (nt). Additional O typing was also performed on all the strains of ST131 clonal group, with a recently described method to detect the ST131-O16 and ST131-O25b clades (Johnson et al., 2014).

4.3.5 Virulence genotypes

Virulence factor (VF) carriage was assessed using an established multiplex PCR assay, which detects 29 ExPEC-associated VF genes (adhesins, toxins, siderophores, capsule genes and miscellaneous genes) (Johnson and Stell, 2000). Virulence score was calculated for each isolate as the sum of positive VFs for each isolate. DNA from 15 isolates belonging to the ST38 clone was screened for the presence of the EAEC transport regulator gene (*aggR*) by PCR, using previously described primers (Chattaway et al., 2014a). PCR was performed in 25 µl reaction using BioMix™ Red (Bioline USA Inc.). Each reaction contained 12.5 µl of 2x BioMix Red, 1 µl of each primer (10pmol/µl), and 1 µl of chromosomal DNA. The amplification parameters were 94°C for 4 min, 30 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min and final extension at 72ºC for 5 min.

4.3.6 Antimicrobial susceptibility testing

Microbiological identification and susceptibility testing for antibiotics was performed using the VITEK II XL automated system with the Gram Negative Susceptibility Card, AST-GN26. A total of 20 antibiotics included in the screen were: penicillins (ampicillin, amoxicillin/clavulanic acid, piperacillin, piperacillintazobactam); cephalosporins (cefalotin, cefuroxime, cefuroxime axetil, cefoxitin, cefpodoxime, cefotaxime, ceftazidime, cefepime); carbapenems (meropenem); aminoglycosides (amikacin, gentamicin, tobramycin); fluoroquinolones (ciprofloxacin, norfloxacin); nitrofurantoin; and trimethoprim-sulfamethoxazole. A resistance score was calculated for each isolate by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics being studied.

4.3.7 Characterization of antibiotic resistance related genes

Isolates from the most frequently detected STs were screened for the presence of β-lactamase genes *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M,}$ *bla*$_{NDM-1}$ and *bla*$_{OXA-1-48}$ by a PCR method using the primers listed in (Table 4.1), as described previously (Dallenne et al., 2010; Lin et al., 2010; Poirel et al., 2011b; Zowawi et al., 2014). In addition, strains from highly quinolone resistant STs (ST131, ST405 and ST410) were examined for mutations in quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC*, using previously described PCR and sequencing primers (Cerquetti et al., 2010; Gibreel et al., 2012b; Yue et al., 2008).

Table 4.1 . PCR primers used to amplify selected genes encoding resistance to beta-lactam antibiotics

PCR target	Primer name	Primer sequence (5'–3')	Product Size (bp)	Annealing temp (°C)	Reference
bla_{TEM}	TEM-F	CATTTCCGTGTCGCCCTTATTC	800	60	(Dallenne et al., 2010)
	TEM-R	CGTTCATCCATAGTTGCCTGAC			
bla_{SHV}	SHV-F	AGCCGCTTGAGCAAATTAAAC	713	60	(Dallenne et al., 2010)
	SHV-R	ATCCCGCAGATAAATCACCAC			
$bla_{CTX-M-14}$	CTX-14-F	TACCGCAGATAATACGCAGGTG	355	48	(Lin et al., 2010)
	CTX-14-R	CAGCGTAGGTTCAGTGCGATCC			
$bla_{CTX-M-15}$	CTX-M-15-F	CACACGTGGAATTTAGGGACT	996	55	(Zowawi et al., 2014)
	CTX-M-15-R	GCCGTCTAAGGCGATAAACA			
bla_{NDM}	NDM-F	GCAGGTTGATCTCCTGCTTG	203	55	(Poirel et al., 2011b)
	NDM-R	ACGGTTTGGCGATCTGGT			
$bla_{OXA-1-like}$	OXA-1-F	GGCACCAGATTCAACTTTCAAG	564	60	(Dallenne et al., 2010)
	OXA-1-R	GACCCCAAGTTTCCTGTAAGTG			
bla_{OXA-48}	OXA-48-F	GCGTGGTTAAGGATGAACAC	438	55	(Zowawi et al., 2014)
	OXA-48-R	CATCAAGTTCAACCCAACCG			

4.3.8 Bioinformatics and phylogenetic analysis

Sequence chromatograms were edited and assembled using CLC Main Workbench 6, including the CLC MLST module (CLC bio, Denmark).

For phylogenetic and nucleotide diversity analyses, the nucleotide sequences of the seven gene fragments of unique STs were concatenated in the order *adk*, *fumC, gyrB, icd, mdh*, *purA* and *recA* using CLC Main workbench 6. The resulting 3423 bp fragments were aligned to allow estimation of the phylogenetic relatedness. Using the same software, the results of the alignment was used to construct a maximum likelihood phylogeny using the Kimura two-parameter (K80) evolution model with the gamma distribution (4 substitution rate categories) (Abdallah et al., 2011; Kimura, 1980; Moura et al., 2009).

The Based upon Related Sequence Types (BURST.v3) clustering algorithm (eburst.mlst.net) was used to analyse the allelic profiles and clonal complexes (CCs) represented by the isolates. The clonal complexes were identified according to the number of single-locus variants (SLVs) and double-locus variants (DLVs) shared between isolates, where only STs that shared six or more loci were assigned to a defined CC (Feil et al., 2004). The minimum spanning tree (MSTree) based on the allelic numbers of the MLST locus was constructed using BioNumerics v.7.1 (Applied Maths) software. MSTree tool generates a graphical representation of the nodes that are linked using unique minimal paths, which minimizes the total summed distance of all branches (Maatallah et al., 2011).

4.4 Results

4.4.1 Molecular typing reveals the true population structure of UPEC in KAMC, Riyadh

MLST was performed on the 202 UPEC isolates in order to examine the phylogenetic relationships and understand the genetic diversity of UPEC from Riyadh, Saudi Arabia. This allowed identification of 51 unique STs. The most commonly detected STs were ST131 (n=35; 17.3% of isolates), ST73 (n=23; 11.4%), ST38 (n=15; 7.4%), ST69 (n=15; 7.4%), ST10 (n=13; 6.4%), ST127 (n=12; 5.9%) and ST95 (n=11; 5.4%), which together accounted for >60% of the isolates (Table 4.2).

ST131 clonal group was the most predominant clone and members of the group were significantly associated with multidrug resistance including ESBL production, and resistance to aminoglycosides and quinolones (See section on antibiotic resistance below). The sub-clonal structure of ST131 was examined based on serotyping and *fimH*-typing. Almost all ST131 isolates 77% (27/35) belonged to O25b-ST131 *fimH30*, 20% (7/35) belonged to O16-ST131 *fimH41* and one isolate belonged to O25b-ST131 *fimH22*.

The clinical and demographic parameters, such as patient age and gender, specimen type and geographic origin were retrieved from laboratory records and examined anonymously. The majority of the strains (87.1%) were isolates from females and patient age ranged from < 2 months to 87 years (mean age 40 years). Among the 202 isolates examined, 141 isolates (69.2%) were recovered from outpatients with a clinical diagnosis of uncomplicated UTIs.

.

Table 4.2 Frequency of detected sequence types, phylogenetic grouping and serotyping

ST (n)	CC	Grouping	Serotype	Frequency %
131 (35)		B2	O25b -O16	17.3
73 (23)	ST-73	B2	O6	11.4
38 (15)	ST-38	D	O1	7.4
69 (15)	ST-69	D	O15-O25	7.4
10 (13)	ST-10	A	O2-O8-O15-O25	6.4
127 (12)	-	B2	O6	5.9
95 (11)	ST-95	B2	O1-O2-O8-O25	5.4
12 (7)	ST-12	B2	O4-O21	3.5
998 (7)	-	B2	-	3.5
405 (6)	ST-405	D	-	3
410 (6)	ST-23	A	-	3
23 (3)	ST-23	A	-	1.5
624 (3)	-	D	-	1.5
14 (2)	ST-14	B2	-	1
58 (2)	ST-155	B1	-	1
101 (2)	ST-101	B1	-	1
162 (2)	ST-469	B1	-	1
167 (2)	ST-10	A	-	1
617 (2)	ST-10	A	-	1
636 (2)	-	B2	-	1
1380 (2)	-	D	-	1
52 (1)	-	A	-	0.5
57 (1)	ST-350	D	-	0.5
62 (1)	-	D	-	0.5
68 (1)	-	D	-	0.5
154 (1)	-	B1	-	0.5
155 (1)	ST-155	B1	-	0.5
210 (1)	-	B1	-	0.5
224 (1)	-	B1	-	0.5
295 (1)	-	B1	-	0.5
315 (1)	ST-38	D	-	0.5
345 (1)	-	B1	-	0.5
347 (1)	-	A	-	0.5
367 (1)	ST-23	A	-	0.5
371 (1)	ST-350	D	-	0.5
449 (1)	ST-31	D	-	0.5
450 (1)	-	A	-	0.5
501 (1)	-	D	-	0.5
537 (1)	ST-14	B2	-	0.5
540 (1)	-	B1	-	0.5
543 (1)	-	A	-	0.5
662 (1)	-	B1	-	0.5
1196 (1)	-	B1	-	0.5
1266 (1)	-	B2	-	0.5
1312 (1)	-	A	-	0.5
1431 (1)	-	B1	-	0.5
1611 (1)	-	B1	-	0.5
2020 (1)	-	D	-	0.5
2659 (1)	ST-38	D	-	0.5
3076 (1)	-	B1	-	0.5
3556 (1)	-	B1	-	0.5
Total (202)				100

4.4.2 Phylogenetic grouping

Phylogenetic group analysis segregated the strains into four major groups (A, B1, B2 and D) (Table 4.2). A total of 103 (50.9%) isolates belonged to group B2, whereas 48 (23.7%) strains belonged to group D, 32 (15.8%) to group A and 19 (9.4%) to group B1. Out of the predominant STs, all the strains of ST73, ST95, ST127, ST131 and one strain of ST38 belonged to phylogenetic group B2. All ST69 and the remaining 14 ST38 isolates belonged to phylogenetic group D. ST10 was composed entirely of isolates from phylogenetic group A.

4.4.3 Clonal relationship of the STs

To compare the level of genetic diversity, as measured by MLST, maximum likelihood phylogenies were constructed showing the genetic relatedness among the 51 STs (Figure 4.1). STs were segregated into three different clusters, which were observed to contain members of the recognized phylogenetic groupings (A, B1, B2, D). Clusters 1 and 3 were observed to exclusively include strains belonging to phylogenetic groups B2 and D, respectively.

However, isolates within Cluster 2 belonged to members of phylogenetic groups A, B1, a single B2 isolate (lineage ST1266) and two isolates from group D (ST57, ST371). ST62 (D) ST662 (B1) strains were the most divergent. Minimum spanning tree (MSTree) and BURST analyses were performed to indicate genetically related strains based on MLST allelic profiles of the seven housekeeping genes.

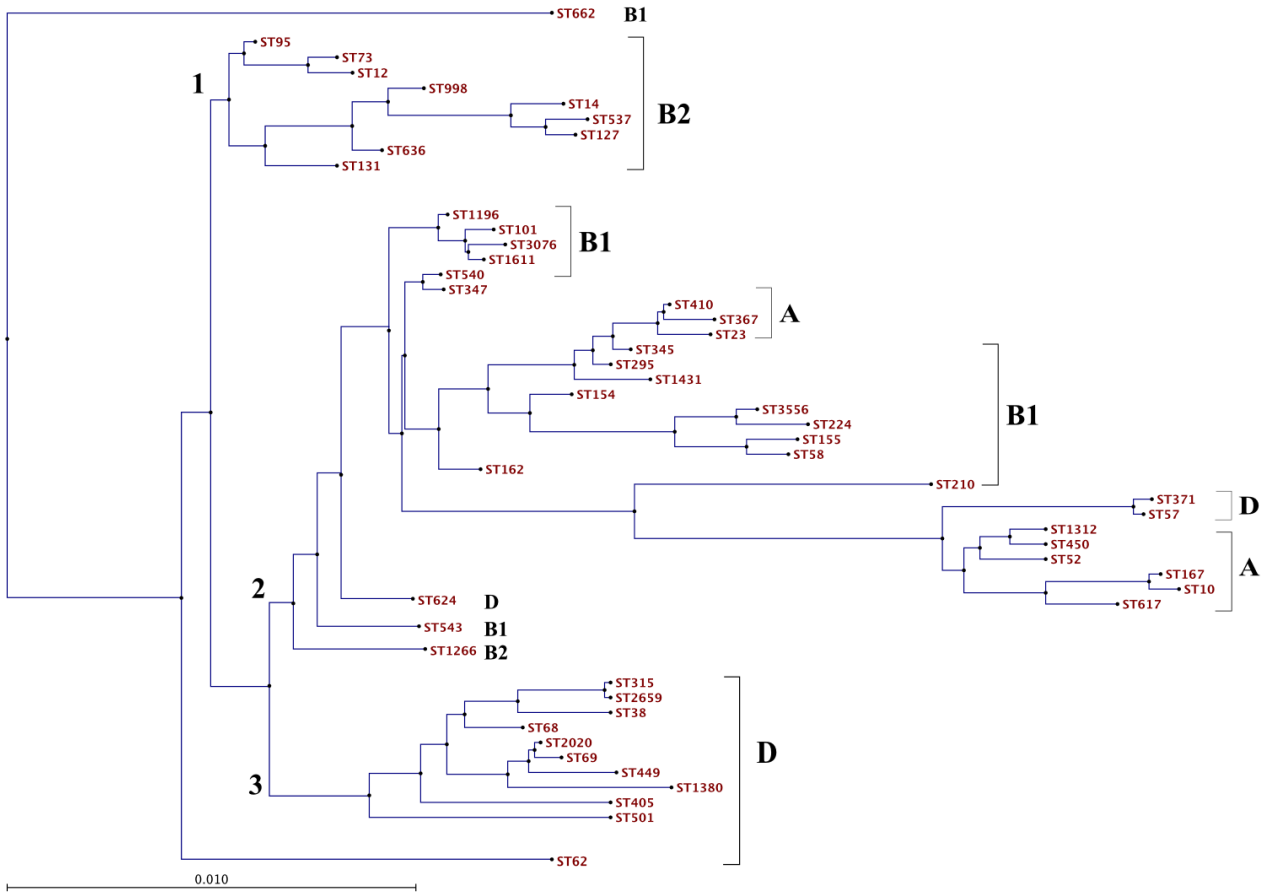

Figure 4.1 Maximum likelihood phylogeny tree of the concatenated nucleotide sequences of the seven gene fragments in the order *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* using the Kimura two-parameter (K80) evolution model with the gamma distribution (4 substitution rate categories). The sequence type number and phylogenetic grouping are indicated at all branches.

Figure 4.2 shows the minimum spanning tree in which each circle corresponds to an ST and the size of the circle is related to the number of isolates found with that profile.

Circles were coloured based the phylogenetic grouping and grey zones encompassing some groups of circles indicate that these profiles belong to the same clonal complex (CC). The relationship between STs is indicated by the connections between the STs. Thick lines connecting pairs of STs indicate that they differ in one allele and two alleles (thin), or three to seven alleles (dashed). The analysis revealed an estimated relationship between STs based on the highest numbers of SLVs and DLVs.

The output from both MSTree and BURST analysis revealed 8 CCs encompassing 22 STs and representing 73 UPEC isolates, with 129 isolates appearing as singletons.

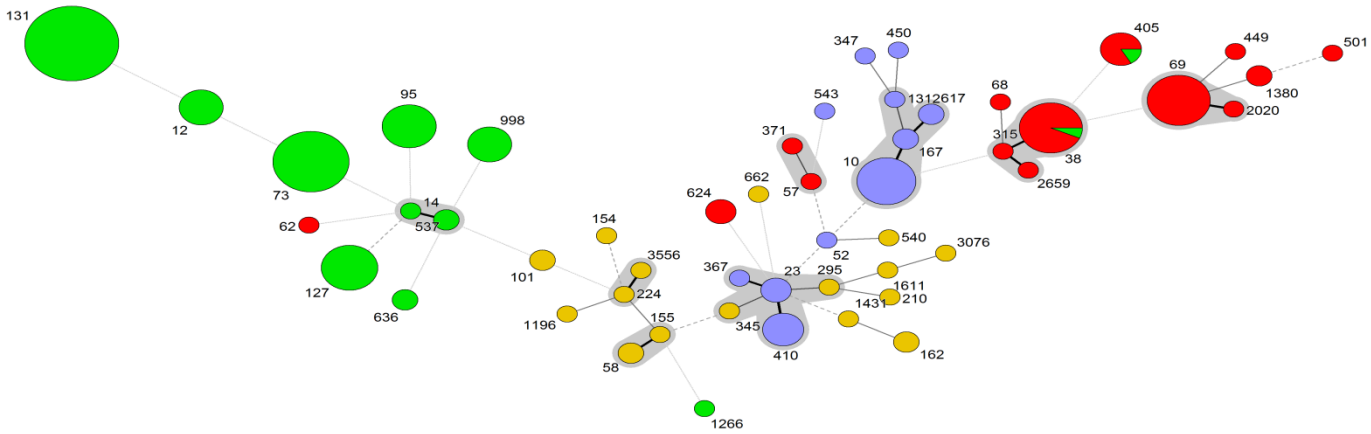

Figure 4.2 Minimum spanning tree analysis of the 202 UPEC strains based on MLST allelic profiles of the seven housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA and recA*. Each circle corresponds to a sequence type and the size of the circle is related to the number of isolates found with that profile. Grey zones between some groups of circles indicate that these profiles belong to the same clonal complex (CC). Circles were coloured based the phylogenetic grouping A (purple), B1 (brown), B2 (green) and D (red).

4.4.4 Prevalence of virulence factors

The virulence profile of the UPEC strains was determined by PCR based surveillance of 29 virulence factors with carriage ranging from 3% (*cvaC* and *bmaE*) to 95% (*fimH*). Virulence factor *papG I, nfaE, gafD, dctB* were not detected in the collection of isolates. Phylogenetic group B2 was significantly associated with a high virulence capacity compared to group A ($P<0.0001$) and were seen to carry significantly more VFs than members of group B1 ($P<0.01$) (Figure 4.3) No significant difference has been seen between group B2 and D.

STs varied considerably in VF content, from ST405, which had the lowest VF score (mean 4.8) to ST127 with the highest VF score (mean 9.4). Table 4.3 shows the virulence profiles of the commonly detected STs that had a significant positive or negative association. The adhesion factors were prevalent virulence determinants, especially the Pap elements in several STs including ST69, ST73, ST95 and ST127. Members of ST131 clone showed a moderate VF score (mean 7.1) compared to other STs. ST131 isolates showed a low carriage of adhesins with significantly low prevalence of *allele-II*. ST69 and ST127 isolates showed a high prevalence of most of the adhesion factors. In addition, ST69 and ST127 were significantly associated with *traT* ($P<0.001$ and $P<0.004$, respectively).

A total of 15 isolates belonging to ST38 were screened for the EAEC transport regulator gene, *aggR*, and PCR results showed four were positive (data not shown).

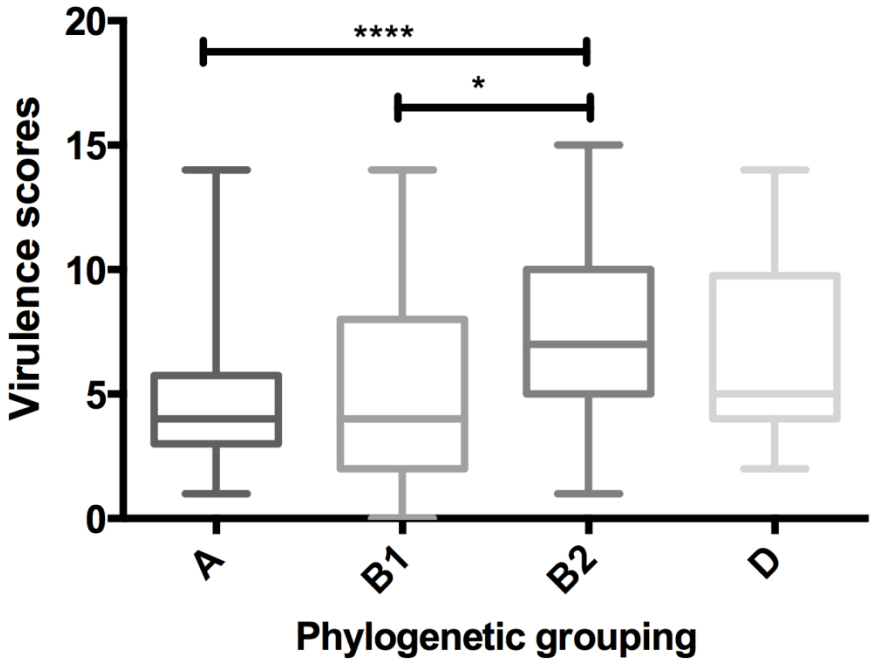

Figure 4.3 Statistical analysis to compare the prevalence of virulence factor scores between the phylogenetic groupings A, B1, B2 and D. Phylogenetic group B2 shows a significant virulence capacity compared to that of other groups.

Table 4.3 Prevalence of various UPEC associated virulence factors within the most commonly detected clonal groups observed in the collection

Category	Specific trait	Total no.	Prevalence (%) of VF by Phylogenetic group				Prevalence (%) of VF by sequence type							
		202	A (n=32)	B1 (n=19)	B2 (n=103)	D (n=48)	ST10 (n=13)	ST38 (n=15)	ST69 (n=15)	ST73 (n=23)	ST95 (n=11)	ST127 (n=12)	ST131 (n=35)	ST405 (n=6)
Adhesion	***papA***	**59 (29)**	**8 (25)**	**2 (11)**	**34 (33)**	**14 (29)**	**5 (38)**	**3 (20)**	**8 (53)**	**8 (35)**	**6 (55)**	**7 (58)**	**8 (23)**	**2 (33)**
	papC	**72 (36)**	**9 (28)**	**6 (32)**	**39 (38)**	**17 (35)**	**6 (46)**	**4 (27)**	**10 (67)**	**8 (35)**	**5 (45)**	**7 (58)**	**11 (31)**	**0 (0)**
	papEF	**56 (28)**	**4 (13)**	**3 (16)**	**35 (34)**	**14 (29)**	**2 (15)**	**2 (13)**	**9 (60)**	**12 (52)**	**5 (45)**	**8 (67)**	**5 (14)**	**1 (17)**
	papGII,III	**15 (7)**	**1 (3)**	**0 (0)**	**13 (13)**	**0 (0)**	**1 (8)**	**0 (0)**	**0 (0)**	**1 (4)**	**6 (55)**	**3 (25)**	**3 (9)**	**0 (0)**
	allele-II	**39 (19)**	**4 (13)**	**3 (16)**	**18 (17)**	**13 (27)**	**2 (15)**	**4 (27)**	**7 (47)**	**6 (26)**	**3 (27)**	**3 (25)**	**2 (6)**	**1 (17)**
	allele-III	**42 (21)**	**7 (22)**	**3 (16)**	**26 (25)**	**6 (13)**	**5 (38)**	**3 (20)**	**2 (13)**	**3 (13)**	**3 (27)**	**4 (33)**	**10 (29)**	**1 (17)**
	sfa/foc DE	**34 (17)**	**2 (6)**	**4 (21)**	**18 (17)**	**9 (19)**	**1 (8)**	**2 (13)**	**2 (13)**	**3 (13)**	**5 (45)**	**2 (17)**	**8 (23)**	**1 (17)**
	afa/draBC	**25 (12)**	**2 (6)**	**3 (16)**	**17 (17)**	**3 (6)**	**0 (0)**	**2 (13)**	**0 (0)**	**1 (4)**	**1 (9)**	**4 (44)**	**4 (11)**	**1 (17)**
	sfaS	**22 (11)**	**2 (6)**	**3 (16)**	**14 (14)**	**2 (4)**	**0 (0)**	**0 (0)**	**1 (7)**	**2 (9)**	**4 (36)**	**3 (25)**	**5 (14)**	**1 (17)**
Toxins	***hlyA***	**14 (7)**	**0 (0)**	**0 (0)**	**12 (12)**	**1 (2)**	**0 (0)**	**1 (7)**	**0 (0)**	**3 (13)**	**4 (36)**	**2 (17)**	**0 (0)**	**0 (0)**
	cnf1	**42 (21)**	**2 (6)**	**3 (16)**	**30 (29)**	**6 (13)**	**2 (15)**	**4 (27)**	**1 (7)**	**10 (43)**	**4 (36)**	**4 (33)**	**9 (26)**	**0 (0)**
Siderophore	***fyuA***	**154 (76)**	**22 (69)**	**13 (68)**	**78 (76)**	**39 (81)**	**8 (62)**	**13 (87)**	**11 (73)**	**19 (83)**	**4 (36)**	**9 (75)**	**30 (86)**	**6 (100)**
	iutA	**118 (58)**	**15 (47)**	**6 (32)**	**66 (64)**	**29 (60)**	**5 (38)**	**10 (67)**	**11 (73)**	**15 (65)**	**8 (73)**	**7 (58)**	**25 (71)**	**1 (17)**
Capsule	***kpsM II***	**76 (38)**	**9 (28)**	**2 (11)**	**45(44)**	**17 (35)**	**4 (31)**	**4 (27)**	**8 (53)**	**9 (39)**	**6 (55)**	**6 (50)**	**20 (57)**	**1 (17)**
	K1	**30 (15)**	**5 (16)**	**0 (0)**	**16 (16)**	**9 (19)**	**3 (23)**	**1 (7)**	**2 (13)**	**2 (9)**	**1 (9)**	**3 (25)**	**8 (23)**	**1 (17)**
	K5	**75 (37)**	**7 (22)**	**6 (32)**	**48(47)**	**12 (25)**	**2 (15)**	**4 (27)**	**6 (40)**	**10 (43)**	**4 (36)**	**9 (75)**	**14 (40)**	**1 (17)**
Miscellaneous	***cvaC***	**6 (3)**	**1 (3)**	**0 (0)**	**3 (3)**	**2 (4)**	**0 (0)**	**0 (0)**	**2 (13)**	**0 (0)**	**2 (18)**	**1 (8)**	**0 (0)**	**0 (0)**
	traT	**128 (63)**	**14 (44)**	**10 (53)**	**71 (69)**	**32 (67)**	**4 (31)**	**10 (67)**	**15 (100)**	**17 (74)**	**7 (64)**	**12 (100)**	**21 (60)**	**2 (33)**
	PAI	**65 (32)**	**2 (6)**	**5 (26)**	**37 (36)**	**19 (40)**	**2 (15)**	**7 (47)**	**9 (60)**	**10 (43)**	**6 (55)**	**4 (33)**	**13 (37)**	**2 (33)**
VFs score [mean, median (range)]			**4.7, 4 (1-14)**	**5, 4 (0-14)**	**7.5, 7 (1-15)**	**6.3, 5 (2-14)**	**5, 5 (1-11)**	**6.2, 5 (2-13)**	**8.1, 7 (2-14)**	**7.9, 7 (2-15)**	**9.2, 10 (2-14)**	**9.4, 9.5 (5-14)**	**7.1, 6 (2-13)**	**4.8, 4 (2-10)**

Bold values indicate significant associations ($P \leq 0.05$); underlining indicates a negative association.

4.4.5 High levels of antibiotic resistance are present in the KAMC isolates

The data relating to susceptibility of strains of different STs towards 20 tested antibiotics was examined using Fisher's exact test. Overall, STs varied considerably in their antibiotic susceptibilities from the most susceptible, ST73, with the lowest resistance scores (median 0.1 and range 0.0-0.3), to the most resistant ST131 (median 0.5 and range 0.2-0.7) (Table 4.4).

The VITEK advanced expert system (AES) showed that 71 isolates (35% of the total) exhibited an ESBL genotype. ST131 isolates ($P \leq 0.002$) and ST405 ($P \leq 0.02$) were significantly associated with ESBL gene carriage compared with other STs (Table 4.4). In addition, ST131 ($P \leq 0.007$) and ST38 ($P \leq 0.03$) were significantly associated with penicillinase production. Table 4.5 shows a comparison of serotyping, phylo-grouping, antimicrobial resistance and virulence factor carriage between ST38 and ST131 clonal groups. ST131 isolates showed the highest antimicrobial resistance (mean= 0.45) and virulence scores (mean= 6) compared to ST38.

Detection of CTX-M like genes was conducted to investigate the prevalence of these ESBL producing isolates among members of the ST38, ST131 and ST405 groups. CTX-M like genes were mostly detected in ST131 (n=21 60% of ST131), with CTX-M-14 (8 isolates belonging to O25b-ST131 *fimH30*) and CTX-M-15 (9 isolates belonging to O25b-ST131 *fimH30* and two isolates from O16-ST131 *fimH41*) being most common. In addition, one isolate belonging to O25b-ST131 *fimH30* were both CTX-M-14 and CTX-M-15 positive. Almost all ESBL producing ST405 isolates were positive for CTX-M-15 (4 isolates) and CTX-M-14 (one isolate). In addition, six isolates of ST38 exhibited ESBL profiles and five of them were positive for CTX-M-15.

Table 4.4 Association of STs with resistance to different antibiotic agents

		β-Lactams											ESBL	Aminoglycosides			Quinolones		Furanes	Trimethoprim-sulfamethoxazole	Resistance-score
		AMP	AMC	PIP	CF	CXM	CXM-AX	FOX	CPD	CTX	CAZ	FEP		AAC(3)	AAC(3), ANT(2)	AAC(6)	CIP	NOR	NIT		[mean, median (range)]
ST	Isolates	(153)	(18)	(153)	(90)	(68)	(68)	(19)	(68)	(62)	(37)	(7)	71	3	26	15	(62)	(88)	(9)	(105)	
ST131	35	33	2	33	27	20	20	0	20	20	14	1	21	1	9	4	25	32	4	23	0.43, 0.5 (0.2-0.7)
P value	—	0.007	—	0.007	0.0002	0.006	0.006	—	0.003	0.001	0.0008	—	0.002	—	0.024	—	0.0001	0.0001	—	—	
ST73	23	15	1	15	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0.11, 0.1 (0.0-0.3)
P value	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
ST38	15	15	4	15	8	6	6	2	6	6	3	0	6	0	1	2	3	8	3	8	0.33, 0.3 (0.1-0.7)
P value		0.02	0.05	0.02	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.02	—	
ST69	15	14	2	14	4	4	4	2	4	3	1	0	4	0	1	0	1	2	0	12	0.23, 0.2 (0.1-0.7)
P value		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.03	
ST10	13	8	1	8	3	3	3	1	3	2	0	0	3	0	0	0	2	2	0	7	0.17, 0.2 (0.0-0.5)
P value	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
ST127	12	7	0	7	3	1	1	0	1	1	0	0	1	0	0	0	0	0	0	2	0.1, 0.1 (0.0-0.4)
P value	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
ST95	11	5	1	5	2	2	2	1	2	1	2	0	2	0	0	0	1	3	0	3	0.14, 0.1 (0.0-0.5)
P value	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
ST12	7	3	0	3	1	1	1	0	1	1	0	0	1	0	1	0	1	4	0	4	0.16, 0.2 (0.0-0.5)
P value	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
ST998	7	6	2	6	5	5	5	2	5	5	2	0	5	1	3	0	0	0	0	3	0.38, 0.4 (0.0-0.7)
P value		—	—	—	—	0.02	0.02	—	0.05	0.04	—	—	—	—	0.048	—	—	—	—	—	
ST405	6	6	0	6	6	5	5	4	5	5	4	0	5	0	0	0	6	6	0	6	0.53, 0.6 (0.4-0.6)
P value		—	—	—	0.04	0.005	0.005	0.001	0.02	0.01	0.009	—	0.02	—	—	—	0.0008	0.007	—	0.03	
ST410	6	6	2	6	2	2	2	1	2	2	2	1	2	1	0	1	5	6	0	4	0.39, 0.2 (0.2-0.8)
P value		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.01	0.007	—	—	

AMP, ampicillin; AMC, amoxicillin/clavulanic acid; PIP, piperacillin; CF, cefalotin; CXM, cefuroxime; CXM-AX, cefuroxime axetil; FOX, cefoxitin; CPD, cefpodoxime; CTX, , cefotaxime; CAZ, ceftazidime FEP, cefepime; AAC, N-acetyltransferase; ANT, O-adenyltransferase; CIP, ciprofloxacin; NOR, norfloxacin; NIT, nitrofurantoin.
P values (by Fisher's exact test) where shown when $P \leq 0.05$ and these relate to differences found when susceptibility profiles for isolates of each ST were compared with those of all other STs combined.

Table 4.5 Serotyping, phylo-grouping, antimicrobial resistance and virulence factor carriage of UPEC lineages ST38 and ST131

ST38					ST131				
Isolate no	**Serotyping**	**Phylo-grouping**	**R-score**	**VF Score**	**Isolate no**	**Serotyping**	**Phylo-grouping**	**R-score**	**VF Score**
SA 007	nt	**D**	**0.15**	**7**	**SA 001**	**O25b**	**B2**	**0.55**	**7**
SA 023	**O1**	**D**	**0.25**	**8**	**SA 011**	**O25b**	**B2**	**0.3**	**8**
SA 034	nt	**D**	**0.15**	**9**	**SA 019**	**O16**	**B2**	**0.2**	**6**
SA 083	**O1**	**D**	**0.55**	**4**	**SA 031**	**O16**	**B2**	**0.15**	**6**
SA 089	nt	**D**	**0.4**	**5**	**SA 037**	**O25b**	**B2**	**0.65**	**7**
SA 133	nt	**D**	**0.3**	**3**	**SA 040**	**O25b**	**B2**	**0.5**	**6**
SA 154	nt	**D**	**0.1**	**2**	**SA 054**	**O25b**	**B2**	**0.2**	**6**
SA 157	nt	**D**	**0.35**	**2**	**SA 057**	**O25b**	**B2**	**0.7**	**9**
SA 160	**O1**	**D**	**0.3**	**5**	**SA 068**	**O25b**	**B2**	**0.65**	**5**
SA 162	nt	**D**	**0.65**	**13**	**SA 071**	**O25b**	**B2**	**0.2**	**5**
SA 165	nt	**B2**	**0.2**	**5**	**SA 073**	**O25b**	**B2**	**0.15**	**10**
SA 182	nt	**D**	**0.15**	**6**	**SA 076**	**O25b**	**B2**	**0.3**	**4**
SA 219	**O1**	**D**	**0.6**	**10**	**SA 077**	**O25b**	**B2**	**0.65**	**11**
SA 223	nt	**D**	**0.35**	**3**	**SA 095**	**O25b**	**B2**	**0.5**	**9**
SA 227	nt	**D**	**0.4**	**11**	**SA 104**	**O16**	**B2**	**0.5**	**10**
					SA 105	**O25b**	**B2**	**0.6**	**12**
					SA 120	**O25b**	**B2**	**0.3**	**5**
					SA 127	**O25b**	**B2**	**0.25**	**4**
					SA 129	**O25b**	**B2**	**0.55**	**10**
					SA 130	**O25b**	**B2**	**0.45**	**10**
					SA 134	**O25b**	**B2**	**0.6**	**10**
					SA 136	**O25b**	**B2**	**0.3**	**10**
					SA 145	**O16**	**B2**	**0.25**	**2**
					SA 171	**O25b**	**B2**	**0.7**	**6**
					SA 186	**O25b**	**B2**	**0.35**	**2**
					SA 194	**O25b**	**B2**	**0.45**	**13**
					SA 195	**O16**	**B2**	**0.45**	**6**
					SA 206	**O25b**	**B2**	**0.7**	**8**
					SA 209	**O16**	**B2**	**0.5**	**6**
					SA 212	**O25b**	**B2**	**0.5**	**5**
					SA 216	**O25b**	**B2**	**0.3**	**6**
					SA 225	**O16**	**B2**	**0.15**	**2**
					SA 228	**O25b**	**B2**	**0.5**	**5**
					SA 240	**O25b**	**B2**	**0.1**	**9**
					SA 242	**O25b**	**B2**	**0.6**	**10**
Average			**0.33**	**6.20**				**0.42**	**7.14**
Median			**0.30**	**5.00**				**0.45**	**6.00**

Most of the ESBL positive ST131 isolates were recovered from outpatients (n= 24, 69% of ST131). However, CTX-M-14 and CTX-M-15 were both detected in isolates from ST131 and ST405 isolates from inpatients and outpatients. A total of 10 isolates of O25b-ST131 *fimH30* and 5 isolates of ST38 were positive for OXA-1-like determinants. Resistance to fluoroquinolones (ciprofloxacin, norfloxacin) was also found to be significantly associated with ST131, ST405 and ST410 (Table 4.4). Among the ST131 isolates, 77% (27/35) belonged to the O25b-ST131 *fimH30* sub-clone, which was statistically associated with ciprofloxacin-resistance ($P \leq 0.0008$) compared to O16-ST131 *fimH41*. The single isolate of the O25b-ST131 *fimH22* sub-clone was resistant to penicillin, aminoglycosides and trimethoprim-sulfamethoxazole.

DNA sequencing of *gyrA* and *parC* genes was performed to screen for mutations leading to amino acid changes associated with fluoroquinolone resistance. Most of the ciprofloxacin-resistant ST131, ST405 and ST410 isolates had multiple mutations in both *gyrA* and *parC* genes (Table 4.6). Isolates that failed to show resistance to ciprofloxacin mostly had a single *gyrA* mutation at codon 83 (Ser $\rightarrow$ Leu), except two isolates of O25b-ST131 *fimH30*, which had multiple mutations in *gyrA* and *parC* genes.

Table 4.6 Molecular characterization of quinolone resistance mechanisms found in UPEC ST131, ST405 and ST410

ST (n)	ESBL production (%)	CTX-M-14 (%)	CTX-M-15 (%)	CIP-R (%)	QRDRb						
					gyrA				*parC*		
					(S)83(L) (%)	(D)87(N) (%)	(D)87(Y) (%)	E(153)K (%)	(S)80(I) (%)	(S)80(R) (%)	(E)84(V) (%)
O25b-ST131 fimH30 (27)	18 (67)	10 (37)	10 (37)	24 (89)	26 (96)	25 (93)	1	1 (4)	26 (96)	0 (0)	26 (96)
O25b-ST131 *fimH22* (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
O16-ST131 *fimH41* (7)	3 (43)	0 (0)	3 (43)	1 (14)	7 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ST405 *fimH27* (3)	2 (67)	0 (0)	1 (33)	3 (100)	3 (100)	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)
ST405 *fimH* nt (3)	3 (100)	0 (0)	3 (100)	3 (100)	3 (100)	3 (100)	0 (0)	0 (0)	0 (0)	3 (100)	0 (0)
ST410 *fimH* nt (6)	2 (33)	0 (0)	1 (17)	5 (83)	3 (50)	2 (33)	1 (17)	1 (17)	3 (50)	0 (0)	0 (0)

4.5 Discussion

The results of this study revealed the presence of multidrug-resistant UPEC strains with a high virulence potential including those from ST38, ST131 ST405 and ST410. Currently, *E. coli* ST131 is a globally dispersed clone of multidrug resistant UPEC responsible for community and hospital-acquired urinary tract and bloodstream infections (Petty et al., 2014; Qureshi and Doi, 2014). Representatives of this clone have been found in six continents as a major clone associated with ESBL production and fluoroquinolone resistance (Al-Agamy et al., 2014; Lau et al., 2008a; Lavollay et al., 2006; Nicolas-Chanoine et al., 2008; Petty et al., 2014; Sidjabat et al., 2010). Extensive studies have characterized ST131 as serotype O25b:H4 and often linked it to the spread of the CTX-M-15 or other ESBLs (Clermont et al., 2008; Johnson et al., 2012; Nicolas-Chanoine et al., 2008). In the UK, the majority of UTIs caused by ESBL producing strains yield members of the O25:H4-ST131 lineage, which includes PFGE strain A that was first identified in January 2003 (Croxall et al., 2011; Gibreel et al., 2012b; Lau et al., 2008a; Woodford et al., 2004).

In 2007, surveillance programs in the United States found that ST131 clonal group was highly prevalent and a major cause of serious multidrug-resistant infections (Johnson et al., 2010). In the work presented here, ST131 (n=35, 17.3%) was the most frequently detected clonal group and members also displayed the highest level of antimicrobial resistance and a high virulence profile.

Additional use of the highly discriminatory *fimH* typing method (Banerjee and Johnson, 2014; Weissman et al., 2012) revealed that O25b-ST131 *fimH30* (also described as Clade C based on whole genome phylogeny) (Petty et al., 2014) was the most prevalent sub-clone (77% of ST131 isolates) and members of this group were associated with a high resistance score. In contrast, the single O25b-ST131 *fimH22* isolate and seven O16-ST131 *fimH41* isolates were observed to have low antimicrobial resistance profiles.

Detection of antimicrobial resistance genes was performed to investigate the determinants responsible for particular resistance patterns in specific clone(s). The results of the current study showed carriage of CTX-M-14 and CTX-M-15 among the most frequently detected clones ST131 and ST405 from both inpatient and outpatient samples. A previous study by Shin and others compared the virulence of CTX-M-15 and CTX-M-14 producing *E. coli.* The results indicated a rapid increase of CTX-M-15 producing *E. coli* isolates was due to the high frequency of virulence determinants (Shin et al., 2011). Our findings show that both CTX-M-14 and CTX-M-15 producing ST131 isolates are significantly associated ($P \leq 0.001$) with a higher rate of virulence determinant carriage, compared to non-CTX-M producing ST131 isolates. Emergence of fluoroquinolone resistance in ESBL-producing UPEC strains has been reported globally with higher resistance rates in specific clones (Giufrè et al., 2012; Matsumura et al., 2013).

In the current study, STs that were significantly associated with fluoroquinolone resistance showed multiple mutations in the chromosomally located *gyrA* and *parC* genes. This pattern of mutations has been reported previously in isolates belonging to ST131, ST405 and ST410 and has been associated with high-level fluoroquinolone resistance, which was consistent with our findings (Table 4.6) (Gibreel et al., 2012b; Johnson et al., 2013; Matsumura et al., 2013; Mavroidi et al., 2012a; Paltansing et al., 2013). However, two fluoroquinolone resistant isolates belonging to O25b-ST131 *fimH30* and ST410 showed mutations in *gyrA* E(153)K, which has not previously been described in *E. coli*, but has been observed once in *Pseudomonas aeruginosa* (Cabot et al., 2014).

Interestingly, ST38 was detected in this study as a high proportion of UPEC isolates (n=15, 7.4%). The diversity with respect to virulence determinant carriage and antibiotic resistance suggests that several ST38 strains are circulating in the population in Riyadh. ST38 isolates have not been widely reported as predominant members in collections of UPEC, but it has been suggested that this group is evolving and is becoming more commonly seen in UTI (Cao et al., 2014; Chattaway et al., 2014a; Riley, 2014; Van der Bij et al., 2012). A key recent observation is the report of uropathogenic ST38 isolates from Germany, the Netherlands and the UK carrying a plasmid mediated transport regulator gene (*aggR*), which is a marker of EAEC (Chattaway et al., 2014a).

Our results showed that four ST38 isolates were *aggR* positive, though we did not determine plasmid carriage in these isolates. Presence of *aggR* may indicate evolution of a strain possessing both UPEC and EAEC characteristics and highlights the diverse mechanisms employed by *E. coli* to ensure fitness and mediate pathogenicity in different ecological niches. The detailed genetic background of some of the ST38 strains studied here is being investigated at the genome level and it will be important to determine whether the 'hybrid' strain is present in isolates collected at KAMC. Six isolates of the ST38 carried CTX-M-15 and four were OXA-1-like positive, though all were NDM-1 negative. Out of the four *aggR* positive isolates, two were CTX-M-15 and one was OXA-1-like positive. In other parts of the world, ST38 isolates have been associated with different combinations of ESBL and/or OXA determinants; OXA-48, and CTX-M group 9 in the UK (Dimou et al., 2012), OXA-48 producers in France (Poirel et al., 2011a), CTX-M-9, -14, and -15 production in Canada (Peirano et al., 2012), CTX-M-14 production in China (Peng and Zong, 2011) and CTX-M-9 and NDM-1 producers in Japan (Suzuki et al., 2009; Yamamoto et al., 2011).

Little is known about the spread of CTX-M-type ESBL among EAEC strains (Chattaway et al., 2014a, 2014b; Y. Chen et al., 2014; Guiral et al., 2011; Okeke et al., 2011), but the surprisingly high detection rate for ST38 UPEC in our study and the suggested emergence of ExPEC with UPEC and EAEC characteristics indicates that increased surveillance for this clone is warranted so that we can fully understand the significance of these pathotypes in UTI. Other STs in this study, including ST405, ST410 and ST998, were found to be distinctive for their high carriage of antibiotic resistance determinants.

A recent study by Matsumura and others proposed the potential for ST405 to become a pandemic clonal group, following on from ST131-O25b (Matsumura et al., 2013). Our results show that ST405 is a commonly detected clonal group, which is associated with a broad multidrug resistance profile. In addition, ST405 has been reported worldwide with various types of $bla_{\text{CTX-M}}$ (Park et al., 2012; Shin et al., 2011; Van der Bij et al., 2012; Zhang et al., 2014). Our results demonstrated that 67% of KAMC ST405 strains were CTX-M-15 positive which is consistent with previous studies (Peirano et al., 2012; Shin et al., 2011).

The analysis of antimicrobial resistance profiles showed 71 isolates (35% of the total) were ESBL-producers. Similar levels of ESBL producing UPEC have been reported in some countries in the Asia-Pacific region and in Middle East (Al-Zarouni et al., 2008; Lu et al., 2012; Zowawi et al., 2013). This is in stark contrast to data for some other regions, where the reported prevalence of ESBL producing isolates in similar collections of UPEC from community and hospital patients is much lower. In the EU and North America, for example, reported rates are often close to or below 5% (Gibreel et al., 2012b; Gündoğdu et al., 2012; Zhanel et al., 2010). Many of the ESBL producing isolates also showed co-resistance to non-beta-lactam antibiotics including ciprofloxacin (n=43, 61% of the ESBL isolates) and trimethoprim-sulfamethoxazole (n=45, 63% of the ESBL isolates) and more than 65% of the CTX-M-15 positive isolates were co-resistant to both ciprofloxacin and trimethoprim-sulfamethoxazole.

These results are a cause of concern, as these organisms will lead to limited treatment options for UPEC infection. The high prevalence of ESBL is already driving high levels of carbapenem prescriptions in the community in Riyadh (S. M. Al Johani, personal communication), which is a genuine threat to the future utility of these antibiotics for clinical therapy

In conclusion, we have described the population structure of UPEC from a major medical centre in Saudi Arabia, which reveals the presence of multidrug-resistant strains with high virulence potential. Members of the ST131 lineage constitute a key UPEC clone, which, in our study, was unusually associated with a profile of high virulence in addition to broad antibiotic resistance. This is in contrast to many previous reports that suggest ST131 isolates are antibiotic resistant, but do not carry extensive virulence capacity. ST38 is a recently evolving lineage that can carry both UPEC and EAEC virulence determinants and warrants close monitoring. Further studies are required to limit the spread of the major UPEC lineages, which can display a high virulence potential and a wide spectrum of drug resistance, including the recent dissemination of carbapenem resistant NDM-1 and OXA-48 *E. coli* strains (Abraham et al., 2014; Yamamoto et al., 2011; Zowawi et al., 2013).

Chapter Five

5 General Discussion

This study presents the molecular epidemiology of ExPEC strains recovered from different infection sites and geographical locations. Major pandemic clonal lineages of ExPEC have been described, based on MLST characterisation. These leading clones are associated with both community-onset and healthcare-associated infections (Lau et al., 2008b; Park et al., 2012; Riley, 2014; Tartof et al., 2005) and include a variety of STs that have been commonly reported as predominant STs (ST69, ST73, ST95 and ST131) (Doumith et al., 2015; Gibreel et al., 2012b). Other STs among the major clonal lineages are characterised by the prevalence of multidrug resistance, high virulence potential and diverse metabolic profiles; these include ST10 (Oteo et al., 2009), ST14 (Lau et al., 2008b), ST23 (Oteo et al., 2009), ST38 (Chattaway et al., 2014a), ST127 (Alghoribi et al., 2014), ST393 (Blanco et al., 2011), ST405 (Matsumura et al., 2012) and ST410 (Mavroidi et al., 2012b). On the basis of the widely acknowledged utility of MLST, this methodology was selected for use in the current study. On the whole, the work revealed the presence of profiles similar to the most commonly detected STs reported in previous epidemiological studies of UPEC.

Interestingly, the collection of UPEC strains from Saudi Arabia showed a high proportion of the ST38 clone (7.4% n=15), which has been reported recently as an evolving extraintestinal enteroaggregative *E. coli* clone (Chattaway et al., 2014a). In the public MLST database, which contains 7,647 *E. coli* entries (as of March 2015), the ST38 clonal group is reported more frequently as UPEC (44%) than EAEC (37%).

However, it was reported that analysis of an in-house MLST database at the Gastrointestinal Bacterial Reference Unit at PHE showed that ST38 is a successful EAEC pathogen (Chattaway et al., 2014a). The other collections of UPEC investigated in this project did contain members of ST38, but at a low frequency. The Saudi ST38 isolates have been investigated for the presence of the EAEC virulence marker, *aggR*. The results indicate that some stains of Saudi ST38 (27%) carry this gene, which is consistent with a previous report that suggested ST38 UPEC may represent an evolving clonal group (Chattaway et al., 2014a). Despite the low number of ST38 isolates detected in blood, urine and biliary sepsis populations, ST38 isolates in this collection showed a high degree of metabolic activity. The metabolic potential of Saudi ST38 strains was not investigated, but the findings reported here support a suggestion that the metabolic potential of ST38 may be a fitness advantage to facilitate persistence in different host niches. A number of studies have highlighted the possible link between metabolism and virulence in pathogenic bacteria (Alteri and Mobley, 2012; Le Bouguénec and Schouler, 2011; Rohmer et al., 2011) with other studies proposed that bacterial metabolic diversity may increase the fitness of *E. coli* during UTI (Alteri et al., 2009; Gibreel et al., 2012a). Bacterial populations in the intestine become established by controlling each other through metabolic competition, limiting nutrients (Freter et al., 1983; Le Bouguénec and Schouler, 2011). This may be the underlying driver for selection of ST38 strains with a fitness advantage conferred through both EAEC and UPEC genetic backgrounds. The epidemiology of ST38 UPEC/EAEC should be the focus of future studies.

Numerous studies show the high prevalence of antimicrobial resistance among UPEC isolates worldwide. In the current studies, an unusually high prevalence of antimicrobial resistance was observed among the Saudi UPEC population in a range of strains, including those from ST38, ST131 and ST405. This included resistance to various types of β-lactam antibiotics, including the third-generation cephalosporins, fluoroquinolones and other antibiotic classes. The spread of multidrug resistant UPEC strains/clones is a real cause for concern, especially given the alarming levels of resistance in the Riyadh region; the strains or mechanisms responsible for resistance may be transmitted to other areas of the globe.

Global spread has been demonstrated for several UPEC clones, including ST131 (Petty et al., 2014; Price et al., 2013). The current results support the previous suggestion that ST38 is an evolving clonal group warranting close monitoring. Continued efforts are required to limit dissemination of highly virulent strains carrying a wide spectrum of antibiotic resistance mechanisms.

In the current study, larvae of the greater wax moth, *G. mellonella* were shown to be susceptible to infection by UPEC, representing an easy and excellent method to investigate bacterial pathogenicity. This infection model can be used to assess and verify the virulence capability of members of the major lineages of UPEC. The findings demonstrate that ST127 isolates are, with one exception, highly virulent. Comparative genomics between virulent and avirulent ST127 strains illustrate that the O antigen is an essential virulence factor for UPEC infection of *Galleria* larvae. Given their virulence, the incidence of ST127 strains should be monitored, as these isolates have not yet been widely reported, but they clearly have a pathogenic potential above that of more widely recognized clones.

It will be interesting to investigate the activity of the ST38 strains in this model, to see if those with an EAEC background differ in virulence to those with no evidence of enteroaggregative virulence factors.

The findings of this work demonstrate that there is much still to learn about the virulence, evolution and the level and dissemination of antimicrobial resistance in UPEC. Variation in virulence determinants in UPEC provides evidence of multiple pathotypes, but the current understanding of virulence is limited. Discovery of novel virulence factors involved in uropathogenesis and urosepsis will further define UPEC pathotypes. This can be facilitated through the use of next generation sequencing approaches for comparative genomic analysis, saturation mutagenesis methods and RNAseq studies. These experiments might help identification of sets of specific virulence or resistance genes that are driving the successful dissemination of the major UPEC lineages and contributing to the emergence of previously under reported strains.

References

6 References

Aballay, A., Drenkard, E., Hilbun, L.R., Ausubel, F.M., 2003. Caenorhabditis elegans innate immune response triggered by Salmonella enterica requires intact LPS and is mediated by a MAPK signaling pathway. Curr. Biol. 13, 47–52.

Abdallah, K.S., Cao, Y., Wei, D.J., 2011. Epidemiologic investigation of extra-intestinal pathogenic E. coli (ExPEC) based on PCR phylogenetic group and fimH single nucleotide polymorphisms (SNPs) in China. Int. J. Mol. Epidemiol. Genet. 2, 339–353.

Abe, C.M., Salvador, F.A., Falsetti, I.N., Vieira, M.A.M., Blanco, J., Blanco, J.E., Blanco, M., MacHado, A.M.O., Elias, W.P., Hernandes, R.T., Gomes, T.A.T., 2008. Uropathogenic Escherichia coli (UPEC) strains may carry virulence properties of diarrhoeagenic E. coli. FEMS Immunol. Med. Microbiol. 52, 397–406.

Abraham, E.P., Chain, E., 1940. An enzyme from bacteria able to destroy penicillin. Nature 146.3713, 837–837.

Abraham, S., Wong, H.S., Turnidge, J., Johnson, J.R., Trott, D.J., 2014. Carbapenemase-producing bacteria in companion animals: A public health concern on the horizon. J. Antimicrob. Chemother. 69, 1155–1157.

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, 490–7.

Ahmed, D., Wahid, S.U.H., Sadique, T., Sultana, N., Islam, M., Halim, F., Islam, N., Hossain, A., 2014. Recurrent urinary tract infection due to co-infection with extended spectrum -lactamase-producer uropathogenic Escherichia coli and enteroaggregative E. coli. JMM Case Reports 1, e001404–e001404.

Aibinu, I., Odugbemi, T., Koenig, W., Ghebremedhin, B., 2012. Sequence Type ST131 and ST10 Complex (ST617) predominant among CTX-M-15-producing Escherichia coli isolates from Nigeria. Clin. Microbiol. Infect. 18, 2011–2013.

Al-Agamy, M.H., Shibl, A.M., Hafez, M.M., Al-Ahdal, M.N., Memish, Z. a, Khubnani, H., 2014. Molecular characteristics of extended-spectrum β-lactamase-producing Escherichia coli in Riyadh: emergence of CTX-M-15-producing E. coli ST131. Ann. Clin. Microbiol. Antimicrob. 13, 4.

Al-Agamy, M.H.M., Shibl, A.M., Radwan, H.H., 2012. Detection of mutations in quinolone-resistant determining regions in clinical isolates of Escherichia coli from Saudi Arabia. AFRICAN J. Biotechnol. 11, 1054–1058.

Al-Zarouni, M., Senok, A., Rashid, F., Al-Jesmi, S.M., Panigrahi, D., 2008. Prevalence and antimicrobial susceptibility pattern of extended-spectrum beta-lactamase-producing enterobacteriaceae in the United Arab Emirates. Med. Princ. Pract. 17, 32–36.

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, e101547.

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, 402.

Alqasim, A., Emes, R., Clark, G., Newcombe, J., La Ragione, R., McNally, A., 2014. Phenotypic microarrays suggest Escherichia coli ST131 is not a metabolically distinct lineage of extra-intestinal pathogenic E. coli. PLoS One 9, e88374.

Alteri, C.J., Mobley, H.L.T., 2012. Escherichia coli physiology and metabolism dictates adaptation to diverse host microenvironments. Curr. Opin. Microbiol. 15, 3–9.

Alteri, C.J., Smith, S.N., Mobley, H.L.T., 2009. Fitness of Escherichia coli during urinary tract infection requires gluconeogenesis and the TCA cycle. PLoS Pathog. 5, e1000448.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–10.

Ananias, M., Yano, T., 2008. Serogroups and virulence genotypes of Escherichia coli isolated from patients with sepsis. Brazilian J. Med. Biol. Res. 41, 877–883.

Arber, W., 2014. Horizontal Gene Transfer among Bacteria and its Role in Biological Evolution. Life 4, 217–224.

Arena, F., Rolfe, P.A., Doran, G., Conte, V., Gruszka, S., Clarke, T., Adesokan, Y., Giani, T., Rossolini, G.M., 2014. Rapid resistome fingerprinting and clonal lineage profiling of carbapenem-resistant Klebsiella pneumoniae isolates by targeted next-generation sequencing. J. Clin. Microbiol. 52, 987–990.

Arthur, M., Johnson, C.E., Rubin, R.H., Arbeit, R.D., Campanelli, C., Kim, C., Steinbach, S., Agarwal, M., Wilkinson, R., Goldstein, R., 1989. Molecular epidemiology of adhesin and hemolysin virulence factors among uropathogenic Escherichia coli. Infect. Immun. 57, 303–313.

Banerjee, R., Johnson, J.R., 2014. A new clone sweeps clean: The enigmatic emergence of Escherichia coli sequence type 131. Antimicrob. Agents Chemother. 58, 4997–5004.

Banerjee, R., Johnston, B., Lohse, C., Chattopadhyay, S., Tchesnokova, V., Sokurenko, E. V, Johnson, J.R., 2013a. The clonal distribution and diversity of extraintestinal Escherichia coli varies according to patient characteristics. Antimicrob. Agents Chemother. 57, 5912–5917.

Banerjee, R., Robicsek, A., Kuskowski, M.A., Porter, S., Johnston, B.D., Sokurenko, E., Tchesnokova, V., Price, L.B., Johnson, J.R., 2013b. Molecular epidemiology of Escherichia coli sequence type 131 and its H30 and H30-Rx subclones among extended-spectrum-beta-lactamase-positive and -negative E. coli clinical isolates from the Chicago region, 2007 to 2010. Antimicrob. Agents Chemother. 57, 6385–6388.

Barillova, P., Tchesnokova, V., Dübbers, A., Küster, P., Peters, G., Dobrindt, U., Sokurenko, E. V., Kahl, B.C., 2014. Prevalence and persistence of Escherichia coli in the airways of cystic fibrosis patients—An unrecognized CF pathogen. Int. J. Med. Microbiol. 304, 415–421.

Barondess, J.J., Beckwith, J., 1995. bor Gene of phage lambda, involved in serum resistance, encodes a widely conserved outer membrane lipoprotein. J. Bacteriol. 177, 1247–1253.

Basu, S., Mukherjee, S.K., Hazra, A., Mukherjee, M., 2013. Molecular characterization of uropathogenic Escherichia coli: Nalidixic acid and ciprofloxacin resistance, virulent factors and phylogenetic background. J. Clin. Diagnostic Res. 7, 2727–2731.

Bauernfeind, A., Grimm, H., Schweighart, S., 1990. A new plasmidic cefotaximase in a clinical isolate of Escherichia coli. Infection 18, 294–298.

Beceiro, A., Tomás, M., Bou, G., 2013. Antimicrobial resistance and virulence: A successful or deleterious association in the bacterial world? Clin. Microbiol. Rev.

Bell, J.R., 2008. A simple way to treat PCR products prior to sequencing using ExoSAP-IT. Biotechniques 44, 834.

Ben Mkaddem, S., Chassin, C., Vandewalle, A., 2010. Contribution of renal tubule epithelial cells in the innate immune response during renal bacterial infections and ischemia-reperfusion injury. Chang Gung Med. J. 33, 225–240.

Bender, J.K., Wille, T., Blank, K., Lange, A., Gerlach, R.G., 2013. LPS structure and PhoQ activity are important for Salmonella Typhimurium virulence in the Galleria mellonella infection model [corrected]. PLoS One 8, e73287.

Bengtsson, S., Naseer, U., Sundsfjord, A., Kahlmeter, G., Sundqvist, M., 2012. Sequence types and plasmid carriage of uropathogenic Escherichia coli devoid of phenotypically detectable resistance. J. Antimicrob. Chemother. 67, 69–73.

Berg, J.M., Tymoczko, J.L., Stryer, L., 2002. Cells Can Respond to Changes in Their Environments. W H Freeman, New York.

Bergsten, G., Wullt, B., Svanborg, C., 2005. Escherichia coli, fimbriae, bacterial persistence and host response induction in the human urinary tract. Int. J. Med. Microbiol. 295, 487–502.

Berry, R.E., Klumpp, D.J., Schaeffer, A.J., 2009. Urothelial cultures support intracellular bacterial community formation by uropathogenic Escherichia coli. Infect. Immun. 77, 2762–72.

Bert, F., Johnson, J.R., Ouattara, B., Leflon-Guibout, V., Johnston, B., Marcon, E., Valla, D., Moreau, R., Nicolas-Chanoine, M.-H., 2010. Genetic diversity and virulence profiles of Escherichia coli isolates causing spontaneous bacterial peritonitis and bacteremia in patients with cirrhosis. J. Clin. Microbiol. 48, 2709–14.

Beutin, L., Kong, Q., Feng, L., Wang, Q., Krause, G., Leomil, L., Jin, Q., Wang, L., 2005. Development of PCR assays targeting the genes involved in synthesis and assembly of the new Escherichia coli O 174 and O 177 O antigens. J. Clin. Microbiol. 43, 5143–9.

Bidet, P., Mahjoub-Messai, F., Blanco, J., Blanco, J., Dehem, M., Aujard, Y., Bingen, E., Bonacorsi, S., 2007. Combined multilocus sequence typing and O serogrouping distinguishes Escherichia coli subtypes associated with infant urosepsis and/or meningitis. J. Infect. Dis. 196, 297–303.

Bien, J., Sokolova, O., Bozko, P., 2012. Role of uropathogenic escherichia coli virulence factors in development of urinary tract infection and kidney damage. Int. J. Nephrol. 10, 15.

Blahna, M.T., Zalewski, C.A., Reuer, J., Kahlmeter, G., Foxman, B., Marrs, C.F., 2006. The role of horizontal gene transfer in the spread of trimethoprim-sulfamethoxazole resistance among uropathogenic Escherichia coli in Europe and Canada. J. Antimicrob. Chemother. 57, 666–672.

Blanco, J., Mora, A., Mamani, R., López, C., Blanco, M., Dahbi, G., Herrera, A., Blanco, J.E., Alonso, M.P., García-Garrote, F., Chaves, F., Orellana, M.Á., Martínez-Martínez, L., Calvo, J., Prats, G., Larrosa, M.N., González-López, J.J., López-Cerero, L., Rodríguez-Baño, J., Pascual, A., 2011. National survey of Escherichia coli causing extraintestinal infections reveals the spread of drug-resistant clonal groups O25b:H4-B2-ST131, O15:H1-D-ST393 and CGA-D-ST69 with high virulence gene content in Spain. J. Antimicrob. Chemother. 66, 2011–21.

Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschäpe, 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, 606–14.

Bower, J.M., Eto, D.S., Mulvey, M.A., 2005. Covert operations of uropathogenic Escherichia coli within the urinary tract. Traffic 6, 18–31.

Brennan, M., Thomas, D.Y., Whiteway, M., Kavanagh, K., 2002. Correlation between virulence of Candida albicans mutants in mice and Galleria mellonella larvae. FEMS Immunol. Med. Microbiol. 34, 153–157.

Brolund, A., Sundqvist, M., Kahlmeter, G., Grape, M., 2010. Molecular characterisation of trimethoprim resistance in Escherichia coli and Klebsiella pneumoniae during a two year intervention on trimethoprim use. PLoS One 5, 1–5.

Brown, S.E., Howard, A., Kasprzak, A.B., Gordon, K.H., East, P.D., 2009. A peptidomics study reveals the impressive antimicrobial peptide arsenal of the wax moth Galleria mellonella. Insect Biochem. Mol. Biol. 39, 792–800.

Browning, D.F., Wells, T.J., França, F.L.S., Morris, F.C., Sevastsyanovich, Y.R., Bryant, J. a, Johnson, M.D., Lund, P. a, Cunningham, A.F., Hobman, J.L., May, R.C., Webber, M. a, Henderson, I.R., 2013. Laboratory adapted Escherichia coli K-12 becomes a pathogen of Caenorhabditis elegans upon restoration of O antigen biosynthesis. Mol. Microbiol. 87, 939–50.

Cabot, G., Bruchmann, S., Mulet, X., Zamorano, L., Moyá, B., Juan, C., Haussler, S., Olivera, A., 2014. Pseudomonas aeruginosa ceftolozane-tazobactam resistance development requires multiple mutations leading to overexpression and structural modification of ampc. Antimicrob. Agents Chemother. 58, 3091–3099.

Cantón, R., Coque, T.M., 2006. The CTX-M β-lactamase pandemic. Curr. Opin. Microbiol. 9, 466–475.

Cantón, R., González-Alba, J.M., Galán, J.C., 2012. CTX-M Enzymes: Origin and Diffusion. Front. Microbiol. 3, 110.

Cao, X., Zhang, Z., Shen, H., Ning, M., Chen, J., Wei, H., Zhang, K., 2014. Genotypic characteristics of multidrug-resistant Escherichia coli isolates associated with urinary tract infections. APMIS 122, 1088–95.

Caroff, N., Espaze, E., Bérard, I., Richet, H., Reynaud, A., 1999. Mutations in the ampC promoter of Escherichia coli isolates resistant to oxyiminocephalosporins without extended spectrum β-lactamase production. FEMS Microbiol. Lett. 173, 459–465.

Carver, T.J., Rutherford, K.M., Berriman, M., Rajandream, M.-A., Barrell, B.G., Parkhill, J., 2005. ACT: the Artemis Comparison Tool. Bioinformatics 21, 3422–3.

Castellani, A., 1919. Manual of tropical medicine,. Baillière Tindall and Cox, London.

Cerquetti, M., Giufrè, M., García-Fernández, A., Accogli, M., Fortini, D., Luzzi, I., Carattoli, A., 2010. Ciprofloxacin-resistant, CTX-M-15-producing Escherichia coli ST131 clone in extraintestinal infections in Italy. Clin. Microbiol. Infect. 16, 1555–8.

Chakupurakal, R., Ahmed, M., Sobithadevi, D.N., Chinnappan, S., Reynolds, T., 2010. Urinary tract pathogens and resistance pattern. J. Clin. Pathol. 63, 652–654.

Champion, O.L., Cooper, I.A.M., James, S.L., Ford, D., Karlyshev, A., Wren, B.W., Duffield, M., Oyston, P.C.F., Titball, R.W., 2009. Galleria mellonella as an alternative infection model for Yersinia pseudotuberculosis. Microbiology 155, 1516–1522.

Chang, J., Yu, J., Lee, H., Ryu, H., Park, K., Park, Y.-J., 2014. Prevalence and characteristics of lactose non-fermenting Escherichia coli in urinary isolates. J. Infect. Chemother. 20, 738–740.

Chaslus-Dancla, E., Pohl, P., Meurisse, M., Marin, M., Lafont, J.P., 1991. High genetic homology between plasmids of human and animal origins ferring resistance to the aminoglycosides gentamicin and apramycin. Antimicrob. Agents Chemother. 35, 590–593.

Chattaway, M.A., Jenkins, C., Ciesielczuk, H., Day, M., DoNascimento, V., Day, M., Rodríguez, I., van Essen-Zandbergen, A., Schink, A.-K., Wu, G., Threlfall, J., Woodward, M.J., Coldham, N., Kadlec, K., Schwarz, S., Dierikx, C., Guerra, B., Helmuth, R., Mevius, D., Woodford, N., Wain, J., 2014a. Evidence of Evolving Extraintestinal Enteroaggregative Escherichia coli ST38 Clone. Emerg. Infect. Dis. 20, 1935–7.

Chattaway, M.A., Jenkins, C., Rajendram, D., Cravioto, A., Talukder, K.A., Dallman, T., Underwood, A., Platt, S., Okeke, I.N., Wain, J., 2014b. Enteroaggregative Escherichia coli Have Evolved Independently as Distinct Complexes within the E. coli Population with Varying Ability to Cause Disease. PLoS One 9, e112967.

Chaudhuri, R.R., Henderson, I.R., 2012. The evolution of the Escherichia coli phylogeny. Infect. Genet. Evol.

Chen, L., Hu, H., Chavda, K.D., Zhao, S., Liu, R., Liang, H., Zhang, W., Wang, X., Jacobs, M.R., Bonomo, R.A., Kreiswirth, B.N., 2014. Complete sequence of a KPC-producing IncN multidrug-resistant plasmid from an epidemic Escherichia coli sequence type 131 strain in China. Antimicrob. Agents Chemother. 58, 2422–2425.

Chen, Y., Chen, X., Zheng, S., Yu, F., Kong, H., Yang, Q., Cui, D., Chen, N., Lou, B., Li, X., Tian, L., Yang, X., Xie, G., Dong, Y., Qin, Z., Han, D., Wang, Y., Zhang, W., Tang, Y.W., Li, L., 2014. Serotypes, genotypes and antimicrobial resistance patterns of human diarrhoeagenic Escherichia coli isolates circulating in southeastern China. Clin. Microbiol. Infect. 20, 52–58.

Clermont, O., Bonacorsi, S., Bingen, E., 2000. Rapid and Simple Determination of the Escherichia coli Phylogenetic Group. Appl. Environ. Microbiol. 66, 4555–4558.

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, 58–65.

Clermont, O., Johnson, J.R., Menard, M., Denamur, E., 2007. Determination of Escherichia coli O types by allele-specific polymerase chain reaction: application to the O types involved in human septicemia. Diagn. Microbiol. Infect. Dis. 57, 129–36.

Clermont, O., Lavollay, M., Vimont, S., Deschamps, C., Forestier, C., Branger, C., Denamur, E., Arlet, G., 2008. The CTX-M-15-producing Escherichia coli diffusing clone belongs to a highly virulent B2 phylogenetic subgroup. J. Antimicrob. Chemother. 61, 1024–1028.

Coque, T.M., Novais, A., Carattoli, A., Poirel, L., Pitout, J., Peixe, L., Baquero, F., Cantón, R., Nordmann, P., 2008. Dissemination of clonally related Escherichia coli strains expressing extended-spectrum beta-lactamase CTX-M-15. Emerg. Infect. Dis. 14, 195–200.

Correia, S., Pacheco, R., Radhouani, H., Diniz, J.C., Ponce, P., Jones-Dias, D., Caniça, M., Igrejas, G., Poeta, P., 2012. High prevalence of ESBL-producing Escherichia coli isolates among hemodialysis patients in Portugal: appearance of ST410 with the bla(CTX-M-14) gene. Diagn. Microbiol. Infect. Dis. 74, 423–5.

Corvec, S., Caroff, N., Espaze, E., Marraillac, J., Reynaud, A., 2002. -11 Mutation in the ampC promoter increasing resistance to beta-lactams in a clinical Escherichia coli strain. Antimicrob. Agents Chemother. 46, 3265–3267.

Cotter, G., Doyle, S., Kavanagh, K., 2000. Development of an insect model for the in vivo pathogenicity testing of yeasts. FEMS Immunol. Med. Microbiol. 27, 163–9.

Croucher, N.J., Thomson, N.R., 2010. Studying bacterial transcriptomes using RNA-seq. Curr. Opin. Microbiol.

Croxall, G., Hale, J., Weston, V., Manning, G., Cheetham, P., Achtman, M., Mcnally, A., 2011. 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, 2501–8.

Cytryńska, M., Mak, P., Zdybicka-Barabas, A., Suder, P., Jakubowicz, T., 2007. Purification and characterization of eight peptides from Galleria mellonella immune hemolymph. Peptides 28, 533–546.

Dahbi, G., Mora, A., Mamani, R., López, C., Pilar, M., Marzoa, J., Blanco, M., Herrera, A., Viso, S., García-garrote, F., Tchesnokova, V., Billig, M., De, F., Toro, M. De, González-lópez, J.J., Prats, G., Chaves, F., Martínez-martínez, L., López-cerezo, L., Denamur, E., Blanco, J., 2014. International Journal of Medical Microbiology Molecular epidemiology and virulence of Escherichia coli O16 : H5-ST131 : Comparison with H 30 and H 30-Rx subclones of O25b : H4-ST131. Int. J. Med. Microbiol. 304, 1247–57.

Dallenne, C., da Costa, A., Decré, D., Favier, C., Arlet, G., 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. J. Antimicrob. Chemother. 65, 490–495.

Davies, J., Courvalin, P., 1977. Mechanisms of resistance to aminoglycosides. Am. J. Med. 62, 868–872.

De Kraker, M.E. a, Wolkewitz, M., Davey, P.G., Koller, W., Berger, J., Nagler, J., Icket, C., Kalenic, S., Horvatic, J., Seifert, H., Kaasch, a., Paniara, O., Argyropoulou, a., Bompola, M., Smyth, E., Skally, M., Raglio, a., Dumpis, U., Kelmere, a. M., Borg, M., Xuereb, D., Ghita, M.C., Noble, M., Kolman, J., Grabljevec, S., Turner, D., Lansbury, L., Grundmann, H., 2011. Burden of antimicrobial resistance in European hospitals: Excess mortality and length of hospital stay associated with bloodstream infections due to Escherichia coli resistant to third-generation cephalosporins. J. Antimicrob. Chemother. 66, 398–407.

DebRoy, C., Roberts, E., Fratamico, P.M., 2011. Detection of O antigens in Escherichia coli. Anim. Health Res. Rev. 12, 169–85.

Desbois, A.P., Coote, P.J., 2012. Utility of Greater Wax Moth Larva (Galleria mellonella) for Evaluating the Toxicity and Efficacy of New Antimicrobial Agents. Adv. Appl. Microbiol. 78, 25–53.

Dhakal, B.K., Mulvey, M.A., 2012. The UPEC pore-forming toxin α-hemolysin triggers proteolysis of host proteins to disrupt cell adhesion, inflammatory, and survival pathways. Cell Host Microbe 11, 58–69.

Diard, M., Baeriswyl, S., Clermont, O., Gouriou, S., Picard, B., Taddei, F., Denamur, E., Matic, I., 2007. Caenorhabditis elegans as a simple model to study phenotypic and genetic virulence determinants of extraintestinal pathogenic Escherichia coli. Microbes Infect. 9, 214–223.

Dias, R.C.S., Moreira, B.M., Riley, L.W., 2010. Use of fimH single-nucleotide polymorphisms for strain typing of clinical isolates of Escherichia coli for epidemiologic investigation. J. Clin. Microbiol. 48, 483–488.

Dimou, V., Dhanji, H., Pike, R., Livermore, D.M., Woodford, N., 2012. Characterization of Enterobacteriaceae producing OXA-48-like carbapenemases in the UK. J. Antimicrob. Chemother. 67, 1660–5.

Dixit, P.D., Pang, T.Y., Studier, F.W., Maslov, S., 2015. Recombinant transfer in the basic genome of Escherichia coli. Proc. Natl. Acad. Sci. 2015, 201510839.

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, 6365–72.

Donnenberg, M., 2002. E. coli: Genomics, Evolution and Pathogenesis.

Dortet, L., Poirel, L., Nordmann, P., 2014. Worldwide dissemination of the NDM-Type carbapenemases in Gram-negative bacteria. Biomed Res. Int. 2014, 12.

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 53, 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, 3108–3110.

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, 18944–51.

DuPont, H.L., Formal, S.B., Hornick, R.B., Snyder, M.J., Libonati, J.P., Sheahan, D.G., LaBrec, E.H., Kalas, J.P., 1971. Pathogenesis of Escherichia coli diarrhea. N. Engl. J. Med. 285, 1–9.

ECDC, 2013. Antimicrobial resistance surveillance in Europe Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2013. Stockholm.

Edwards, P.R., Ewing, W.H., 1986. Edwards and Ewing's identification of Enterobacteriaceae. Elsevier, Michigan.

Egea, P., López-Cerero, L., Torres, E., Gómez-Sánchez, M.D.C., Serrano, L., Navarro Sánchez-Ortiz, M.D., Rodriguez-Baño, J., Pascual, A., 2012. Increased raw poultry meat colonization by extended spectrum beta-lactamase-producing Escherichia coli in the south of Spain. Int. J. Food Microbiol. 159, 69–73.

Ejrnæs, K., 2011. Bacterial characteristics of importance for recurrent urinary tract infections caused by Escherichia coli. Dan. Med. Bull. 58, B4187.

Emody, L., Kerényi, M., Nagy, G., 2003. Virulence factors of uropathogenic Escherichia coli. Int. J. Antimicrob. Agents 22, 29–33.

Eriksson-Grennberg, K.G., Boman, H.G., Jansson, J.A.T., Thoren, S., 1965. Resistance of Escherichia coli to Penicillins I. Genetic Study of Some Ampicillin-Resistant Mutants. J. Bacteriol. 90, 54–62.

Escherich, T., 1989. The intestinal bacteria of the neonate and breast-fed infant. 1884. Rev. Infect. Dis. 10, 1220–1225.

ESPAUR, 2014. English surveillance programme for antimicrobial utilisation and resistance (ESPAUR). London, Public Health England.

Evans, B.A., Rozen, D.E., 2012. A Streptococcus pneumoniae infection model in larvae of the wax moth Galleria mellonella. Eur. J. Clin. Microbiol. Infect. Dis. 31, 2653–60.

Ewers, C., Bethe, A., Semmler, T., Guenther, S., Wieler, L.H., 2012. Extended-spectrum β-lactamase-producing and AmpC-producing Escherichia coli from livestock and companion animals, and their putative impact on public health: a global perspective. Clin. Microbiol. Infect. 18, 646–55.

Farmer, J.J., Davis, B.R., Hickman-Brenner, F.W., McWhorter, A., Huntley-Carter, G.P., Asbury, M.A., Riddle, C., Wathen-Grady, H.G., Elias, C., Fanning, G.R., 1985. Biochemical identification of new species and biogroups of Enterobacteriaceae isolated from clinical specimens. J. Clin. Microbiol. 21, 46–76.

Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., Spratt, B.G., 2004. eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data. J. Bacteriol. 186, 1518–1530.

Ferguson, W.W., June, R.C., 1952. Experiments on feeding adult volunteers with Escherichia coli 111, B4, a coliform organism associated with infant diarrhea. Am. J. Hyg. 55, 155–169.

Fiett, J., Baraniak, A., Izdebski, R., Sitkiewicz, I., Żabicka, D., Meler, A., Filczak, K., Hryniewicz, W., Gniadkowski, M., 2014. The first NDM metallo-β-lactamase-producing Enterobacteriaceae isolate in Poland: evolution of IncFII-type plasmids carrying the bla(NDM-1) gene. Antimicrob. Agents Chemother. 58, 1203–7.

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, 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, 1589–1596.

Foxman, B., 2003. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Dis. Mon. 49, 53–70.

Freter, R., Brickner, H., Botney, M., Cleven, D., Aranki, A., 1983. Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. Infect. Immun. 39, 676–85.

Funke, G., Monnet, D., DeBernardis, C., Von Graevenitz, A., Freney, J., 1998. Evaluation of the VITEK 2 system for rapid identification of medically relevant gram-negative rods. J. Clin. Microbiol. 36, 1948–1952.

Furuya, E.Y., Lowy, F.D., 2006. Antimicrobial-resistant bacteria in the community setting. Nat. Rev. Microbiol. 4, 36–45.

Garcı, a, 2010. producing Escherichia coli ST131 clone in extraintestinal infections in Italy. October 16, 21–24.

Gavin, P.J., Warren, J.R., Obias, A.A., Collins, S.M., Peterson, L.R., 2002. Evaluation of the Vitek 2 system for rapid identification of clinical isolates of gram-negative bacilli and members of the family Streptococcaceae. Eur. J. Clin. Microbiol. Infect. Dis. 21, 869–74.

Gibreel, 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, 3202–7.

Gibreel, 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, 346–356.

Gibreel, T.M., 2011. Molecular Epidemiology of Uropathogenic Escherichia coli in North West England and Characterisation of the ST131 clone in the Region. The University of Manchester, Manchester, UK.

Giufrè, M., Graziani, C., Accogli, M., Luzzi, I., Busani, L., Cerquetti, M., 2012. Escherichia coli of human and avian origin: Detection of clonal groups associated with fluoroquinolone and multidrug resistance in Italy. J. Antimicrob. Chemother. 67, 860–867.

Goller, C.C., Seed, P.C., 2010. Revisiting the Escherichia coli polysaccharide capsule as a virulence factor during urinary tract infection: contribution to intracellular biofilm development. Virulence 1, 333–337.

Griebling, T.L., 2005. Urologic diseases in america project: trends in resource use for urinary tract infections in men. J. Urol. 173, 1288–1294.

Guenther, S., Aschenbrenner, K., Stamm, I., Bethe, A., Semmler, T., Stubbe, A., Stubbe, M., Batsajkhan, N., Glupczynski, Y., Wieler, L.H., Ewers, C., 2012. Comparable High Rates of Extended-Spectrum-Beta-Lactamase-Producing Escherichia coli in Birds of Prey from Germany and Mongolia. PLoS One 7, e53039.

Guiral, E., Mendez-Arancibia, E., Soto, S.M., Salvador, P., Fabrega, A., Gascon, J., Vila, J., 2011. CTX-M-15-producing enteroaggregative Escherichia coli as cause of travelers' diarrhea. Emerg. Infect. Dis. 17, 1950–3.

Gündoğdu, A., Long, Y.B., Katouli, M., 2012. Prevalence and pathogenesis of extended-spectrum beta-lactamase producing Escherichia coli causing urinary tract infection in hospitalized patients. Eur. J. Clin. Microbiol. Infect. Dis. 31, 3107–16.

Gutmann, L., Ferre, B., Goldstein, F.W., Rizk, N., Pinto-Schuster, E., Acar, J.F., Collatz, E., 1989. SHV-5, a novel SHV-type β-lactamase that hydrolyzes broad-spectrum cephalosporins and monobactams. Antimicrob. Agents Chemother. 33, 951–956.

Hacker, J., Bender, L., Ott, M., Wingender, J., Lund, B., Marre, R., Goebel, W., 1990. Deletions of chromosomal regions coding for fimbriae and hemolysins occur in vitro and in vivo in various extraintestinal Escherichia coli isolates. Microb. Pathog. 8, 213–25.

Harada, K., Nakai, Y., Kataoka, Y., 2012. Mechanisms of resistance to cephalosporin and emergence of O25b-ST131 clone harboring CTX-M-27 β-lactamase in extraintestinal pathogenic Escherichia coli from dogs and cats in Japan. Microbiol. Immunol. 56, 480–5.

Harris, S.R., Cartwright, E.J.P., Török, M.E., Holden, M.T.G., Brown, N.M., Ogilvy-Stuart, A.L., Ellington, M.J., Quail, M.A., Bentley, S.D., Parkhill, J., Peacock, S.J., 2013. Whole-genome sequencing for analysis of an outbreak of meticillin-resistant Staphylococcus aureus: A descriptive study. Lancet Infect. Dis. 13, 130–136.

Hasman, H., Schembri, M. a., Klemm, P., 2000. Antigen 43 and type 1 fimbriae determine colony morphology of Escherichia coli K-12. J. Bacteriol. 182, 1089–1095.

Herzog, K., Engeler Dusel, J., Hugentobler, M., Beutin, L., Sägesser, G., Stephan, R., Hächler, H., Nüesch-Inderbinen, M., 2014. Diarrheagenic enteroaggregative Escherichia coli causing urinary tract infection and bacteremia leading to sepsis. Infection 42, 441–444.

Ho, P.-L., Wong, R.C., Lo, S.W., Chow, K.-H., Wong, S.S., Que, T.-L., 2010. Genetic identity of aminoglycoside-resistance genes in Escherichia coli isolates from human and animal sources. J. Med. Microbiol. 59, 702–707.

Ho, P.L., Ho, P.L., Chu, Y.P.-S., Lo, W.-U., Chow, K.-H., Law, P.Y., Tse, C.W.-S., Ng, T.-K., Cheng, V.C.-C., Que, T.-L., 2015. High prevalence of Escherichi coli sequence type 131 among antimicrobial-resistant E . coli isolates from geriatric patients JMM-D-14-00091 revised High Escherichia sequence. J. Med. Microbiol. 243–247, 0–22.

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, 584–95.

Hodkinson, B.P., Grice, E.A., 2014. Next-Generation Sequencing : A Review of Technologies and Tools for Wound Microbiome Research. Adv. wound care 4, 50–58.

Hounsom, L., Grayson, K., Melzer, M., 2011. Mortality and associated risk factors in consecutive patients admitted to a UK NHS trust with community acquired bacteraemia. Postgrad. Med. J. 87, 757–762.

Huovinen, P., Pulkkinen, L., Helin, H.L., Mäkilä, M., Toivanen, P., 1986. Emergence of trimethoprim resistance in relation to drug consumption in a Finnish Hospital from 1971 through 1984. Antimicrob. Agents Chemother. 29, 73–76.

Jacoby, G. a., 2009. AmpC beta-lactamases. Clin. Microbiol. Rev. 22, 161–182.

Jadhav, S., Hussain, A., Devi, S., Kumar, A., Parveen, S., Gandham, N., Wieler, L.H., Ewers, C., Ahmed, N., 2011. Virulence characteristics and genetic affinities of multiple drug resistant uropathogenic Escherichia coli from a semi urban locality in India. PLoS One 6, e18063.

Jander, G., Rahme, L.G., Ausubel, F.M., 2000. Positive correlation between virulence of Pseudomonas aeruginosa mutants in mice and insects. J. Bacteriol. 182, 3843–5.

Jarlier, V., Nicolas, M.H., Fournier, G., Philippon, A., 1988. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. 10, 867–878.

Jaurin, B., Grundström, T., 1981. ampC cephalosporinase of Escherichia coli K-12 has a different evolutionary origin from that of beta-lactamases of the penicillinase type. Proc. Natl. Acad. Sci. U. S. A. 78, 4897–901.

Johnson, a P., Malde, M., Woodford, N., Cunney, R.J., Smyth, E.G., 1995. Urinary isolates of apramycin-resistant Escherichia coli and Klebsiella pneumoniae from Dublin. Epidemiol. Infect. 114, 105–112.

Johnson, J.R., 1991. Virulence factors in Escherichia coli urinary tract infection. Clin. Microbiol. Rev. 4, 80–128.

Johnson, J.R., Bryan, T.T.O., Kuskowski, M., Maslow, J.N., 2001. Ongoing Horizontal and Vertical Transmission of Virulence Genes and papA Alleles among Escherichia coliBlood Isolates from Patients with Diverse-Source Bacteremia Ongoing Horizontal and Vertical Transmission of Virulence Genes and papA Alleles among Esche. Infect. Immun. 69, 5363.

Johnson, J.R., Clermont, O., Johnston, B., Clabots, C., Tchesnokova, V., Sokurenko, E., Junka, A.F., Maczynska, B., Denamur, E., 2014. Rapid and Specific Detection, Molecular Epidemiology, and Experimental Virulence of the O16 Subgroup within Escherichia coli Sequence Type 131. J. Clin. Microbiol. 52, 1358–65.

Johnson, J.R., Gajewski, A., Lesse, A.J., Russo, T. a, 2003a. Extraintestinal Pathogenic Escherichia coli as a Cause of Invasive Nonurinary Infections Extraintestinal Pathogenic Escherichia coli as a Cause of Invasive Nonurinary Infections. Society 41, 5798–5802.

Johnson, J.R., Johnston, B., Clabots, C., Kuskowski, M. a, Castanheira, M., 2010. Escherichia coli sequence type ST131 as the major cause of serious multidrug-resistant E. coli infections in the United States. Clin. Infect. Dis. 51, 286–294.

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, 417–22.

Johnson, J.R., Manges, A.R., O'Bryan, T.T., Riley, L.W., 2002a. A disseminated multidrug-resistant clonal group of uropathogenic Escherichia coli in pyelonephritis. Lancet 359, 2249–51.

Johnson, J.R., Menard, M., Johnston, B., Kuskowski, M.A., Nichol, K., Zhanel, G.G., 2009. Epidemic clonal groups of Escherichia coli as a cause of antimicrobial-resistant urinary tract infections in Canada, 2002 to 2004. Antimicrob. Agents Chemother. 53, 2733–9.

Johnson, J.R., Murray, A.C., Gajewski, A., Sullivan, M., Snippes, P., Kuskowski, M.A., Smith, K.E., 2003b. Isolation and molecular characterization of nalidixic acid-resistant extraintestinal pathogenic Escherichia coli from retail chicken products. Antimicrob. Agents Chemother. 47, 2161–2168.

Johnson, J.R., Murray, A.C., Kuskowski, M.A., Schubert, S., Prère, M.-F., Picard, B., Colodner, R., Raz, R., 2005. Distribution and characteristics of Escherichia coli clonal group A. Emerg. Infect. Dis. 11, 141–5.

Johnson, J.R., Nicolas-Chanoine, M.-H., DebRoy, C., Castanheira, M., Robicsek, A., Hansen, G., Weissman, S., Urban, C., Platell, J., Trott, D., Zhanel, G., Clabots, C., Johnston, B.D., Kuskowski, M.A., 2012. Comparison of Escherichia coli ST131 pulsotypes, by epidemiologic traits, 1967-2009. Emerg. Infect. Dis. 18, 598–607.

Johnson, J.R., Orskov, I., Orskov, F., Goullet, P., Picard, B., Moseley, S.L., Roberts, P.L., Stamm, W.E., 1994. O, K, and H Antigens Predict Virulence Factors, Carboxylesterase B Pattern, Antimicrobial Resistance, and Host Compromise among Escherichia coli Strains Causing Urosepsis. J. Infect. Dis. 169, 119–126.

Johnson, J.R., Roberts, P.L., Stamm, W.E., 1987. P fimbriae and other virulence factors in Escherichia coli urosepsis: association with patients' characteristics. J. Infect. Dis. 156, 225–229.

Johnson, J.R., Russo, T.A., 2005. Molecular epidemiology of extraintestinal pathogenic (uropathogenic) Escherichia coli. Int. J. Med. Microbiol. 295, 383–404.

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, 261–72.

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., 2002b. Global molecular epidemiology of the O15:K52:H1 extraintestinal pathogenic Escherichia coli clonal group: evidence of distribution beyond Europe. J. Clin. Microbiol. 40, 1913–23.

Johnson, J.R., Tchesnokova, V., Johnston, B., Clabots, C., Roberts, P.L., Billig, M., Riddell, K., Rogers, P., Qin, X., Butler-Wu, S., Price, L.B., Aziz, M., Nicolas-Chanoine, M.H., Debroy, C., Robicsek, A., Hansen, G., Urban, C., Platell, J., Trott, D.J., Zhanel, G., Weissman, S.J., Cookson, B.T., Fang, F.C., Limaye, A.P., Scholes, D., Chattopadhyay, S., Hooper, D.C., Sokurenko, E. V, 2013. Abrupt emergence of a single dominant multidrug-resistant strain of Escherichia coli. J. Infect. Dis. 207, 919–928.

Juhas, M., 2013. Horizontal gene transfer in human pathogens. Crit. Rev. Microbiol. 7828, 1–8.

Kalita, A., Hu, J., Torres, A.G., 2014. Recent advances in adherence and invasion of pathogenic Escherichia coli. Curr. Opin. Infect. Dis. 459–464.

Kaper, J.B., Nataro, J.P., Mobley, H.L., 2004. Pathogenic Escherichia coli. Nat. Rev. Microbiol. 2, 123–140.

Karmali, M.A., Gannon, V., Sargeant, J.M., 2010. Verocytotoxin-producing Escherichia coli (VTEC). Vet. Microbiol. 140, 360–370.

Kass, E.H., 1956. Asymptomatic infections of the urinary tract. Trans. Assoc. Am. Physicians 69, 56–64.

Katouli, M., 2010. Population structure of gut Escherichia coli and its role in development of extra-intestinal infections. Iran. J. Microbiol. 2, 59–72.

Katouli, M., Kühn, I., Möllby, R., 1990. Evaluation of the stability of biochemical phenotypes of Escherichia coli upon subculturing and storage. J. Gen. Microbiol. 136, 1681–1688.

Kauffmann, F., 1947. The serology of the coli group. J. Immunol. 57, 71–100.

Kauffmann, F., 1964. Serology and Chemistry of Enterobacteriaceae. Arb. Paul. Ehrlich. Inst. Georg. Speyer. Haus. Ferdinand. Blum. Inst. Frankf. A. M. 61, 17–28.

Kavanagh, K., Reeves, E.P., 2004. Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. FEMS Microbiol. Rev. 28, 101–12.

Kawamura-Sato, K., Yoshida, R., Shibayama, K., Ohta, M., 2010. Virulence genes, quinolone and fluoroquinolone resistance, and phylogenetic background of uropathogenic Escherichia coli strains isolated in Japan. Jpn. J. Infect. Dis. 63, 113–115.

Kayser, F.H., Bienz, K.A., Eckert, J., 2011. Medical Microbiology. Thieme, New York.

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, 3541–7.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–20.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1.

Köhler, C.D., Dobrindt, U., 2011. What defines extraintestinal pathogenic Escherichia coli? Int. J. Med. Microbiol. 301, 642–647.

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. Infect. Immun. 54, 322–7.

Köser, C.U., Holden, M.T.G., Ellington, M.J., Cartwright, E.J.P., Brown, N.M., Ogilvy-Stuart, A.L., Hsu, L.Y., Chewapreecha, C., Croucher, N.J., Harris, S.R., Sanders, M., Enright, M.C., Dougan, G., Bentley, S.D., Parkhill, J., Fraser, L.J., Betley, J.R., Schulz-Trieglaff, O.B., Smith, G.P., Peacock, S.J., 2012. Rapid Whole-Genome Sequencing for Investigation of a Neonatal MRSA Outbreak. N. Engl. J. Med. 366, 2267–2275.

Kühn, I., 1985. Biochemical fingerprinting of Escherichia coli: a simple method for epidemiological investigations. J. Microbiol. Methods 3, 159–170.

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, 1241–4.

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, 1076–80.

Lavollay, M., Mamlouk, K., Frank, T., Akpabie, A., Burghoffer, B., Ben Redjeb, S., Bercion, R., Gautier, V., Arlet, G., 2006. Clonal dissemination of a CTX-M-15 beta-lactamase-producing Escherichia coli strain in the Paris area, Tunis, and Bangui. Antimicrob. Agents Chemother. 50, 2433–8.

Le Bouguénec, C., Schouler, C., 2011. Sugar metabolism, an additional virulence factor in enterobacteria. Int. J. Med. Microbiol. 301, 1–6.

Lee, J.C., Oh, J.Y., Cho, J.W., Park, J.C., Kim, J.M., Seol, S.Y., Cho, D.T., 2001. The prevalence of trimethoprim-resistance-conferring dihydrofolate reductase genes in urinary isolates of Escherichia coli in Korea. J. Antimicrob. Chemother. 47, 599–604.

Lee, S., Yu, J.K., Park, K., Oh, E.J., Kim, S.Y., Park, Y.J., 2010. Phylogenetic groups and virulence factors in pathogenic and commensal strains of escherichia coli and their association with blaCTX-M. Ann. Clin. Lab. Sci. 40, 361–367.

Leffler, H., Edén, C.S., 1980. Chemical identification of a glycosphingolipid receptor for Escherichia coli attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. FEMS Microbiol. Lett. 8, 127–134.

Leflon-Guibout, V., Blanco, J., Amaqdouf, K., Mora, A., Guize, L., Nicolas-Chanoine, M.H., 2008. Absence of CTX-M enzymes but high prevalence of clones, including clone ST131, among fecal Escherichia coli isolates from healthy subjects living in the area of Paris, France. J. Clin. Microbiol. 46, 3900–3905.

Leuko, S., Raivio, T.L., 2012. Mutations that impact the enteropathogenic Escherichia coli Cpx envelope stress response attenuate virulence in Galleria mellonella. Infect. Immun. 80, 3077–85.

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, 71–7.

Lin, C.-F., Hsu, S.-K., Chen, C.-H., Huang, J.-R., Lo, H.-H., 2010. Genotypic detection and molecular epidemiology of extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in a regional hospital in central Taiwan. J. Med. Microbiol. 59, 665–71.

Liu, B., Wu, F., Li, D., Beutin, L., Chen, M., Cao, B., Wang, L., 2010. Development of a serogroup-specific DNA microarray for identification of Escherichia coli strains associated with bovine septicemia and diarrhea. Vet. Microbiol. 142, 373–8.

Liu, Y., DebRoy, C., Fratamico, P., 2007. Sequencing and analysis of the Escherichia coli serogroup O117, O126, and O146 O-antigen gene clusters and development of PCR assays targeting serogroup O117-, O126-, and O146-specific DNA sequences. Mol. Cell. Probes 21, 295–302.

Livermore, D.M., 2003. The threat from the pink corner. Ann. Med. 35, 226–34.

Livermore, D.M., Woodford, N., 2006. The beta-lactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter. Trends Microbiol. 14, 413–420.

Lloyd, A.L., Rasko, D. a., Mobley, H.L.T., 2007. Defining genomic islands and uropathogen-specific genes in uropathogenic Escherichia coli. J. Bacteriol. 189, 3532–46.

Lowe, P., Haswell, H., Lewis, K., 2006. Use of various common isolation media to evaluate the new VITEK 2 colorimetric GN card for identification of Burkholderia pseudomallei. J. Clin. Microbiol. 44, 854–856.

Lu, P.L., Liu, Y.C., Toh, H.S., Lee, Y.L., Liu, Y.M., Ho, C.M., Huang, C.C., Liu, C.E., Ko, W.C., Wang, J.H., Tang, H.J., Yu, K.W., Chen, Y.S., Chuang, Y.C., Xu, Y., Ni, Y., Chen, Y.H., Hsueh, P.R., 2012. Epidemiology and antimicrobial susceptibility profiles of Gram-negative bacteria causing urinary tract infections in the Asia-Pacific region: 2009-2010 results from the Study for Monitoring Antimicrobial Resistance Trends (SMART). Int. J. Antimicrob. Agents 40, S37–43.

Lu, Y., Iyoda, S., Satou, H., Satou, H., Itoh, K., Saitoh, T., Watanabe, H., 2006. A new immunoglobulin-binding protein, EibG, is responsible for the chain-like adhesion phenotype of locus of enterocyte effacement-negative, shiga toxin-producing Escherichia coli. Infect. Immun. 74, 5747–55.

Maatallah, M., Cheriaa, J., Backhrouf, A., Iversen, A., Grundmann, H., Do, T., Lanotte, P., Mastouri, M., Elghmati, M.S., Rojo, F., Mejdi, S., Giske, C.G., 2011. Population structure of Pseudomonas aeruginosa from five Mediterranean countries: evidence for frequent recombination and epidemic occurrence of CC235. PLoS One 6, e25617.

Magnet, S., Blanchard, J.S., 2005. Molecular insights into aminoglycoside action and resistance. Chem. Rev. 105, 477–497.

Maiden, M.C.J., 2006. Multilocus sequence typing of bacteria. Annu. Rev. Microbiol. 60, 561–88.

Maiden, M.C.J., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., others, 1998. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. 95, 3140.

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, 1007–1013.

Manges, A.R., Tabor, H., Tellis, P., Vincent, C., Tellier, P.-P., 2008. Endemic and epidemic lineages of Escherichia coli that cause urinary tract infections. Emerg. Infect. Dis. 14, 1575–83.

Marre, R., Kreft, B., Hacker, J., 1990. Genetically engineered S and F1C fimbriae differ in their contribution to adherence of Escherichia coli to cultured renal tubular cells. Infect. Immun. 58, 3434–7.

Martin, G.S., Mannino, D.M., Eaton, S., Moss, M., 2003. The epidemiology of sepsis in the United States from 1979 through 2000. N. Engl. J. Med. 348, 1546–1554.

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, 2803–12.

Martínez-Martínez, L., Pascual, A., Jacoby, G.A., 1998. Quinolone resistance from a transferable plasmid. Lancet 351, 797–799.

Maslow, J.N., Mulligan, M.E., Arbeit, R.D., 1993. Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. Clin. Infect. Dis. 17, 153–62; quiz 163–4.

Matsumura, Y., Yamamoto, M., Nagao, M., Hotta, G., Matsushima, A., Ito, Y., Takakura, S., Ichiyama, S., 2012. Emergence and spread of B2-st131-o25b, B2-st131-o16 and d-st405 clonal groups among extended-spectrum-β-lactamase-producing escherichia coli in Japan. J. Antimicrob. Chemother. 67, 2612–2620.

Matsumura, Y., Yamamoto, M., Nagao, M., Ito, Y., Takakura, S., Ichiyama, S., 2013. Association of fluoroquinolone resistance, virulence genes, and IncF plasmids with extended-spectrum-β-lactamase-producing escherichia coli sequence type 131 (ST131) and ST405 clonal groups. Antimicrob. Agents Chemother. 57, 4736–4742.

Mavroidi, A., Miriagou, V., Liakopoulos, A., Tzelepi, E., Stefos, A., Dalekos, G.N., Petinaki, E., 2012a. Ciprofloxacin-resistant Escherichia coli in Central Greece: mechanisms of resistance and molecular identification. BMC Infect. Dis. 12, 371.

Mavroidi, A., Miriagou, V., Malli, E., Stefos, A., Dalekos, G.N., Tzouvelekis, L.S., Petinaki, E., 2012b. Emergence of Escherichia coli sequence type 410 (ST410) with KPC-2 β-lactamase. Int. J. Antimicrob. Agents 39, 247–50.

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, E328–34.

Mellmann, A., Harmsen, D., Cummings, C.A., Zentz, E.B., Leopold, S.R., Rico, A., Prior, K., Szczepanowski, R., Ji, Y., Zhang, W., McLaughlin, S.F., Henkhaus, J.K., Leopold, B., Bielaszewska, M., Prager, R., Brzoska, P.M., Moore, R.L., Guenther, S., Rothberg, J.M., Karch, H., 2011. Prospective genomic characterization of the german enterohemorrhagic Escherichia coli O104:H4 outbreak by rapid next generation sequencing technology. PLoS One 6, e22751.

Miajlovic, H., Cooke, N.M., Moran, G.P., Rogers, T.R.F., Smith, S.G., 2014. Response of Extraintestinal Pathogenic Escherichia coli to Human Serum Reveals a Protective Role for Rcs-Regulated Exopolysaccharide Colanic Acid. Infect. Immun. 82, 298–305.

Milne, I., Stephen, G., Bayer, M., Cock, P.J.A., Pritchard, L., Cardle, L., Shaw, P.D., Marshall, D., 2013. Using Tablet for visual exploration of second-generation sequencing data. Brief. Bioinform. 14, 193–202.

Mingeot-Leclercq, M.P., Glupczynski, Y., Tulkens, P.M., 1999. Aminoglycosides: Activity and resistance. Antimicrob. Agents Chemother. 43, 727–737.

Mokady, D., Gophna, U., Ron, E.Z., 2005. Virulence factors of septicemic Escherichia coli strains. Int. J. Med. Microbiol. 295, 455–62.

Moll, A., Manning, P.A., Timmis, K.N., 1980. Plasmid-determined resistance to serum bactericidal activity: A major outer membrane protein, the traT gene product, is responsible for plasmid-specified serum resistance in Escherichia coli. Infect. Immun. 28, 359–367.

Mora, A., López, C., Dabhi, G., Blanco, M., Blanco, J.E., Alonso, M.P., Herrera, A., Mamani, R., Bonacorsi, S., Moulin-Schouleur, M., Blanco, J., 2009. Extraintestinal pathogenic Escherichia coli O1:K1:H7/NM from human and avian origin: detection of clonal groups B2 ST95 and D ST59 with different host distribution. BMC Microbiol. 9, 132.

Mora, A., Viso, S., López, C., Alonso, M.P., García-Garrote, F., Dabhi, G., Mamani, R., Herrera, A., Marzoa, J., Blanco, M., Blanco, J.E., Moulin-Schouleur, M., Schouler, C., Blanco, J., 2013. Poultry as reservoir for extraintestinal pathogenic Escherichia coli O45:K1:H7-B2-ST95 in humans. Vet. Microbiol. 167, 506–12.

Moreno, E., Andreu, A., Pérez, T., Sabaté, M., Johnson, J.R., Prats, G., 2006. Relationship between Escherichia coli strains causing urinary tract infection in women and the dominant faecal flora of the same hosts. Epidemiol. Infect. 134, 1015–23.

Morris, D., Boyle, F., Ludden, C., Condon, I., Hale, J., O'Connell, N., Power, L., Boo, T.W., Dhanji, H., Lavallee, C., Woodford, N., Cormican, M., 2011. Production of KPC-2 Carbapenemase by an Escherichia coli Clinical Isolate Belonging to the International ST131 Clone. Antimicrob. Agents Chemother. 55, 4935–4936.

Morris, D., McGarry, E., Cotter, M., Passet, V., Lynch, M., Ludden, C., Hannan, M.M., Brisse, S., Cormican, M., 2012. Detection of OXA-48 carbapenemase in the pandemic clone Escherichia coli O25b:H4-ST131 in the course of investigation of an outbreak of OXA-48-producing Klebsiella pneumoniae. Antimicrob. Agents Chemother. 56, 4030–1.

Morrison, D.C., Ryan, J.L., 1987. Endotoxins and disease mechanisms. Annu. Rev. Med. 38, 417–32.

Moura, R.A., Sircili, M.P., Leomil, L., Matté, M.H., Trabulsi, L.R., Elias, W.P., Irino, K., Pestana de Castro, A.F., 2009. Clonal relationship among atypical enteropathogenic Escherichia coli strains isolated from different animal species and humans. Appl. Environ. Microbiol. 75, 7399–408.

Mukherjee, K., Altincicek, B., Hain, T., Domann, E., Vilcinskas, A., Chakraborty, T., 2010. Galleria mellonella as a model system for studying Listeria pathogenesis. Appl. Environ. Microbiol. 76, 310–317.

Mulvey, M.A., 2002. Adhesion and entry of uropathogenic Escherichia coli. Cell. Microbiol. 4, 257–71.

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, 1494–1497.

Mulvey, M.R., Bryce, E., Boyd, D.A., Ofner-Agostini, M., Land, A.M., Simor, A.E., Paton, S., 2004. Molecular Characterization of Cefoxitin-Resistant Escherichia coli from Canadian Hospitals. Antimicrob. Agents Chemother. 49, 358–365.

Mulvey, M.R., Bryce, E., Boyd, D.A., Ofner-Agostini, M., Land, A.M., Simor, A.E., Paton, S., 2005. Molecular characterization of cefoxitin-resistant Escherichia coli from Canadian hospitals. Antimicrob. Agents Chemother. 49, 358–365.

Mushtaq, S., Irfan, S., Sarma, J.B., Doumith, M., Pike, R., Pitout, J., Livermore, D.M., Woodford, N., 2011. Phylogenetic diversity of Escherichia coli strains producing NDM-type carbapenemases. J. Antimicrob. Chemother. 66, 2002–5.

Mylonakis, E., Moreno, R., El Khoury, J.B., Idnurm, A., Heitman, J., Calderwood, S.B., Ausubel, F.M., Diener, A., 2005. Galleria mellonella as a model system to study Cryptococcus neoformans pathogenesis. Infect. Immun. 73, 3842–3850.

Neu, H.C., 1992. Urinary Tract Infections. Am. Jounal Med. 92, 63–70.

Nicolas-Chanoine, M.-H.H., Blanco, J., Leflon-Guibout, V., Demarty, R., Alonso, M.P., Caniça, M.M., Park, Y.-J.J., Lavigne, J.-P.P., Pitout, J., Johnson, J.R., 2008. Intercontinental emergence of Escherichia coli clone O25:H4-ST131 producing CTX-M-15. J. Antimicrob. Chemother. 61, 273–81.

Noh, J.Y., Patnaik, B.B., Tindwa, H., Seo, G.W., Kim, D.H., Patnaik, H.H., Jo, Y.H., Lee, Y.S., Lee, B.L., Kim, N.J., Han, Y.S., 2014. Genomic organization, sequence characterization and expression analysis of Tenebrio molitor apolipophorin-III in response to an intracellular pathogen, Listeria monocytogenes. Gene 534, 204–17.

Nordmann, P., 2014. Carbapenemase-producing Enterobacteriaceae: Overview of a major public health challenge. Med. Mal. Infect. 44, 51–56.

Nordmann, P., Poirel, L., Walsh, T.R., Livermore, D.M., 2011. The emerging NDM carbapenemases. Trends Microbiol. 19, 588–595.

Okeke, I.N., Macfarlane-Smith, L.R., Fletcher, J.N., Snelling, A.M., 2011. IS3 profiling identifies the enterohaemorrhagic Escherichia coli O-island 62 in a distinct enteroaggregative E. coli lineage. Gut Pathog. 3, 4.

Okeke, I.N., Wallace-Gadsden, F., Simons, H.R., Matthews, N., Labar, A.S., Hwang, J., Wain, J., 2010. Multi-locus sequence typing of enteroaggregative Escherichia coli isolates from Nigerian children uncovers multiple lineages. PLoS One 5, e14093.

Olive, D.M., Bean, P., 1999. Principles and Applications of Methods for DNA-Based Typing of Microbial Organisms. J. Clin. Microbiol. 37, 1661–1669.

Olsen, R.J., Watkins, M.E., Cantu, C.C., Beres, S.B., Musser, J.M.,. Virulence of serotype M3 Group A Streptococcus strains in wax worms (Galleria mellonella larvae). Virulence 2, 111–119.

Ørskov, F., Ørskov, I., Evans, D.J., Sack, R.B., Sack, D.A., Wadström, T., 1976. Special Escherichia coli serotypes among enterotoxigenic strains from diarrhoea in adults and children. Med. Microbiol. Immunol. 162, 73–80.

Orskov, I., Orskov, F., 1985. Escherichia coli in extra-intestinal infections. J. Hyg. (Lond). 95, 551–575.

Orskov, I., Orskov, F., Jann, B., Jann, K., 1977. Serology, chemistry, and genetics of O and K antigens of Escherichia coli. Bacteriol. Rev. 41, 667–710.

Ortega, X., Silipo, A., Saldías, M.S., Bates, C.C., Molinaro, A., Valvano, M.A., 2009. Biosynthesis and structure of the Burkholderia cenocepacia K56-2 lipopolysaccharide core oligosaccharide: truncation of the core oligosaccharide leads to increased binding and sensitivity to polymyxin B. J. Biol. Chem. 284, 21738–51.

Oteo, J., Diestra, K., Juan, C., Bautista, V., Novais, A., Pérez-Vázquez, M., Moyá, B., Miró, E., Coque, T.M., Oliver, A., Cantón, R., Navarro, F., Campos, J., 2009. Extended-spectrum beta-lactamase-producing Escherichia coli in Spain belong to a large variety of multilocus sequence

typing types, including ST10 complex/A, ST23 complex/A and ST131/B2. Int. J. Antimicrob. Agents 34, 173–6.

Oztug, M., Martinon, D., Weers, P.M.M., 2012. Characterization of the apoLp-III/LPS complex: insight into the mode of binding interaction. Biochemistry 51, 6220–7.

Paltansing, S., Kraakman, M.E.M., Ras, J.M.C., Wessels, E., Bernards, A.T., 2013. Characterization of fluoroquinolone and cephalosporin resistance mechanisms in Enterobacteriaceae isolated in a Dutch teaching hospital reveals the presence of an Escherichia coli ST131 clone with a specific mutation in parE. J. Antimicrob. Chemother. 68, 40–45.

Palusińska-Szysz, M., Zdybicka-Barabas, A., Pawlikowska-Pawlęga, B., Mak, P., Cytryńska, M., 2012. Anti-Legionella dumoffii Activity of Galleria mellonella Defensin and Apolipophorin III. Int. J. Mol. Sci. 13, 17048–64.

Papanicolaou, G.A., Medeiros, A.A., Jacoby, G.A., 1990. Novel plasmid-mediated beta-lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy beta-lactams in clinical isolates of Klebsiella pneumoniae. Antimicrob. Agents Chemother. 34, 2200–2209.

Park, S.H., Byun, J.-H., Choi, S.-M., Lee, D.-G., Kim, S.-H., Kwon, J.-C., Park, C., Choi, J.-H., Yoo, J.-H., 2012. Molecular epidemiology of extended-spectrum β-lactamase-producing Escherichia coli in the community and hospital in Korea: emergence of ST131 producing CTX-M-15. BMC Infect. Dis. 12, 149.

Park, Y.S., Bae, I.K., Kim, J., Jeong, S.H., Hwang, S., Seo, Y.-H., Cho, Y.K., Lee, K., Kim, J.M., 2014. Risk factors and molecular epidemiology of community-onset extended-spectrum β-lactamase-producing Escherichia coli bacteremia. Yonsei Med. J. 55, 467–75.

Paterson, D.L., Bonomo, R. a, 2005. Extended-Spectrum beta -Lactamases : a Clinical Update. Society 18, 657–686.

Pavez, M., Neves, P., Dropa, M., Matté, M.H., Grinbaum, R.S., Elmor De Araújo, M.R., Mamizuka, E.M., Lincopan, N., 2008. Emergence of carbapenem-resistant Escherichia coli producing CMY-2-type AmpC β-lactamase in Brazil. J. Med. Microbiol. 57, 1590–1592.

Peigne, C., Bidet, P., Mahjoub-Messai, F., Plainvert, C., Barbe, V., Médigue, C., Frapy, E., Nassif, X., Denamur, E., Bingen, E., Bonacorsi, S., 2009. The plasmid of Escherichia coli strain S88 (O45:K1:H7) that causes neonatal meningitis is closely related to avian pathogenic E. coli plasmids and is associated with high-level bacteremia in a neonatal rat meningitis model. Infect. Immun. 77, 2272–84.

Peirano, G., Ahmed-Bentley, J., Fuller, J., Rubin, J.E., Pitout, J.D.D., 2014a. Travel-related carbapenemase-producing Gram-negative bacteria in Alberta, Canada: the first 3 years. J. Clin. Microbiol. 52, 1575–81.

Peirano, G., Bradford, P.A., Kazmierczak, K.M., Badal, R.E., Hackel, M., Hoban, D.J., Pitout, J.D.D., 2014b. Global incidence of carbapenemase-producing Escherichia coli ST131. Emerg. Infect. Dis. 20, 1928–31.

Peirano, G., van der Bij, A.K., Gregson, D.B., Pitout, J.D.D., 2012. Molecular epidemiology over an 11-year period (2000 to 2010) of extended-spectrum β-lactamase-producing Escherichia coli causing bacteremia in a centralized Canadian region. J. Clin. Microbiol. 50, 294–9.

Peleg, A.Y., Jara, S., Monga, D., Eliopoulos, G.M., Moellering, R.C., Mylonakis, E., 2009. Galleria mellonella as a model system to study Acinetobacter baumannii pathogenesis and therapeutics. Antimicrob. Agents Chemother. 53, 2605–9.

Peng, C., Zong, Z., 2011. Sequence type 38 Escherichia coli carrying blaCTX-M-14. J. Med. Microbiol. 60, 694–695.

Perlhagen, M., Forsberg, T., Perlhagen, J., Nivesjö, M., 2007. Evaluating the specificity of a new type of urine collection bag for infants. J. Pediatr. Urol. 3, 378–381.

Petty, N.K., Ben Zakour, N.L., Stanton-Cook, M., Skippington, E., Totsika, M., Forde, B.M., Phan, M.-D., Gomes Moriel, D., Peters, K.M., Davies, M., Rogers, B.A., Dougan, G., Rodriguez-Baño, J., Pascual, A., Pitout, J.D.D., Upton, M., Paterson, D.L., Walsh, T.R., Schembri, M.A., Beatson, S.A., 2014. Global dissemination of a multidrug resistant Escherichia coli clone. Proc. Natl. Acad. Sci. U. S. A. 111, 5694–9.

Phan, M., Peters, K.M., Sarkar, S., Forde, B.M., Lo, A.W., Stanton-cook, M., Roberts, L.W., Upton, M., Beatson, S.A., Schembri, M.A., 2015. Third-generation cephalosporin resistance conferred by a chromosomally encoded bla CMY-23 gene in the Escherichia coli ST131 reference strain EC958. dkv066 Epu.

Phan, M.-D.D., Peters, K.M., Sarkar, S., Lukowski, S.W., Allsopp, L.P., Gomes Moriel, D., Achard, M.E.S., Totsika, M., Marshall, V.M., Upton, M., Beatson, S.A., Schembri, M.A., Moriel, D.G., Achard, M.E.S., 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, e1003834.

Philippon, A., Arlet, G., Jacoby, G.A., 2002. Plasmid-Determined AmpC-Type beta -Lactamases. Antimicrob. Agents Chemother. 46, 1–11.

Philippon, A., Labia, R., Jacoby, G., 1989. MINIREVIEW Extended-Spectrum Beta-Lactamases. Antimicrob. Agents Chemother. 33, 1131–1136.

Phipps, D.J., Chadwick, J.S., Peter Aston, W., 1994. Gallysin-1, an antibacterial protein isolated from hemolymph of Galleria mellonella. Dev. Comp. Immunol. 18, 13–23.

Piatti, G., Mannini, A., Balistreri, M., Schito, A.M., 2008. Virulence factors in urinary Escherichia coli strains: Phylogenetic background and quinolone and fluoroquinolone resistance. J. Clin. Microbiol. 46, 480–487.

Picard, B., Garcia, J.S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., Elion, J., Denamur, E., 1999. The link between phylogeny and virulence in Escherichia coli extraintestinal infection. Infect. Immun. 67, 546–53.

Pitout, J.D.D., 2012. Extraintestinal pathogenic Escherichia coli: A combination of virulence with antibiotic resistance. Front. Microbiol. 3, 1–7.

Pitout, J.D.D., Laupland, K.B., 2008. Extended-spectrum β-lactamase-producing Enterobacteriaceae: an emerging public-health concern 8, 159–166.

Pitout, J.D.D., Nordmann, P., Laupland, K.B., Poirel, L., 2005. Emergence of Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) in the community. J. Antimicrob. Chemother. 56, 52–59.

Poirel, L., Barbosa-Vasconcelos, A., Simões, R.R., Da Costa, P.M., Liu, W., Nordmann, P., 2012. Environmental KPC-producing Escherichia coli isolates in Portugal. Antimicrob. Agents Chemother. 56, 1662–1663.

Poirel, L., Bernabeu, S., Fortineau, N., Podglajen, I., Lawrence, C., Nordmann, P., 2011a. Emergence of OXA-48-producing Escherichia coli clone ST38 in France. Antimicrob. Agents Chemother. 55, 4937–8.

Poirel, L., Walsh, T.R., Cuvillier, V., Nordmann, P., 2011b. Multiplex PCR for detection of acquired carbapenemase genes. Diagn. Microbiol. Infect. Dis. 70, 119–23.

Prats, G., Navarro, F., Mirelis, B., Dalmau, D., Margall, N., Coll, P., Stell, A., Johnson, J.R., 2000. Escherichia coli serotype O15:K52:H1 as a uropathogenic clone. J. Clin. Microbiol. 38, 201–9.

Price, L.B., Johnson, J.R., Aziz, M., 2013. The Epidemic of Extended-Spectrum-β -Lactamase-Producing Escherichia coli ST131 Is Driven by a Single Highly Pathogenic Subclone, H 30-Rx. MBio 4, e00377–13.

Qi, C., Pilla, V., Yu, J.H., Reed, K., 2010. Changing prevalence of Escherichia coli with CTX-M-type extended-spectrum β-lactamases in outpatient urinary E. coli between 2003 and 2008. Diagn. Microbiol. Infect. Dis. 67, 87–91.

Qureshi, Z.A., Doi, Y., 2014. Escherichia coli sequence type 131: epidemiology and challenges in treatment. Expert Rev. Anti. Infect. Ther. 12, 597–609.

Ragnarsdóttir, B., Svanborg, C., 2012. Susceptibility to acute pyelonephritis or asymptomatic bacteriuria: Host-pathogen interaction in urinary tract infections. Pediatr. Nephrol. 27, 2017–2029.

Ramamoorthy, A., Rosenfeld, Y., Shai, Y., 2006. Lipopolysaccharide (Endotoxin)-host defense antibacterial peptides interactions: Role in bacterial resistance and prevention of sepsis. Biochim. Biophys. Acta - Biomembr. 1758, 1513–1522.

Ratcliffe, N.A., 1985. Invertebrate immunity--a primer for the non-specialist. Immunol. Lett. 10, 253–70.

Reyes, R.E., Andrade, A.A., González, C.R., Herrera, M.O., Jiménez, R.C., 2012. Mechanisms of O-Antigen Structural Variation of Bacterial Lipopolysaccharide (LPS). INTECH Open Access Publishe.

Riley, L.W., 2014. Pandemic lineages of extraintestinal pathogenic Escherichia coli. Clin. Microbiol. Infect. 20, 380–90.

Rogers, B.A., Sidjabat, H.E., Paterson, D.L., 2011. Escherichia coli O25b-ST131: A pandemic, multiresistant, community-associated strain. J. Antimicrob. Chemother. 66, 1–14.

Rohmer, L., Hocquet, D., Miller, S.I., 2011. Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. Trends Microbiol. 19, 341–348.

Ronald, A., 2003. The etiology of urinary tract infection: traditional and emerging pathogens. Dis. Mon. 49, 71–82.

Rosen, D. a, Hooton, T.M., Stamm, W.E., Humphrey, P. a, Hultgren, S.J., 2007. Detection of intracellular bacterial communities in human urinary tract infection. PLoS Med. 4, 1949–1958.

Russo, T.A., Johnson, J.R., 2003. Medical and economic impact of extraintestinal infections due to Escherichia coli: Focus on an increasingly important endemic problem. Microbes Infect. 5, 449–456.

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. 114.

Salipante, S.J., Sengupta, D.J., Rosenthal, C., Costa, G., Spangler, J., Sims, E.H., Jacobs, M.A., Miller, S.I., Hoogestraat, D.R., Cookson, B.T., McCoy, C., Matsen, F.A., Shendure, J., Lee, C.C., Harkins, T.T., Hoffman, N.G., 2013. Rapid 16S rRNA next-generation sequencing of polymicrobial clinical samples for diagnosis of complex bacterial infections. PLoS One 8, e65226.

Salzet, M., 2001. Vertebrate innate immunity resembles a mosaic of invertebrate immune responses. Trends Immunol. 22, 285–8.

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, 2503–19.

Sánchez, S., Llorente, M.T., Echeita, M.A., Herrera-León, S., 2015. Development of Three Multiplex PCR Assays Targeting the 21 Most Clinically Relevant Serogroups Associated with Shiga Toxin-Producing E. coli Infection in Humans. PLoS One 10, e0117660.

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, e94786.

Schink, A.K., Kadlec, K., Kaspar, H., Mankertz, J., Schwarz, S., 2013. Analysis of extended-spectrum-β-lactamase-producing Escherichia coli isolates collected in the GERM-Vet monitoring programme. J. Antimicrob. Chemother. 68, 1741–1749.

Schneider, G., Dobrindt, U., Brüggemann, H., Nagy, G., Janke, B., Blum-Oehler, G., Buchrieser, C., Gottschalk, G., Emödy, L., Hacker, J., 2004. The pathogenicity island-associated K15 capsule determinant exhibits a novel genetic structure and correlates with virulence in uropathogenic Escherichia coli strain 536. Infect. Immun. 72, 5993–6001.

Sheng, W.H., Badal, R.E., Hsueh, P.R., 2013. Distribution of extended-spectrum beta-lactamases, AmpC beta-lactamases, and carbapenemases among Enterobacteriaceae isolates causing intra-abdominal infections in the Asia-pacific region: Results of the study for monitoring antimicrobial resistance trend. Antimicrob. Agents Chemother. 57, 2981–2988.

Sherry, N.L., Porter, J.L., Seemann, T., Watkins, A., Stinear, T.P., Howden, B.P., 2013. Outbreak investigation using high-throughput genome sequencing within a diagnostic microbiology laboratory. J. Clin. Microbiol. 51, 1396–401.

Shiga, K., 1897. Sekiri byogen kenkyu hokoku dai-ichi (first report on etiologic research on dysentery). Saikingaku Zasshi 25, 790–810.

Shin, J., Kim, D.H., Ko, K.S., 2011. Comparison of CTX-M-14- and CTX-M-15-producing Escherichia coli and Klebsiella pneumoniae isolates from patients with bacteremia. J. Infect. 63, 39–47.

Shulman, S.T., Friedmann, H.C., Sims, R.H., 2007. Theodor Escherich: the first pediatric infectious diseases physician? Clin. Infect. Dis. 45, 1025–1029.

Sidjabat, H.E., Derrington, P., Nimmo, G.R., Paterson, D.L., 2010. Escherichia coli ST131 producing CTX-M-15 in Australia. J. Antimicrob. Chemother. 65, 1301–3.

Siu, L.K., Lu, P.-L., Chen, J.-Y., Lin, F.M., Chang, S.-C., 2003. High-Level Expression of AmpC -Lactamase Due to Insertion of Nucleotides between -10 and -35 Promoter Sequences in Escherichia coli Clinical Isolates: Cases Not Responsive to Extended-Spectrum-Cephalosporin Treatment. Antimicrob. Agents Chemother. 47, 2138–2144.

Skjøt-Rasmussen, L., Olsen, S.S., Jakobsen, L., Ejrnæs, K., Scheutz, F., Lundgren, B., Frimodt-Møller, N., Hammerum, a. M., Ejrnaes, K., Scheutz, F., Lundgren, B., Frimodt-Møller, N., Hammerum, a. M., 2013. Escherichia coli clonal group A causing bacteraemia of urinary tract origin. Clin. Microbiol. Infect. 19, 656–661.

Sköld, O., 2001. Resistance to trimethoprim and sulfonamides, in: Veterinary Research. EDP Sciences, pp. 261–273.

Smith, J.L., Fratamico, P.M., Gunther, N.W., 2007. Extraintestinal pathogenic Escherichia coli. Foodborne Pathog. Dis. 4, 134–163.

Smith, S.N., Hagan, E.C., Lane, M.C., Mobley, H.L.T., 2010. Dissemination and systemic colonization of uropathogenic Escherichia coli in a murine model of bacteremia. MBio 1, e00262–10.

Staley, T.E., Norris, H.T., Staley, J.A., 1972. E. coli diarrhoea. Lancet 2, 597.

Stamm, W.E., Counts, G.W., Running, K.R., Fihn, S., Turck, M., Holmes, K.K., 1982. Diagnosis of coliform infection in acutely dysuric women. N. Engl. J. Med. 307, 463–468.

Stewardson, A., Fankhauser, C., De Angelis, G., Rohner, P., Safran, E., Schrenzel, J., Pittet, D., Harbarth, S., 2013. Burden of bloodstream infection caused by extended-spectrum β-lactamase-producing enterobacteriaceae determined using multistate modeling at a Swiss University Hospital and a nationwide predictive model. Infect. Control Hosp. Epidemiol. 34, 133–43.

Stone, P.W., 2009. Economic burden of healthcare-associated infections: an American perspective. Expert Rev. Pharmacoecon. Outcomes Res. 9, 417–22.

Strahilevitz, J., Jacoby, G.A., Hooper, D.C., Robicsek, A., 2009. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin. Microbiol. Rev. 22, 664–89.

Sullivan, M.J., Petty, N.K., Beatson, S.A., 2011. Easyfig: a genome comparison visualizer. Bioinformatics 27, 1009–10.

Suzuki, S., Shibata, N., Yamane, K., Wachino, J., Ito, K., Arakawa, Y., 2009. Change in the prevalence of extended-spectrum-beta-lactamase-producing Escherichia coli in Japan by clonal spread. J. Antimicrob. Chemother. 63, 72–9.

Takahashi, A., Muratani, T., Yasuda, M., Takahashi, S., Monden, K., Ishikawa, K., Kiyota, H., Arakawa, S., Matsumoto, T., Shima, H., Kurazono, H., Yamamoto, S., 2009. Genetic profiles of fluoroquinolone-resistant Escherichia coli isolates obtained from patients with cystitis: Phylogeny, virulence factors, PAIusp subtypes, and mutation patterns. J. Clin. Microbiol. 47, 791–795.

Takahashi, H., Hayakawa, I., Akimoto, T., 2003. The history of the development and changes of quinolone antibacterial agents. Yakushigaku Zasshi. 38, 161–179.

Tang, S.S., Apisarnthanarak, A., Hsu, L.Y., 2014. Mechanisms of β-lactam antimicrobial resistance and epidemiology of major community- and healthcare-associated multidrug-resistant bacteria. Adv. Drug Deliv. Rev. 78, 3–13.

Tartof, S.Y., Solberg, O.D., Manges, A.R., Riley, L.W., 2005. Analysis of a uropathogenic Escherichia coli clonal group by multilocus sequence typing. J. Clin. Microbiol. 43, 5860–5864.

Tenover, F.C., Arbeit, R.D., Goering, R. V, 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Molecular Typing Working Group of the Society for Healthcare Epidemiology of America. Infect. Control Hosp. Epidemiol. 18, 426–439.

Tichaczek-Goska, D., Witkowska, D., Cisowska, A., Jankowski, S., Hendrich, A.B., 2012. The bactericidal activity of normal human serum against Enterobacteriaceae rods with lipopolysaccharides possessing O-antigens composed of mannan. Adv. Clin. Exp. Med. 21, 289–299.

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, e26578.

Tracz, D.M., Boyd, D.A., Hizon, R., Bryce, E., McGeer, A., Ofner-Agostini, M., Simor, A.E., Paton, S., Mulvey, M.R., 2007. ampC gene expression in promoter mutants of cefoxitin-resistant Escherichia coli clinical isolates. FEMS Microbiol. Lett. 270, 265–271.

Van Belkum, A., Struelens, M., De Visser, A., Verbrugh, H., Tibayrenc, M., 2001. Role of genomic typing in taxonomy, evolutionary genetics, and microbial epidemiology. Clin. Microbiol. Rev. 14, 547–560.

Van Den Bogaard, A.E., Stobberingh, E.E., 2000. Epidemiology of resistance to antibiotics: Links between animals and humans. Int. J. Antimicrob. Agents 14, 327–335.

Van Der Bij, A.K., Peirano, G., Goessens, W.H.F., Van Der Vorm, E.R., Van Westreenen, M., Pitout, J.D.D., 2011. Clinical and molecular characteristics of extended-spectrum-beta- lactamase-producing Escherichia coli causing bacteremia in the Rotterdam Area, Netherlands. Antimicrob. Agents Chemother. 55, 3576–3578.

Van der Bij, A.K., Peirano, G., Pitondo-Silva, A., Pitout, J.D.D., 2012. The presence of genes encoding for different virulence factors in clonally related Escherichia coli that produce CTX-Ms. Diagn. Microbiol. Infect. Dis. 72, 297–302.

Van Elsas, J.D., Semenov, A. V, Costa, R., Trevors, J.T., 2011. Survival of Escherichia coli in the environment: fundamental and public health aspects. ISME J. 5, 173–183.

Velasco, M., Horcajada, J.P., Mensa, J., Moreno-Martinez, A., Vila, J., Martinez, J.A., Ruiz, J., Barranco, M., Roig, G., Soriano, E., 2001. Decreased invasive capacity of quinolone-resistant Escherichia coli in patients with urinary tract infections. Clin. Infect. Dis. 33, 1682–6.

Vellinga, A., Murphy, A.W., Hanahoe, B., Bennett, K., Cormican, M., 2010. A multilevel analysis of trimethoprim and ciprofloxacin prescribing and resistance of uropathogenic Escherichia coli in general practice. J. Antimicrob. Chemother. 65, 1514–20.

Vila, J., Simon, K., Ruiz, J., Horcajada, J.P., Velasco, M., Barranco, M., Moreno, A., Mensa, J., 2002. Are quinolone-resistant uropathogenic Escherichia coli less virulent? J. Infect. Dis. 186, 1039–42.

Vincent, C., Boerlin, P., Daignault, D., Dozois, C.M., Dutil, L., Galanakis, C., Reid-Smith, R.J., Tellier, P.P., Tellis, P.A., Ziebell, K., Manges, A.R., 2010. Food reservoir for Escherichia coli causing urinary tract infections. Emerg. Infect. Dis. 16, 88–95.

Vincent, C., Duclos, B., Grangeasse, C., Vaganay, E., Riberty, M., Cozzone, A.J., Doublet, P., 2000. Relationship between exopolysaccharide production and protein-tyrosine phosphorylation in gram-negative bacteria. J. Mol. Biol. 304, 311–21.

Vos, M., Didelot, X., 2009. A comparison of homologous recombination rates in bacteria and archaea. ISME J. 3, 199–208.

Walker, R.C., 1999. The fluoroquinolones. Mayo Clin. Proc. 74, 1030–1037.

Wallace-Gadsden, F., Johnson, J.R., Wain, J., Okeke, I.N., 2007. Enteroaggregative Escherichia coli related to uropathogenic clonal group A. Emerg. Infect. Dis. 13, 757–60.

Walters, J.B., Ratcliffe, N.A., 1983. Studies on the in vivo cellular reactions of insects: Fate of pathogenic and non-pathogenic bacteria in Galleria mellonella nodules. J. Insect Physiol. 29, 417–424.

Walters, M.S., Chelsea Lane, M., Vigil, P.D., Smith, S.N., Walk, S.T., Mobley, H.L.T., 2012. Kinetics of uropathogenic Escherichia coli metapopulation movement during urinary tract infection. MBio 3, 1–11.

Wand, M.E., McCowen, J.W.I., Nugent, P.G., Sutton, J.M., 2013. Complex interactions of Klebsiella pneumoniae with the host immune system in a Galleria mellonella infection model. J. Med. Microbiol. 62, 1790–1798.

Wand, M.E., Müller, C.M., Titball, R.W., Michell, S.L., 2011. Macrophage and Galleria mellonella infection models reflect the virulence of naturally occurring isolates of B. pseudomallei, B. thailandensis and B. oklahomensis. BMC Microbiol. 11, 11.

Wang, L., Reeves, P.R., 1998. Organization of Escherichia coli O157 O antigen gene cluster and identification of its specific genes. Infect. Immun. 66, 3545–51.

Wang, M.-C., Tseng, C.-C., Chen, C.-Y., Wu, J.-J., Huang, J.-J., 2002. The role of bacterial virulence and host factors in patients with Escherichia coli bacteremia who have acute cholangitis or upper urinary tract infection. Clin. Infect. Dis. 35, 1161–1166.

Wang, Q., Ruan, X., Wei, D., Hu, Z., Wu, L., Yu, T., Feng, L., Wang, L., 2010. Development of a serogroup-specific multiplex PCR assay to detect a set of Escherichia coli serogroups based on the identification of their O-antigen gene clusters. Mol. Cell. Probes 24, 286–290.

Weintraub, A., 2007. Enteroaggregative Escherichia coli: Epidemiology, virulence and detection. J. Med. Microbiol. 56, 4–8.

Weiser, J.N., Gotschlich, E.C., 1991. Outer-Membrane Protein-a (Ompa) Contributes to Serum Resistance and Pathogenicity of Escherichia-Coli K-1. Infect. Immun. 59, 2252–2258.

Weissman, S.J., Johnson, J.R., Tchesnokova, V., Billig, M., Dykhuizen, D., Riddell, K., Rogers, P., Qin, X., Butler-Wu, S., Cookson, B.T., Fang, F.C., Scholes, D., Chattopadhyay, S., Sokurenko, E., 2012. High-resolution two-locus clonal typing of extraintestinal pathogenic Escherichia coli. Appl. Environ. Microbiol. 78, 1353–1360.

Wiles, T.J., Kulesus, R.R., Mulvey, M. a., 2008. Origins and virulence mechanisms of uropathogenic Escherichia coli. Exp. Mol. Pathol. 85, 11–9.

Wilson, M., 2002. Bacterial adhesion to host tissues. Cambridge University Press, Cambridge.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C.J., Ochman, H., Achtman, M., 2006. Sex and virulence in Escherichia coli: An evolutionary perspective. Mol. Microbiol. 60, 1136–1151.

Woodford, N., Ward, M.E., Kaufmann, M.E., Turton, J., Fagan, E.J., James, D., Johnson, A.P., Pike, R., Warner, M., Cheasty, T., Pearson, A., Harry, S., Leach, J.B., Loughrey, A., Lowes, J.A., Warren, R.E., Livermore, D.M., 2004. Community and hospital spread of Escherichia coli producing CTX-M extended-spectrum beta-lactamases in the UK. J. Antimicrob. Chemother. 54, 735–43.

Wright, G.D., 2011. Molecular mechanisms of antibiotic resistance. Chem. Commun. (Camb). 47, 4055–4061.

Yamamoto, T., Takano, T., Iwao, Y., Hishinuma, A., 2011. Emergence of NDM-1-positive capsulated Escherichia coli with high resistance to serum killing in Japan. J. Infect. Chemother. 17, 435–9.

Yong, D., Toleman, M.A., Giske, C.G., Cho, H.S., Sundman, K., Lee, K., Walsh, T.R., 2009. Characterization of a new metallo-beta-lactamase gene, bla NDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob. Agents Chemother. 53, 5046–5054.

Yue, L., Jiang, H.-X., Liao, X.-P., Liu, J.-H., Li, S.-J., Chen, X.-Y., Chen, C.-X., Lü, D.-H., Liu, Y.-H., 2008. Prevalence of plasmid-mediated quinolone resistance qnr genes in poultry and swine clinical isolates of Escherichia coli. Vet. Microbiol. 132, 414–20.

Zdybicka-Barabas, A., Stączek, S., Mak, P., Skrzypiec, K., Mendyk, E., Cytryńska, M., 2013. Synergistic action of Galleria mellonella apolipophorin III and lysozyme against Gram-negative bacteria. Biochim. Biophys. Acta 1828, 1449–56.

Zerbino, D.R., Birney, E., 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18, 821–9.

Zhanel, G.G., DeCorby, M., Adam, H., Mulvey, M.R., McCracken, M., Lagacé-Wiens, P., Nichol, K.A., Wierzbowski, A., Baudry, P.J., Tailor, F., Karlowsky, J.A., Walkty, A., Schweizer, F., Johnson, J., Hoban, D.J., 2010. Prevalence of antimicrobial-resistant pathogens in Canadian hospitals: Results of the Canadian Ward Surveillance study (CANWARD 2008). Antimicrob. Agents Chemother. 54, 4684–4693.

Zhang, J., Zheng, B., Zhao, L., Wei, Z., Ji, J., Li, L., Xiao, Y., 2014. Nationwide high prevalence of CTX-M and an increase of CTX-M-55 in Escherichia coli isolated from patients with community-onset infections in Chinese county hospitals. BMC Infect. Dis. 14, 659.

Zhang, L., Foxman, B., Manning, S.D., Tallman, P., Marrs, C.F., 2000. Molecular epidemiologic approaches to urinary tract infection gene discovery in uropathogenic Escherichia coli. Infect. Immun. 68, 2009–15.

Zhang, X., Lou, D., Xu, Y., Shang, Y., Li, D., Huang, X., Li, Y., Hu, L., Wang, L., Yu, F., 2013. First identification of coexistence of blaNDM-1 and blaCMY-42 among Escherichia coli ST167 clinical isolates. BMC Microbiol. 13, 282.

Ziebell, K., Steele, M., Zhang, Y., Benson, A., Taboada, E.N., Laing, C., McEwen, S., Ciebin, B., Johnson, R., Gannon, V., 2008. Genotypic characterization and prevalence of virulence factors among Canadian Escherichia coli O157:H7 strains. Appl. Environ. Microbiol. 74, 4314–23.

Zowawi, H.M., Balkhy, H.H., Walsh, T.R., Paterson, D.L., 2013. β-lactamase production in key gram-negative pathogen isolates from the Arabian Peninsula. Clin. Microbiol. Rev. 26, 361–380.

Zowawi, H.M., Sartor, A.L., Balkhy, H.H., Walsh, T.R., Al Johani, S.M., AlJindan, R.Y., Alfaresi, M., Ibrahim, E., Al-Jardani, A., Al-Abri, S., Al Salman, J., Dashti, A.A., Kutbi, A.H., Schlebusch, S., Sidjabat, H.E., Paterson, D.L., 2014. Molecular characterization of carbapenemase-producing Escherichia coli and Klebsiella pneumoniae in the countries of the Gulf cooperation council: dominance of OXA-48 and NDM producers. Antimicrob. Agents Chemother. 58, 3085–90.