Extra-intestinal pathogenic Escherichia coli in the UK

The importance in bacteraemia versus urinary tract infection, colonisation of widespread clones and specific virulence factors

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Submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy

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Abstract

Extra-intestinal pathogenic *Escherichia coli* (ExPEC) are a significant cause of urinary tract infections and bacteraemia in the UK and around the world. These *E. coli* primarily belong to phylogenetic groups B2 and D, with the clones ST131, ST127, ST95, ST73 and ST69 responsible for the majority of these infections.
In the UK, studies of ExPEC have focused on isolates from the North of England, ST131 strains and ExPEC that possess extended-spectrum beta-lactamase (ESBL) enzymes. Therefore, very little is understood about the UK ExPEC population as a whole, the breadth of virulence factors contributing to these infections and the differences between urinary and bloodstream-derived ExPEC.

In this study ST131 was more frequently detected in bloodstream isolates and ST95 was most prevalent in urinary isolates. Comparative virulence of the major clones in the *Galleria mellonella* infection model revealed ST131 isolates to effect the highest mortality, although serogroup O6, which is linked with ST73, was also associated with high mortality, potentially explaining the success of ST73-O6 in bacteraemia. Analysis of virulence factors identified *pap*, *afa/dra* and *kpsMT*II as important determinants in isolates causing urosepsis and those of ST131, while *fyuA* and *fimH* were distinctly lacking, demonstrating their role as colonisation factors rather than virulence factors.

Although these findings are important, with appropriate treatment of urinary tract infections they can become redundant, as ExPEC would be eradicated before causing a severe infection such as bacteraemia or urosepsis. In urinary isolates, resistance to trimethoprim approached 50% and ampicillin resistance was >70%, while ST131 isolates as a whole demonstrated ciprofloxacin and trimethoprim resistance >50%. Together these indicate that empirical UTI guidelines need to be revisited, to prevent recurrence of infection and ascension to the kidneys and bloodstream. In addition, data from this study can be used to develop a point-of-care test to detect ST131, to guide appropriate treatment, without the delay associated with referring a urine specimen for microbiological investigation.

Contents

Abbreviations

ABC	ATP-binding cassette
ABU	Asymptomatic bacteruria
AIEC	Adherent invasive *E. coli*
AME	Aminoglycoside modifying enzyme
AMK	Amikacin
AMP	Ampicillin
AMRHAI	Antimicrobial Resistance and Healthcare Associated Infections reference unit
APEC	Avian-pathogenic *E. coli*
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUG	Augmentin (amoxicillin-clavulanate)
AZT	Aztreonam
BFP	Bundle forming pilus
BLC	Blood culture
BSAC	British Society for Antimicrobial Chemotherapy
CAI	Community associated infection
CAZ	Ceftazidime
CFU	Colony forming units
CHL	Chloramphenicol
CIP	Ciprofloxacin
CPR	Cefpirome
CSF	Cerebral spinal fluid
CTX	Cefotaxime
COMP	Complicated urinary tract infection
DAEC	Diffusely adherent *E. coli*
DD	Disc diffusion

DEC	Diarrhoeagenic *E. coli*
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
EAST1	Enteroaggregative *E. coli* heat-stable enterotoxin 1
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ERP	Ertapenem
ESBL	Extended spectrum beta lactamase
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extra-intestinal pathogenic *E. coli*
FOX	Cefoxitin
FQ	Fluoroquinolone
GEN	Gentamicin
GIT	Gastrointestinal tract
GP	General Practitioner
GU	Genitourinary tract
HAI	Hospital associated infection
HNM	H-antigen Non-motile
HPA	Health Protection Agency
HUS	Haemolytic uremic syndrome
IM	Imipenem
LEE	Locus of enterocyte effacement
MALDI-Tof	Matrix assisted laser desorption/ionisation – time of flight
MEM	Meropenem
MIC	Minimum inhibitory concentration
MIN	Minocycline

MLEE	Multi locus enzyme electrophoresis
MLST	Multi locus sequence typing
MS	Mass spectroscopy
NCTC	National Collection of Type Cultures
NIT	Nitrofurantoin
NM	Non-motile
NMEC	Neonatal meningitis-causing *E. coli*
NT	Non typeable
OD	Optical density
ORF	Open Reading Frame
*P*ABA	Para-aminobenzoic acid
PAI	Pathogenicity island
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PHE	Public Health England (formerly HPA)
PMQR	Plasmid mediated quinolone resistance
PTZ	Tazocin (piperacillin-tazobactam)
RLH	Royal London Hospital
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SNP	Single nucleotide polymorphism
SPATE	Serine protease autotransporter of Enterobacteriaceae
ST	Sequence type
STEC	Shiga toxin-producing *E. coli*
SXT	Co-trimoxazole (trimethoprim-sulfamethoxazole)
T3SS	Type III secretion system
TEM	Temocillin
TIG	Tigecycline

Tm	Melting temperature
TOB	Tobramycin
TRIM	Trimethoprim
UC	Uncomplicated urinary tract infection
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
VF	Virulence factor

Publications and Presentations

H. Ciesielczuk, J. Betts, L. Phee, M. Doumith, R. Hope, N. Woodford & D. W. Wareham. Comparative virulence of urinary and bloodstream isolates of extra-intestinal pathogenic *Escherichia coli* in a *Galleria mellonella* model. Virulence (2014): *Accepted*

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M. Chattaway, C. Jenkins, H. Ciesielczuk, M. Day, V. DoNascimento, M. Day, I. Rodríguez, A. van Essen-Zandbergen, A. Schink, G. Wu, J. Threlfall, N. Woodford & J. Wain on behalf of the SAFEFOODERA_ESBL Consortium. Evidence of evolving extraintestinal enteroaggregative *Escherichia coli* ST38 clone. Emerging Infectious Diseases (2014): 20 (11)

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H. Ciesielczuk, M. Doumith, R. Hope. N. Woodford & D. W. Wareham. Characterisation of the ST131-O25b clone in London and the UK. Presented at the ESCMID Conference on *Escherichia coli*: an old friend with new tidings (2013)

H. Ciesielczuk, M. Doumith, R. Hope. N. Woodford & D. W. Wareham. *Escherichia coli* urosepsis: Determined by virulence determinants or antibiotic resistance? Presented at the 53rd ICAAC (2013)

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1. Introduction

1.1 *Escherichia coli*

Escherichia coli is a Gram-negative, facultative anaerobic bacillus and the type species of the genus *Escherichia*, within the Enterobacteriaceae family (Colman *et al*. 1993). The *E. coli* Genome Project measured the genome at 4,639,221 base pairs in length, with a GC content of 51%. Initial comparative gene analysis, using multiple gene and peptide databases, suggested that 60% of the genome was *E. coli*- or Enterobacteriaceae-specific (Blattner *et al*. 1997). However, a later study reported only 40% of the *E. coli* genome to be conserved, with the remaining 60% conferring the pathotype, virulence and resistance (Welch *et al*. 2002). First described by the German physician Theodor Escherich in 1885, *E. coli* was isolated from the faeces of healthy newborns and named *Bacterium coli commune* after its shape and natural habitat. However, it was not until 1954 that the name *Escherichia coli* was recognised (Cowan, 1954). Subsequent isolation from urinary tract infections (UTI) suggested ascension of bacteria from the colon to the bladder. Despite this finding, *E. coli* was long considered the major commensal of intestinal flora and of low virulence, before becoming established as a human pathogen (Crichton and Old 1985; Sussman, 1985).

As in humans, *E. coli* is also an intestinal commensal and a pathogen of animals (Bettelheim, 1997). In companion animals *E. coli* is a frequent cause of UTIs (Ewers *et al*. 2010; Johnson *et al*. 2008b), while in farm and food animals *E. coli* causes colibacillosis, post-weaning diarrhoea, mastitis and septicaemia (Ghanbarpour and Oswald, 2010; Johnson *et al*. 2009b; Ruan and Zhang, 2013).

1.1.1 Laboratory Identification

E. coli produce round, flat, opaque colonies on non-selective media (e.g. blood agar). Some strains also produce a zone of haemolysis due to haemolysin production. On selective media such as MacConkey

agar and cysteine lactose electrolyte-deficient (CLED) agar, *E. coli* ferment the lactose, producing pink and yellow colonies respectively. Although 10% of *E. coli* are lactose non-fermenters, including most diarrhoeal pathotypes, a positive indole reaction distinguishes these *E. coli* from other Enterobacteriaceae (Nataro and Kaper, 1998). A summary of the biochemical tests used to distinguish *E. coli* from other genera can be found in table 1.

Chromogenic agars are now routinely employed for isolation and identification of urinary tract pathogens, including *E. coli*, to speed up identification. These agars contain either β-glucuronidase or β-galactosidase, producing blue or pink *E. coli* colonies, depending on the manufacturer. CHROMagar is as sensitive (98-100%) as traditional selective agars employed for *E. coli* identification (Scarparo *et al*. 2002).

In clinical microbiology laboratories a combination of solid-culture, semi-automated biochemical analysers, PCR and/or matrix-assisted laser desorption ionisation time of flight (MALDI-Tof) mass spectroscopy (MS) may be employed to identify *E. coli* (Harris and Hartley 2003; Menozzi *et al*. 2006; Persson *et al*. 2007) in all specimen types. These systems are time-efficient, improving the time to bacterial identification and, in some instances, associated antibiotic resistance profile.

Table 1 Biochemical tests differentiating *Escherichia coli* from closely related genera

Organism	Lactose	ONPG	Indole	Urease	Hydrogen sulphide
E. coli	+	+	+	-	-
Shigella species	-	-	-	-	-
Salmonella species	-	-	-	-	+
K. pneumoniae	+	+	-	+	-
E. aerogenes	+	+	-	-	-
S. marcescens	-	+	-	-	-

Adapted from Medical Microbiology, 2nd edition (Mims, 1998; Boadi *et al.* 2010; Guentzel, 1996; Hale and Keusch, 1996; Rosa *et al*. 1980)

1.2 Intestinal pathogenic *Escherichia coli*

E. coli are broadly categorised into intestinal and extra-intestinal. The seven intestinal pathogenic *E. coli* variants (or pathotypes) include enterohaemorrhagic *E. coli* (EHEC), a subset of shiga-toxin-producing *E. coli* (STEC) also known as verotoxin-producing *E. coli* (VTEC); enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroinvasive *E. coli* (EIEC); enteroaggregative *E. coli* (EAEC); diffusely-adherent *E. coli* (DAEC) and adherent-invasive *E. coli* (AIEC).

These pathotypes cause diarrhoeal disease by invasion (EIEC); toxins (STEC, ETEC); or rearrangement of the intestinal epithelial cytoskeleton (EPEC), mediated by pathotype-specific virulence factors (e.g. shiga toxin (*Stx*) 1 or 2, STEC).

The prevalence of each pathotype varies according to geographical region and patient population (Croxen *et al*. 2013; Nataro and Kaper, 1998). In the UK bloody diarrhoea caused by *E. coli* is associated with food-borne outbreaks of EHEC O157:H7 and O26, although frontline laboratories tend to limit their detection to the former. Incidence is higher in the summer months, when faecal shedding of EHEC O157:H7 in cattle is at its highest, and infection is frequently more severe in children, occasionally leading to haemorrhagic colitis and haemolytic uraemic syndrome (HUS) if not diagnosed and treated promptly (Croxen, *et al.* 2013; Gormley *et al*. 2011; Jenkins *et al*. 2008; Money *et al*. 2010). DAEC is thought to account for approximately 10%-11% of cases of diarrhoea in UK children (Knutton *et al*. 2001), while EPEC strains account for approximately 4% of suspected VTEC isolates referred to Public Health England's (PHE) Gastrointestinal Bacterial Reference Unit (GBRU). Similarly to EHEC, EPEC was isolated mostly (86%) from children <15 years with bloody diarrhoea (Sakkejha *et al*. 2013). Travellers' diarrhoea has traditionally been associated with ETEC (Hill and Ryan, 2008), but in the UK EAEC and EIEC have been implicated in a significant proportion of cases (Perry *et al*. 2010). In addition to diarrhoeal disease, EAEC has been associated with UTIs and Clonal group A strains (uropathogenic clone associated with trimethoprim-sulfamethoxazole resistance), that suggest EAEC possesses extra-intestinal traits

(Olesen *et al*. 2012; Wallace-Gadsden *et al* 2007). AIEC strains also possess extra-intestinal traits, including serotypes and virulence factors. Unlike the other diarrhoeal pathotypes AIEC is associated with persistent infection of Crohn's disease patients, rather than self-limiting diarrhoeal disease of healthy patients (Martinez-Medina *et al*. 2009).

1.3 Extra-intestinal Pathogenic *Escherichia coli*

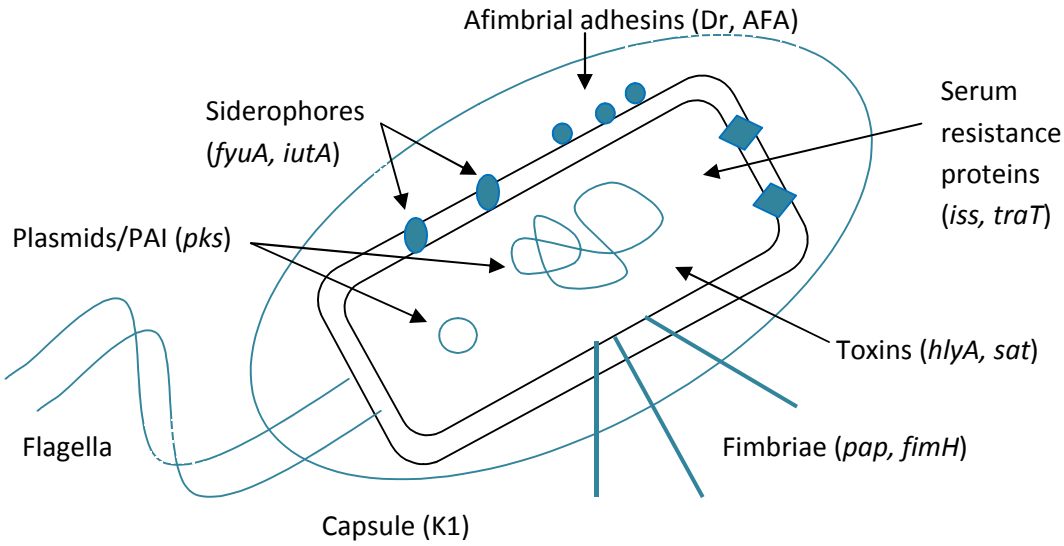
Extra-intestinal pathogenic *E. coli* (ExPEC) primarily cause UTIs and bacteraemia, but also neonatal meningitis, osteomyelitis and intra-abdominal infections, among others. They have also been characterised by phylogenetic group (predominantly groups B2 and D) and the presence of specific virulence factors: P-fimbriae, type 1 fimbriae, haemolysin, yersiniabactin, aerobactin, type II capsule, serum resistance proteins and the virulence-associated pathogenicity island *malX* (Johnson and Stell, 2000; Johnson *et al*. 2012b; Russo and Johnson, 2000; Smith *et al*. 2007; Venier *et al*. 2007).

The uropathogenic *E. coli* (UPEC) type strain, CFT073, was originally isolated from the blood and urine of a woman with pyelonephritis from Maryland, USA in 1997 (Kao *et al*. 1997). Full genome sequencing of CFT073 identified these virulence factors on virulence-associated DNA fragments, including pathogenicity islands (PAI), plasmids, prophages and insertion sequences (Parham *et al*. 2005a; Welch *et al.* 2002; Guyer *et al*. 1998). Since this initial study, resistance determinants such as CTX-M-15 and *aac(6')-Ib-cr* have been identified on these mobile genetic elements, along with these virulence factors (Huang *et al*. 2012; Woodford *et al*. 2004).

Other ExPEC pathotypes, in addition to UPEC, include neonatal meningitis-causing *E. coli* (NMEC) and avian pathogenic *E. coli* (APEC), which share genomic similarities and sequence types with human strains (Mora *et al*. 2009; Wiles *et al*. 2008). The prevalence of virulence and resistance determinants varies among these pathotypes and among commensal *E. coli*, although commensals are reported to possess fewer determinants (Obata-Yasuoka *et al*. 2002).

Different types of *E. coli* virulence factors are displayed in figure 1. Isolates in this study were investigated for a variety of virulence factors described by Johnson and Stell (2000), as detailed below (Johnson & Stell, 2000).

Figure 1 Mobile genetic elements and virulence factors possessed by extra-intestinal pathogenic *Escherichia coli*



1.3.1 Virulence determinants

1.3.1.1 Adhesins

E. coli adhesins can be broadly categorised as fimbrial (type I, P, S, F1C, G) or afimbrial (AFA, Dr, Hra, Tsh, Iha, M and CS31A). As their name infers, these virulence factors perform the important primary stage of infection: attachment to host cells or tissues. This binding facilitates the establishment of a bacterial community and/or biofilm, in order to initiate infection.

Of the fimbrial adhesins, type 1 fimbriae are by far the most common, typically identified in

88%-100% of *E. coli* strains, plus other members of the Enterobacteriaceae family, with a higher proclivity in virulent strains (Blum *et al*. 1991; Cooke *et al*. 2010; Duguid and Campbell, 1969; Johnson, 1991; Johnson *et al*. 2005b; Moreno *et al*. 2005). Key factors in adhesion, these fimbriae also aid establishment of intracellular reservoirs within bladder cells, which act as a source for recurrent UTI (Anderson *et al*. 2003).

Traditionally more widespread in UPEC causing pyelonephritis, P-fimbriae or pap (pyelonephritis-associated pili) are the second-most prevalent fimbrial type (Blum *et al.* 1991; Narciso *et al*. 2011; Norinder *et al*. 2011; Piatti *et al*. 2008). These fimbriae exist in three forms, with different binding affinities affecting the cells (uroepithelials and erythrocytes) and the host (human or animal) that they bind (Stromberg *et al*. 1990).

In contrast with type I and P-fimbriae, S- and F1C-fimbriae are reported to have a specific function in binding to the brain endothelium and facilitating the development of *E. coli* meningitis (Huang *et al*. 1995; Johnson, 1991; Korhonen *et al*. 1985; Schnaar *et al*. 2009). However, these fimbriae have also been indentified in ExPEC causing UTI and bacteraemia (Blanco *et al*. 1997; Norinder *et al.* 2011; Spurbeck *et al*. 2011), but at a lower prevalence of 21%-54% (Blum *et al.* 1991; Johnson and Stell, 2000; Johnson *et al.* 2005b; Mitsumori *et al*. 1999).

Rarely detected in human ExPEC infections (Johnson & Stell, 2000; Karisik *et al*. 2008), G-fimbriae (*gafD*) have been identified in EPEC, ETEC and occasional *E. coli* strains causing septicaemia, in humans and animals (el Mazouari *et al*. 1994; Lintermans *et al*. 1988). These fimbriae bind erythrocytes and intestinal villi, enabling toxin secretion directly adjacent to the target tissue (Lintermans *et al.* 1988; Saarela *et al*. 1995; Tanskanen *et al*. 2001).

Afimbrial cell surface adhesins function in cell binding, but also in pore formation and as serine proteases (Guignot *et al*. 2000; Kobayashi *et al*. 2010).

One of the more prevalent afimbrial adhesins is the heat-resistant agglutinin (Hra), also known as Hek, which has been identified in human and animal ExPEC. Identified in 28%-55% of human ExPEC strains, this adhesin is encoded on the J96 II PAI, but is not an absolute requirement for colonisation. However, its PAI location and association with other PAIs leads to the *hra* gene often being detected alongside the virulence factors type I fimbriae (*fimH*), haemolysin A (*hlyA*), cytotoxic necrotising factor 1 (*cnf1*), P-fimbriae (*pap*), uropathogenic specific protein (*usp*), the siderophore *iroN* and the *pks* island (Bhargava *et al*. 2009; Bidet *et al*. 2005; Johnson *et al*. 2008a; Lutwyche *et al*. 1994; Srinivasan *et al*. 2003).

The AFA/Dr family of adhesins bind different epitopes of collagen and blood group antigens in the attachment process (Labigne-Roussel *et al*. 1984; Nowicki *et al*. 1990). Unlike fimbrial adhesins this family of proteins has been identified in <20% ExPEC strains (Lopes *et al*. 2005; Venier *et al.* 2007), as well as DAEC and commensal *E. coli* isolates (Obata-Yasuoka *et al.* 2002).

More prevalent (40%) in ExPEC than the AFA/Dr adhesins (Johnson *et al.* 2008a), the iron-regulated gene A (*IrgA*) homologue adhesin (Iha), which was originally identified by deletion mutants of an adhesion-conferring operon, has been associated with virulent *E. coli* strains (Johnson *et al*. 2005a), including EHEC O157:H7 and the UPEC type strain CFT073 (Tarr *et al*. 2000). Iha appears to be upregulated in iron-depleted environments, acting as a siderophore receptor (Hagan and Mobley, 2007; Leveille *et al*. 2006).

Identified by transposon mutagenesis, the *E. coli* temperature-sensitive haemagglutinin (Tsh) so named due to its optimal expression at 26^{o}C, rather than the conventional 37^{o}C, has also been identified in multiple pathotypes, including ExPEC (7%-9%) and APEC (33%). With a broad binding specificity (erythrocytes, haemoglobin, fibronectin and collagen), this adhesin also functions as a serine protease autotransporter of Enterobacteriaceae (SPATE) protein in strains causing cystitis, pyelonephritis, bacteraemia and neonatal meningitis (Heimer *et al*. 2004; Mora *et al.* 2009; Provence and Curtiss, 1994).

Co-localised with G-fimbriae (Rhen *et al*. 1986), the M adhesin is similarly rarely detected in ExPEC strains (Johnson *et al.* 2005b), suggesting it functions more as a colonisation factor than a virulence factor (Johnson & Stell, 2000; Karisik *et al.* 2008).

Another adhesin, identified predominantly in bovine *E. coli*, is the coli-surface-associated antigen, CS31A (Korth *et al*. 1991), which also appears to be rarely detected in human ExPEC. However, this virulence factor is encoded on a highly transmissible plasmid (Jallat *et al*. 1994). Given that several *E. coli* virulence factors appear to be expressed by both animal and human ExPEC strains, it would be interesting to determine if the prevalence of this adhesin in human ExPEC has changed in recent years.

1.3.1.2 Toxins

Once attachment to host cells has been initiated, *E. coli* secrete or inject toxins that serve to damage host cells, facilitate invasion or destroy host tissues; completing the second stage of infection.

Haemolysin A (HlyA) is perhaps the principal toxin of most ExPEC strains, identified in 29%-52% of isolates (Cooke *et al.* 2010; Johnson & Stell, 2000; Mahjoub-Messai *et al*. 2011; Moreno *et al.* 2005). HlyA lyses erythrocytes allowing the producing strain to obtain iron for growth and survival in the absence of siderophores (Beutin, 1991; Blanco *et al.* 1997; Cavalieri *et al*. 1984; Mitsumori *et al.* 1999; Norinder *et al.* 2011). Encoded on a number of ExPEC PAIs, *hlyA* is often detected in combination with *pap*, *hra* and *cnf1* (Guyer *et al.* 1998; Bidet *et al.* 2005; Blanco *et al.* 1997). APEC haemolysins, *hlyE* and *hlyF*, meanwhile, are encoded on plasmids and are also occasionally identified in human ExPEC strains (del Castillo *et al*. 1997; Reingold *et al*. 1999).

Secreted autotransporter toxin (Sat) is another dominant ExPEC toxin, which had been identified in 39%-94% of investigated strains (Johnson *et al* 2008a; Mahjoub-Messai *et al* 2011; Pitout *et al*. 2005), although this toxin has rarely been screened for, likely due to its recent discovery (Guyer *et al*. 2000). Encoded on the 536 III PAI, this toxin interferes with the cytoskeleton of epithelial cells and acts as a

serine protease, with homology to two diarrhoeagenic SPATE proteins, as determined by comparison of deletion mutants and wildtype strains in cytotoxicity assays (Dobrindt *et al*. 2002; Guyer *et al.* 2000).

Vacuolating autotransporter toxin (Vat) is another SPATE protein, with homology to the *tsh* and *vat* of APEC. In addition, it has also rarely been screened for, but a few studies demonstrated high prevalence in phylogenetic group B2 and ExPEC strains causing bacteraemia and UTIs (Johnson *et al.* 2008a; Parham *et al*. 2005b).

EAEC and *Shigella flexneri* encode the SPATE 'Protein involved in Intestinal Colonisation' (Pic), which lyses mucin as part of diarrheal disease and mediates serum resistance and weak haemagglutination during extra-intestinal infection (Henderson *et al*. 1999; Olesen *et al.* 2012). However, Pic has also been identified in ExPEC strains causing cystitis and pyelonephritis, but these studies suggest that Pic is not required for extra-intestinal virulence despite its function (Heimer *et al.* 2004).

Another EAEC virulence factor is the heat-stable enterotoxin, EAST1 (Savarino *et al*. 1993). Identified in 6%-88% of diarrhoeagenic strains, EAST1 has also been detected in 16%-43% of ExPEC strains causing UTIs and bacteraemia (Girardeau *et al*. 2003; Lopes *et al.* 2005; Paiva de and Dubreuil, 2001).

Cyclomodulins are a group of toxins that interfere with the cytoskeleton of Host epithelials to mediate tissue invasion and damage. Colibactin is the most frequently detected cyclomodulin in human ExPEC strains (Dubois *et al*. 2010; Johnson *et al.* 2008a; Johnson & Stell, 2000), inducing megalocytosis: the enlargement of the cytoplasm and nucleus of cells (Nougayrede *et al*. 2006). This genotoxin is encoded on the pks island, along with *clbA*, which is reported to facilitate colibactin production and synthesis of the siderophore yersiniabactin (FyuA), together ensuring the virulence of ExPEC (Martin *et al*. 2013).

Cytotoxic necrotising factor 1 (Cnf1) functions similarly to AB-type toxins, generating multinucleated cells that develop into necrotic lesions (Caprioli *et al*. 1983; Johnson, 1991). Encoded on

the J96 II PAI (Blum *et al*. 1995), *cnf1* has been detected in 15%-54% of human ExPEC strains causing UTI and bacteraemia (Cooke *et al.* 2010; Johnson *et al.* 2005b; Mahjoub-Messai *et al.* 2011; Venier *et al.* 2007).

Cycle inhibiting factor (Cif) is common in EPEC and EHEC, but has rarely been detected in ExPEC strains (Dubois *et al.* 2010; Marches *et al*. 2003). Cif is another cyclomodulin with cysteine protease, transglutaminase and acetyltransferase activity that induces cell cycle arrest (Jubelin *et al*. 2009; Samba-Louaka *et al*. 2009; Taieb *et al*. 2006). Cytolethal distending toxin B (CdtB) is also rarely detected in human ExPEC. As with the other cyclomodulins, this toxin induces cell cycle arrest and production of large mononucleated cells (Johnson and Stell, 2000; Mahjoub-Messai *et al.* 2011; Taieb *et al.* 2006; Toth *et al*. 2003).

1.3.1.3 Siderophores

Unlike the adhesins and toxins of *E. coli*, there are only four siderophores that have been routinely screened for in human ExPEC. Siderophores are used to chelate iron from the host for various cell processes during infection (Johnson, 1991).

Yersiniabactin (FyuA) is perhaps the most well known and also the primary siderophore of ExPEC, identified in ≤98% ExPEC strains (Johnson *et al.* 2005b; Johnson and Stell, 2000; Mahjoub-Messai *et al.* 2011; Moreno *et al.* 2005). Encoded on the 'high pathogenicity island' (Riley *et al*. 2006; Schubert *et al*. 1998), FyuA has been implicated in bacteraemia-associated mortality and has been proposed to be one of, if not *the*, most important ExPEC virulence factor (Martin *et al.* 2013; Mora-Rillo *et al*. 2013).

Aerobactin (IutA) is another highly efficient siderophore of ExPEC that has been identified in *Salmonella* and *Shigella* species (Johnson, 1991). Encoded on the colV plasmid or the chromosome (de, V *et al*. 1986; Johnson, 1991), IutA has been reported in 41%-68% of ExPEC causing UTI and bacteraemia, but slightly less frequently than FyuA (Johnson *et al.* 2005b; Johnson and Stell, 2000;Mahjoub-Messai *et*

al. 2011; Moreno *et al.* 2005).

Both the iron-responsive element (IreA) and iron-regulated locus (IroN) are less frequently reported in ExPEC than IutA and FyuA (Girardeau *et al*. 1991; Johnson *et al.* 2005b; Mahjoub-Messai *et al.* 2011). However, these siderophores, identified by transposon mutagenesis, have only recently been discovered (Russo *et al*. 2001). Both IreA and IroN have been associated with APEC virulence (Jeong *et al*. 2012; Kariyawasam *et al*. 2006), but IreA has also been linked to antibiotic resistance (Johnson *et al.* 2012b; Pitout *et al.* 2005).

1.3.1.4 Capsule

E. coli produces more than 80 different capsule types, or K antigens, which are composed of acidic polysaccharides. Capsules are reported to facilitate invasion of tissues and evasion of the host immune system (Ananias and Yano, 2008; Huang *et al*. 1999; Orskov and Orskov, 1992). *E. coli* capsules were previously categorised into three groups (I-III), based on thermostability during serotyping, biochemical and genetic characteristics. However, a new capsule classification scheme has since been described which includes four capsular groups (1-4) composed of O-antigens and/or K antigens. Not all strains of *E. coli* express K antigens, but the majority of ExPEC isolates are capsulated, supporting the notion of capsules as an important virulence factor (Orskov & Orskov, 1992; Whitfield and Roberts, 1999).

The K1 capsule has dominated ExPEC infections, including neonatal meningitis and APEC, often being the most frequently reported capsule type in strains causing UTIs and bacteraemia (Johnson *et al.* 2005b; Johnson and Stell, 2000; Kariyawasam *et al.* 2006; Mahjoub-Messai *et a.l* 2011; Mora *et al.* 2009; Obata-Yasuoka *et al.* 2002). As with K1, K5 is also commonly identified in *E. coli* causing neonatal meningitis and bacteraemia (Johnson and Stell, 2000; Karisik *et al.* 2008).

Specific K-antigens are often associated with particular O-antigens, including K15, which has

been identified in O6 strains (Johnson *et al.* 2008b). Other capsular types traditionally associated with ExPEC infections included K2, K3, K8, K12, K13, K15, K52 and K53 (McCabe *et al*. 1978).

1.3.1.5 Miscellaneous

Finally, ExPEC express a variety of virulence factors, previously of hypothetical function, that do not conform to any of the above groups.

These include uropathogenic specific protein (*usp*), which was originally thought to be limited to strains causing UTIs (Kurazono *et al*. 2000). Described as an important factor in infectivity (Yamamoto *et al*. 2001), Usp has recently been described as a genotoxin with DNase activity, disrupting tight junctions and the actin cytoskeleton of host cells (Nipic *et al*. 2013). While *usp* has been identified in 80%-94% of urinary *E. coli*, this genotoxin appears to be absent from Clonal group A strains, which comprise a significant UPEC clone (Johnson *et al*. 2009a; Kurazono *et al.* 2000; Skjot-Rasmussen *et al*. 2012b).

The function of outer membrane protein T (OmpT) in ExPEC infections, on the other hand, remains unclear, although siderophore and protease activity have been suggested (Lundrigan and Webb, 1992; Rupprecht *et al*. 1983). Despite this unknown, OmpT is proposed to be a very important virulence factor of ExPEC, with a high prevalence (41%-83%) in ExPEC strains (Johnson *et al.* 2005b; Mahjoub-Messai *et al.* 2011).

In contrast to OmpT, IbeA has a defined function in the invasion of the brain endothelium during neonatal meningitis, which was determined by transposon mutagenesis (Huang *et al.* 1995; Huang *et al.* 1999). Encoded on the *gimA* genomic island (Homeier *et al*. 2010; Huang *et al*. 2001), IbeA is thought to facilitate invasion of multiple other extra-intestinal sites, as it has been detected in strains causing cystitis, pyelonephritis and prostatitis (Johnson *et al.* 2005b; Moreno *et al.* 2005). In addition, IbeA is highly prevalent in APEC strains, with a hypothetical avian origin (Homeier *et al.* 2010).

The outer membrane protein TraT confers surface exclusion (preventing cells with the same plasmid type from conjugating), has a minor role in serum resistance and is thought to prevent opsonisation, thereby inhibiting phagocytosis (Achtman *et al*. 1977; Binns *et al*. 1982; Kanukollu *et al*. 1985; Waters and Crosa, 1991). Encoded chromosomally on colV plasmids along with *iutA* (Waters and Crosa, 1991), *traT* is highly prevalent (65%-74%) among ExPEC strains causing bacteraemia and UTI (Cooke *et al.* 2010; Johnson *et al.* 2005b).

As with TraT, the increased serum survival (Iss) protein has been detected on colV plasmids, with roles in serum resistance and surface exclusion (Binns *et al.* 1982; Waters and Crosa, 1991). Despite co-localisation on colV plasmids, *iss* is less prevalent than *traT* in isolates causing bacteraemia (23% vs. 68%), UTI (17% vs. 65%) and in APEC strains, probably due to the location of the *traT* gene in the plasmid transfer region of the plasmid (Johnson *et al.* 2005b; Olesen *et al.* 2012; Skjot-Rasmussen *et al.* 2012b; Waters and Crosa, 1991).

The fourth virulence factor encoded on colV plasmids is the bacteriocin colicin V, which is thought to function as an adhesin and cause cell lysis by disrupting the cell membrane (Waters and Crosa, 1991). Like *iss*, colicin V is also less frequently detected than the other plasmid-encoded virulence factors in human ExPEC strains, suggesting colicin V is more important in APEC virulence, but not human ExPEC virulence; that these virulence factors may also be chromosomally encoded; or a mobile element within the plasmid is being transmitted that encodes *traT* and *iutA* more frequently than *iss* and colicin V (Johnson and Stell, 2000; Mora *et al.* 2009; Wang *et al*. 2010).

Finally, the enzyme encoded by *malX* functions in the metabolism of maltose and glucose (Reidl and Boos, 1991), but has no role in ExPEC virulence. However, it is located on the CFT073 II PAI, which is frequently identified in virulent ExPEC strains (Ostblom *et al*. 2011; Parham *et al.* 2005a). Therefore, *malX* is considered a marker of virulence, with high detection rates (60%-87%) in urinary and bacteraemia strains (Johnson *et al.* 2005b; Johnson and Stell, 2000; Moreno *et al.* 2005).

While new *E. coli* virulence factors continue to be discovered, data from this study is limited to the determinants described above, which are included in the updated PCR protocol of Johnson and Stell (2000) (Johnson and Stell, 2000).

1.3.1.6 *Galleria mellonella* model

Previous studies have used various cell lines (Stromberg *et al*. 1990; Guignot *et al*. 2000; Nipic *et al*. 2013) and mouse models to characterise various ExPEC virulence factors (Johnson *et al*. 2005a; Anderson *et al.* 2003), but more recently a new animal model has been proposed. *Galleria mellonella* larvae have been used to study the pathogenesis, virulence and antibiotic susceptibility of various bacteria including *Klebsiella*, *Acinetobacter*, *Stenotrophomonas* species and EPEC (Leuko and Raivio, 2012; Wand *et al*. 2013; Hornsey *et al*. 2013; Betts *et al*. 2014). In 2014 this model was validated for studying the virulence of ExPEC (Williamson *et al*, 2014) and was used in this study to analyse 40 clinical ExPEC strains. This is detailed in chapter 8.

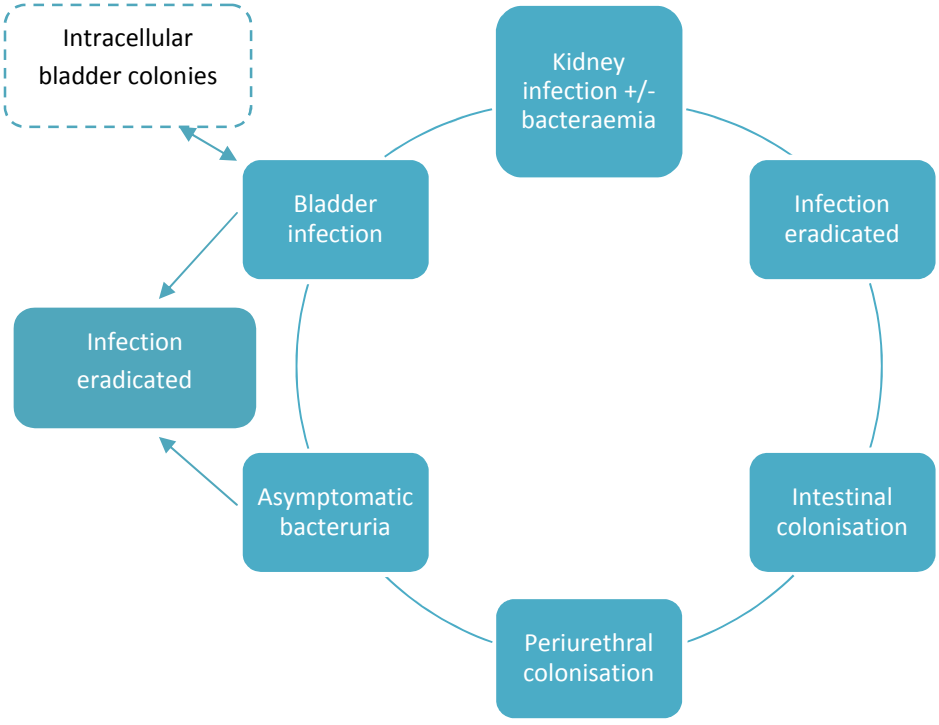
1.4 Urinary Tract Infection

It is well established in the medical community that UTIs are one of the most common community- and hospital-associated infections, affecting most women at least once in their lifetime. Approximately, 12%-20% of men will also suffer a UTI, while in children UTI prevalence ranges from <1% to 8% depending on age (Newcastle asymptomatic bacteruria research group, 1975; Foxman, 2002; Jakovljevic *et al*. 2013; Lipsky, 1989).

The commensal flora of the periurethral area is comprised of a range of bacteria, most of which are intestinal in origin: Enterobacteriaceae, *Gardnerella vaginalis*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Corynebacterium*, *Bacteroides*, *Fusobacterium* and *Veillonella* species; with *E. coli* accounting for 1%-12% (Hooton and Stamm, 1996). However, any of these bacteria may gain entry

into the bladder, via the urethra, through instrumentation or sexual activity. Host and bacterial factors (e.g. pH change, antibiotics, fimbriae) then determine whether bacteria are removed by micturition shortly after causing a temporary, asymptomatic bacteruria; or if they colonise the bladder epithelium, establishing a bladder infection termed cystitis or a lower UTI. Conditions that enable bacteria to ascend the ureters (e.g. ureteric reflux, pregnancy) result in a kidney infection, also known as pyelonephritis or an upper UTI (Mobley *et al*. 1990). This pathophysiology (Figure 2) lends to women developing a UTI more frequently than men, due to their shorter urethras and proximity of faecal commensals and pathogens, which can be transmitted easily from the perineal region to the urethral opening. Hormonal changes in women also increase susceptibility to infection at early and later stages of life (Hooton and Stamm, 1996). In men however, the urethral meatus is further from the perineal region, the skin is drier and the urethra is longer, making colonisation and ascension to the bladder less likely (Lipsky, 1989).

Figure 2 Diagram demonstrating the pathophysiology of developing a urinary tract infection and subsequent bacteraemia



1.4.1 Asymptomatic bacteruria

Asymptomatic bacteruria (ABU) is the presence of bacteria in urine in the absence of symptoms. Transient bacteruria occurs at a low frequency in children, but is common in pregnant women and the elderly. Signs of infection may include pyuria and/or a positive dipstick test. Therefore, culture is the gold standard for confirming ABU (Nicolle *et al*. 2005; Rubin *et al*. 1992).

Approximately 2%-15% of pregnant women develop ABU and/or symptomatic UTI due to changes in the position of their bladder and ureters, which results in increased urine retention and urine reflux. This results in a 20- to 30-fold increased risk of developing pyelonephritis, with potentially serious consequences for the mother and the baby (e.g. premature labour, low birth weight). Therefore all pregnant women are screened and, if warranted, treated in the early stages of pregnancy for ABU (Ipe *et al*. 2013; Nicolle *et al.* 2005).

Elderly patients are predisposed to ABU due to anatomical obstruction (e.g. prostatitis), hormonal changes and poor hygiene. However, antibiotic treatment is discouraged, as most cases will resolve naturally or go undiagnosed. In addition, antibiotic treatment in the elderly may not clear the infection; may cause the infection to become symptomatic; can lead to the infecting isolate developing resistance; may select for a resistant strain; or the patient may suffer adverse side effects (Fraser *et al*. 2012; Nicolle *et al.* 2005; O'Sullivan *et al*. 2013; Werner *et al*. 2011).

1.4.2 Uncomplicated cystitis

Uncomplicated cystitis refers to symptomatic bladder infection in healthy patients lacking any complications or abnormalities. Symptoms in women include dysuria (pain on urination), frequency, urgency and lower abdominal pain. Men may also suffer with slow-stream micturition and dribbling, while in children symptoms are often non-specific. Therefore, malaise, incontinence, vomiting, abdominal pain and nocturnal incontinence in the absence of systemic signs, such as fever, may be

suggestive of infection. In catheterised patients new costovertebral tenderness, new onset of delirium, rigors and a fever greater than 37.9°C are indicative of a symptomatic UTI (Newcastle asymptomatic bacteruria research group, 1975; Kudinha *et al*. 2013b; SIGN 2006; Lipsky, 1989).

Uncomplicated cystitis is typically reported (86%) in sexually active, non-pregnant women between the ages of 18 and 84 years, with a mean age of 40 years (Bean *et al*. 2008; Lipsky, 1989). Men usually present when aged 58 to 63 years, although UTIs attributed to instrumentation, such as catheters, have been diagnosed in men <60 years. Despite gender differences in age of presentation, cystitis is reported to increase with age, irrespective of sex (Cornia *et al*. 2006; De Backer *et al*. 2008; Kahlmeter, 2003; Koeijers *et al*. 2010; McNulty *et al*. 2004; Lipsky, 1989).

Recent studies suggested 7%-8% of children (<18 years) were diagnosed with a UTI, compared to an early study reporting a prevalence of 2%, with young girls tending to suffer more frequently than young boys. However, UTIs in young boys peak in those aged <3 months and are often attributed to congenital genitourinary disorders, frequent incomplete emptying of the bladder, greater risk of faecal contamination than their older counterparts and being uncircumcised. Renal scarring, a major complication of UTI in children, has been observed more frequently in girls (Shaikh *et al*. 2008; Newcastle asymptomatic bacteruria research group, 1975; Jakovljevic *et al.* 2013; Lipsky, 1999; McNulty *et al.* 2004).

1.4.3 Complicated cystitis and pyelonephritis

Complicated cystitis refers to a lower UTI in the presence of comorbidity, or a structural or functional abnormality that increases the patient's susceptibility to UTI. These include catheterisation or other instrumentation, diabetes, neurogenic bladder, immunocompromising conditions (e.g. lupus, HIV), recurrent UTI, infection with a multi-drug-resistant isolate, prostatitis and urogenital surgery.

Pyelonephritis is also considered a complicated UTI, which is caused by an ascending infection or

by haematogenous spread. Delays in diagnosis and/or treatment may result in impaired kidney function due to renal scarring or development of bacteraemia and sepsis, which is associated with significant mortality (Newcastle asymptomatic bacteruria research group, 1975; Ronald and Ludwig, 2001; SIGN, 2006). Reports suggest pyelonephritis occurs more often in women (60% of cases) than men, affects ≤30% pregnant women and is generally more likely to affect both kidneys (53%) in women (Lipsky, 1989; Nicolle *et al.* 2005; SIGN, 2006).

Symptoms are the same as for uncomplicated cystitis, but may be accompanied by loin pain, flank tenderness, fever, rigors or other systemic inflammatory response symptoms. In children symptoms include fever and vomiting, with or without flank pain (Balsara *et al*. 2013; Gupta *et al*. 2011; Krieger *et al*. 1999; Kudinha *et al* 2013b; Leydon *et al*. 2009; SIGN, 2006).

Men are generally considered as having a complicated UTI, due to the increased likelihood of functional/structural abnormalities (e.g. tumours, enlarged prostate), genitourinary instrumentation (e.g. catheter) or surgery. Bacterial prostatitis, occurring in men between the ages of 40 and 60 years (<1%, acute infection) or 50 and 80 years (10%, chronic infection), is the most commonly reported reason for (recurrent) UTI in elderly men. UTI symptoms may be accompanied by fever, chills, myalgia and lower back pain, although some patients remain asymptomatic (Krieger *et al.* 1999; Lipsky, 1999; Lipsky *et al*. 2010; Williamson *et al*. 2013a). In addition, 35% of men presenting with a community-associated UTI have a long-term catheter (Cornia *et al.* 2006). Despite the association between recurrent UTI and prostatitis, recurrence is more frequent in women and 78% of cases are due to *E. coli* (Czaja *et al*. 2009). In addition to the typical UTI pathophysiology, formation of intracellular pod-like reservoirs within the bladder mucosa, mediated by type 1 fimbriae, where *E. coli* are protected from antibiotics and the host immune system, are also thought to be a source for recurrent infection (Anderson *et al.* 2003; Barber *et al*. 2013).

1.4.4 Bacterial aetiology

E. coli is the most common cause of UTI (Newcastle asymptomatic bacteruria research group, 1975; Kahlmeter, 2003; Lipsky, 1999; Schito *et al*. 2009). Other bacteria isolated from women and young girls include *Klebsiella pneumoniae*, *Proteus mirabilis* and *Staphylococcus saprophyticus*, with *S. saprophyticus* more common in those aged 18 to 49 years (Newcastle asymptomatic bacteruria research group, 1975; De Backer *et al.* 2008). UTI aetiology in pregnant women is similar to non-pregnant women, although a higher prevalence (26%) of *Streptococcus agalactiae* in women with gestational diabetes has been reported (Ipe *et al.* 2013).

In men, UTI aetiology is varied. *E. coli* remains the dominant pathogen in community-associated UTIs, but other species causing the remainder of infections vary with age. For example, *Pseudomonas* species are more frequently detected in >70 years, *Enterococcus* species predominate in men aged 18 to 50 years, whilst other Gram-negative bacteria (e.g. *Serratia*, *Proteus*, *Klebsiella* and *Enterobacter* species) occur more frequently in men aged 51 to 70 years (Jakovljevic *et al.* 2013; Koeijers *et al.* 2010; Lipsky, 1999)

1.4.5 Clinical and laboratory diagnosis

Most UTIs are diagnosed clinically by a GP and empirical antibiotics are prescribed according to local guidelines and resistance rates. Only with treatment failure, pregnancy or a complicated infection are urine specimens routinely referred for microbiological investigation. However, this referral practice varies according to, and within, GP practices (Fahey *et al*. 2003; Hillier *et al*. 2006; McNulty *et al*. 2003; Olesen and Oestergaard, 1995). Therefore, resistance data derived from these specimens is often biased (Hryniewicz *et al*. 2001).

Indicators of UTI include the presence of leucocyte esterase and/or nitrites (Chien *et al*. 2007), plus erythrocytes, leucocytes (≥10^3 cells/L), cellular casts or epithelial cells, which also predict kidney

damage and the degree of specimen contamination during collection (Health Protection Agency, 2012b; Lipsky, 1989). With quantitative culture,$\geq 10^3$ or $\geq 10^5$ bacterial colony-forming units (CFU) per millilitre (ml) of urine is indicative of UTI in men and women, respectively, with lower counts considered to indicate probable contamination (Kass, 1957; Lipsky, 1999; Lipsky, 1989). Cultures of catheter bag urine are more difficult to interpret, due to permanent colonisation of the catheter. However, $\geq 10^5$CFU/ml may be considered significant, in conjunction with a new symptomatic episode, from a well-collected specimen (Health Protection Agency, 2012b; Hooton *et al*. 2010).

1.5 Bacteraemia

Presence of bacteria in the blood, or bacteraemia, is classified as primary (bacteria directly enter the bloodstream) or secondary (enter from a primary infected focus) (Mims, 1998). Bacteraemia may be transient, intermittent or continuous following tooth extraction, pneumococcal pneumonia or endocarditis, for example. Primary bacteraemia is more common in hospitalised patients with a weakened immune system or undergoing invasive procedures, while secondary bacteraemia is typically a community-associated infection (Health Protection Agency, 2012a).

1.5.1 Signs and symptoms

Fever (>38°C or <36°C) is the most common symptom of bacteraemia and may be accompanied by hypotension, tachycardia, tachypnea, confusion, rigors, oliguria, lactic acidosis, leukocytosis, leukopenia and thrombocytopenia. Examples of source-specific symptoms include pneumonia (chest source) or dysuria (urinary source). Catheter-associated bacteraemia is also difficult to diagnose, but signs include isolation of the same organism from blood cultures and catheter/tip culture; sepsis resolution on removal of the catheter; and quantitative culture comparison between catheter-drawn and alternative line/vein blood cultures.

Pregnant women often present with a fever and a raised C-reactive protein. Complications include septic shock (4%), caesarean section (29%), pre-term labour (29%), neonatal/foetal death (10%) and neonatal infection (5%). Foetal mortality was highest if diagnosis occurred in the second trimester (40%) and lowest in the third trimester (1%) (Surgers *et al*. 2012).

1.5.2 Incidence and prevalence

Bacteraemia occurs primarily in patients >65 years, in men and in those with co-morbidities (Health Protection Agency, 2011; Sogaard *et al*. 2011), followed by children <5 years (Eykyn *et al*. 1990), with a rate of 0.3% in pregnant women (Surgers *et al.* 2012).

E. coli is the leading cause, accounting for 47% of reports in Europe, with a significant increase in isolates resistant to third-generation cephalosporins. In addition, the urinary tract is the most common source (45%) of bacteraemia (de Kraker *et al*. 2012; Health Protection Agency and Health, 2012; Laupland *et al*. 2008). *Staphylococcus aureus* (29%) is also common, followed by *Streptococcus pneumoniae*, *Enterococcus faecalis* and *Enterococcus faecium* (de Kraker *et al.* 2012; Lawes *et al*. 2012). In the developing world, *E. coli* and *S. aureus* commonly cause bacteraemia, but *Salmonella typhi* (India, Nepal, Laos), *Cryptococcus neoformans*, *Mycobacterium* species (both Thailand), *Streptococcus pneumoniae* and *Haemophilus influenza* (both Indian sub-continent) are also frequent causes of bacteraemia (Deen *et al*. 2012).

In the UK, reports of *E. coli* bacteraemia have increased yearly. Between 2009 and 2013 reporting of *E. coli* bacteraemia was voluntary, with rates increasing from 27.2% to 31.5% of all reported bacteraemias. In June 2011 reporting became mandatory, with approximately 18,000 cases reported for that year. This increased to 32,000 cases in 2012 and 33,000 in 2013, compared to the 25,000 voluntarily reported in 2009 (Health Protection Agency, 2013; Health Protection Agency, 2014; Public Health England, 2014b). This rise in *E. coli* bacteraemia has been linked to antibiotic pressure and

immunosuppression facilitating *E. coli* success and evasion of the host's immune system (Health Protection Agency, 2011; Schlackow *et al*. 2012).

An Oxford (UK) study reported a 28-day mortality of 17%, far higher than a Danish study reporting a 30-day mortality of 6%, but similar to the 30-day mortality of 20% reported by a Dutch group. Inappropriate empirical therapy was hypothesised as the main reason for high mortality in Oxford, supported by the Dutch study, which reported lower mortality rates if appropriate empirical therapy was administered within 24 hours of admission (Frakking *et al*. 2013; Schlackow *et al.* 2012; Sogaard *et al.* 2011). An NHS trust in Essex (UK) reported higher 30-day mortality rates for both community-associated (25%) and hospital-associated (35%) cases of bacteraemia, which covered all bacterial causes. *E. coli* specific 30-day mortality was reported for community-associated bacteraemia only at 15.5%; with age, PITT score (system based on mental status, fever, hypotension, mechanical ventilation and history of heart attack), Charlson comorbidity index (score based on age and presence of specific diseases) and undefined source of infection significantly associated with 30-day mortality. Despite the prevalence of *E. coli* in causing bacteraemia, mortality attributed to *E. coli* was lower than that for *S. aureus* (17.1%) and *S. pneumoniae* (20%) bacteraemias (Hounsom *et al*. 2011; Melzer and Welch, 2013).

1.5.3 Clinical and laboratory diagnosis

Blood culture is the gold standard for diagnosing bacteraemia, enabling rapid detection and targeted antibiotic treatment (following empirical antibiotic therapy), to reduce mortality (Health Protection Agency, 2012a; Pradipta *et al*. 2013). However, only 7%-12% of blood cultures sent to the laboratory are positive. This may be because blood cultures were taken as a precaution, as it is not always possible to distinguish between an infectious cause and non-infectious cause from symptoms alone; the patient may have been given antibiotics prior to cultures being collected; and a low volume of blood can cause

false-negatives. This also means multiple blood culture sets may be required to identify a pathogen causing bacteraemia (Deen *et al.* 2012; Eykyn *et al.* 1990; O'Grady *et al*. 2008; Sogaard *et al.* 2011). The source of infection is confirmed by culturing the same pathogen from a non-blood site showing signs of infection, such as the urinary tract (Bouza *et al*. 2002; Horan *et al*. 2008; O'Grady *et al.* 2008). Elevated C-reactive protein (secreted in response to acute inflammation) and procalcitonin (increased in serum during severe bacterial infections) levels may also indicate infection (Tunkel *et al*. 2004).

1.6 Treatment and prevention

1.6.1 Urinary tract infection

UTIs are predominantly diagnosed clinically and treated empirically, using the recommended antibiotics listed in table 2. Unless the patient has had a previous infection, it is assumed that the urinary strain will be fully antibiotic-susceptible. Generally a short course of antibiotics is used to treat cystitis and a longer course is prescribed for complicated infections (e.g. prostatitis) and pyelonephritis. Patients not receiving antibiotics include the elderly (ABU only) and catheter-associated bacteruria, unless a new symptomatic episode arises.

Infection with a resistant isolate is more likely to be diagnosed microbiologically, and is often associated with recent antibiotic therapy. Ampicillin and trimethoprim prescriptions have been significantly associated with subsequent UTIs caused by resistant *E. coli*. A similar trend has been documented with low-dose ciprofloxacin inducing resistance-conferring mutations (Hillier *et al*. 2007; Marcusson *et al*. 2005; Nicolle *et al.* 2005). In response many GPs have reduced unnecessary prescribing, especially if the patient's symptoms are mild (Butler *et al*. 2007). The GP may also refer a urine specimen for culture to confirm the absence of a UTI or suggest self-management, such as increased fluid intake and cranberry supplements (Fahey *et al.* 2003; Leydon *et al.* 2009; McNulty *et al.* 2003; SIGN, 2006).

Table 2 Recommended antibiotic treatment for *Escherichia coli* urinary tract infections

Infection type	Antibiotic(s)
Asymptomatic bacteruria, not pregnant	No treatment
Asymptomatic bacteruria, pregnant	Nitrofurantoin (avoid at term) or co-trimoxazole (avoid in first trimester), 3-7 days
Cystitis	Nitrofurantoin or co-trimoxazole or fosfomycin (one dose) or pivmecillinam, 3-5 days (7 days for men); amoxicillin or oral cephalosporin if pregnant
Pyelonephritis	Augmentin or ciprofloxacin or co-trimoxazole, 7 days (women) or 14 days (men); add IV amikacin or IV ceftriaxone if hospital-acquired, patient has a co-morbidity or received antibiotics in last 3 months
Catheter-associated bacteruria	Ciprofloxacin or augmentin, 7-10 days
Prostatitis	Ciprofloxacin, 14-28 days; trimethoprim as an alternative

(Gupta *et al.* 2011; Joint Formulary Committee 2013; Lipsky *et al.* 2010; SIGN, 2006; Barts and the London Trust Antimicrobial Review Group, 2011)

1.6.2 Bacteraemia

National and local (Bart's and the London Trust) recommendations for antibiotic treatment of *E. coli* bacteraemia can be found in table 3. Empirical treatment is started within one to two hours of diagnosis due to the high morbidity and mortality rates. Treatment may also involve catheter removal, fluid drainage or surgical debridement. If severe sepsis is diagnosed, fluids and vasopressors may also be administered to facilitate tissue recovery. Treatment length varies according to antibiotic resistance, source of infection and complicating factors. If the source of infection can be removed (e.g. catheter) treatment can last 7 days, whilst bacteraemia secondary to endocarditis, liver abscess, brain abscess, prosthetic device-related infection or disciitis may be extended to 4-6 weeks to achieve optimal tissue penetration. In pregnant women, treatment for 14 days with amoxicillin, augmentin or a third-generation cephalosporin has been reported (Surgers *et al.* 2012). However, with the high rates (70%) of

augmentin resistance reported in *E. coli*, a third-generation cephalosporin with low-level resistance would be more appropriate (Cooke *et al.* 2010; Health Protection Agency, 2011; Schlackow *et al.* 2012).

Table 3 Recommended antibiotic treatment for *Escherichia coli* bacteraemia

Treatment type	Antibiotic(s)	Infection source
Empirical	Broad-spectrum cephalosporin (e.g. cefuroxime) or anti-pseudomonas penicillin (e.g. piperacillin-tazobactam)	Unknown
Suspected resistant bacteria	Use carbapenem (e.g. meropenem)	
Escherichia coli **bacteraemia**	Augmentin or piperacillin-tazobactam or ciprofloxacin, 14 days. Add amikacin if severe	Genitourinary tract
	Meropenem, 14 days	Pancreatitis
	Meropenem, 21 days. De-escalate to ceftriaxone (if required) once antibiogram known	Meningitis
	Piperacillin-tazobactam or ciprofloxacin or ceftriaxone, 14 days	Cholecystitis, peritonitis
	Augmentin, 14 days. Add amikacin if severe	Biliary, (post-surgical) intra-abdominal
	Ciprofloxacin, 4-6 weeks	Osteomyelitis

(Bouza *et al.* 2002; Dellinger *et al*. 2013; Phee, 2013; Pradipta *et al.* 2013; Barts and the London Trust Antimicrobial Review Group, 2011)

1.6.3 Vaccines

Vaccines targeting *E. coli* are currently licensed for use in cattle and poultry only (e.g. SCOURMUNE®, Merck, NJ, USA; PROSYSTEM® RCE, Merck). An *E. coli* vaccine for preventing human UTI/ExPEC infections is required, because recurrent UTI does not confer a protective effect, increasing resistance is limiting treatment options, and the ExPEC population is highly heterogeneous. However, this heterogeneity also makes designing an *E. coli* vaccine difficult.

Various UPEC strains, outer membrane proteins and virulence factors have been trialled in *E. coli*

vaccines including Urovac® (SolcoBasel, Basel, Switzerland and Protein Express, Cincinnati, OH, USA), Uro-Vaxom® (OM Pharma, Myerin, Switzerland), Urvakol® (Institute of Microbiology, Olomouc, Czech Republic) and Urostim® (Bulbio, National Centre for Infectious and Parasitic Diseases, Sofia, Bulgaria). However, these were poorly immunogenic, did not confer long-term protection and compliance issues were raised (Brumbaugh and Mobley, 2012). A novel vaccine (Medimmune, Gaithersburg, MD, USA) containing the *fimH* adhesin and *fimC* chaperone protein was evaluated in monkey and human trials. While promising in monkeys, protective antibody levels in women were comparable to previous vaccine trials, requiring further vaccine development (Langermann *et al*. 2000; Meiland *et al*. 2004).

Bacterial interference by means of a 2-hour indwelling bladder suspension with non-pathogenic *E. coli* provides an alternative option, with>50% patients remaining infection-free one year after treatment. However, the once-daily dosing will likely result in compliance issues (Darouiche *et al*. 2005).

1.7 Antibiotic Resistance

1.7.1 Antibiotic resistance by age and sex

Antibiotic resistance rates in *E. coli* are reported to be higher in men, or women, depending on the study. This is likely due to the chosen study population and/or regional differences in prescription practices (Bean *et al.* 2008; De Backer *et al.* 2008; den Heijer *et al.* 2013; Health Protection Agency, 2008; Koeijers *et al.* 2010; Linhares *et al*. 2013; Schito *et al.* 2009).

In children, the antibiotic-naive may carry *E. coli* that is resistant to trimethoprim (34%) and amoxicillin-clavulanate (48%), representative of community strains and resistance rates (Chakupurakal *et al*. 2010). Resistance increases into adulthood as patients are prescribed antibiotics, acquire resistant community strains and are hospitalised (Fahey *et al.* 2003; Hillier *et al.* 2007; Sahm *et al*. 2001). Antibiotic resistance peaks in patients aged >65 years and isolates are more likely to be multi-drug

resistant (Gobernado *et al*. 2007; Goettsch *et al*. 2000).

1.7.2 Antibiotic resistance in urinary tract infections

Empirical treatment has evolved as resistance has increased. Short courses of ampicillin or trimethoprim have been replaced with β-lactam/β-lactamase inhibitor combinations, early-generation cephalosporins and trimethoprim-sulfamethoxazole (co-trimoxazole). Transmission of plasmid-mediated extended-spectrum β-lactamases (ESBLs) with *aac(6')-Ib-cr* and spread of strains with chromosomal fluoroquinolone resistance, has also led to the re-evaluation of ciprofloxacin in treating complicated UTIs, with dual therapy proposed. Nitrofurantoin and fosfomycin may be considered for uncomplicated cystitis (Den Heijer *et al*. 2010; Dyer *et al.* 1998). Fortunately community-associated infections (CAI) tend to demonstrate lower rates of resistance than hospital-associated infections (HAI) (Bean *et al.* 2008). Antibiotic resistance rates and associated resistance mechanisms, for urinary and bloodstream *E. coli* isolates, can be found in table 4.

1.7.3 Antibiotic resistance in bacteraemia

E. coli causing bacteraemia are often more resistant than urinary strains (Bean *et al.* 2008; Livermore *et al*. 2008), as demonstrated by the resistance rates in table 4. In addition, ESBLs are more frequently isolated from bloodstream infections than UTIs (e.g. 12% vs. 2%) (Livermore *et al.* 2008; Schito *et al.* 2009). Despite this, carbapenem resistance remains low and carbapenemase enzymes are rarely detected in *E. coli* compared to other Enterobacteriaceae and non-fermenting Gram-negative rods (European Centre for Disease Prevention and Control, 2011; Woodford *et al*. 2013). However, this will probably change as plasmid-mediated carbapenemases start to circulate within the *E. coli* population.

Table 4 UK non-susceptibility rates for the antibiotics used to eradicate *Escherichia coli* causing urinary tract infection and bacteraemia

Antibiotic	Mechanism of action*	Resistance mechanism*	Percentage resistance (UTI)#	Percentage resistance (BLC) #
Ampicillin	Binds penicillin-binding proteins, inhibiting cell wall synthesis	Penicillinase enzymes (TEM-1, OXA-1) or ESBLs (especially CTX-M types, particularly CTX-M-15 in UK); increased expression of efflux pumps	**31.8 - 55**	**63 – 91**
Amoxicillin-clavulanate	Clavulanate inhibits β-lactamase enzymes; amoxicillin binds penicillin-binding proteins		**2 – 48**	**9 – 43.8**
Piperacillin-tazobactam	Tazobactam inhibits β-lactamase enzymes; piperacillin binds penicillin-binding proteins	TEM and OXA ESBLS and carbapenemase enzymes	**NK**	**8 – 10.3**
Cefuroxime†	Binds penicillin-binding proteins, inhibiting cell wall synthesis	Expression of ESBLs or carbapenemase enzymes e.g. KPC	**0.5 – 7.5**	**4 – 19.4**
Meropenem	Binds penicillin-binding proteins, inhibiting cell wall synthesis	Expression of carbapenemase enzymes; porin loss; upregulation of efflux pumps	**NK**	**0 – 0.1**
Trimethoprim	Binds DHFR enzymes involved in folate metabolism	Increased enzyme activity or alternative enzyme e.g. *dfrA*	**14.9 – 40.2**	**40**
Trimethoprim-sulfamethoxazole	Binds DHFR and *P*ABA enzymes involved in folate metabolism	Increased enzyme activity or alternative enzyme e.g. *dfrA, sul1*	**14.4**	**40 - 54**
Nitrofurantoin	Inhibit enzymes of the citric acid cycle, DNA and protein synthesis	unknown	**0 – 11**	**NK**
Ciprofloxacin	Binds DNA gyrase, preventing rejoining of DNA strands	Mutations in DNA gyrase (*gyrA*) and topoisomerase IV(*parC*) enzymes; DNA gyrase protection proteins (*qnrA/B/C*); efflux pumps (*qepA*, *oqxAB*); other enzymes e.g. *aac(6')-Ib-cr*	**0.5 - 12**	**14 – 42**
Gentamicin‡	Binds 30S ribosomal subunit, inhibiting transcription and thus protein synthesis	Aminoglycoside modifying enzymes e.g. the N-acetyltransferase encoded by *aac(6')-Ib*	**0.5 – 6.3**	**7.5 – 12**

†representative of cephalosporin resistance; ‡representative of aminoglycoside resistance

NB. DHFR – dihydrofolate reductase, *P*ABA – *para*-aminobenzoic acid, UTI – urinary tract infection, BLC – bacteraemia, NK - unknown

*(Drawz and Bonomo, 2010; Joint Formulary Committee, 2013; Lacey, 1982; McOsker and Fitzpatrick, 1994; Neu and Gootz, 1996; Pan *et al*. 1996; Strahilevitz *et al.* 2009)

#(Bean *et al.* 2008; Chakupurakal *et al.* 2010; Cooke *et al.* 2010; Health Protection Agency, 2011; Horner *et al*. 2014; Kahlmeter and Poulsen, 2012; Livermore *et al.* 2008)

1.8 Typing

Traditionally *E. coli* strains were differentiated by phenotypic and biochemical methods, but these have been surpassed by various molecular methods. Typing of *E. coli* was originally performed to improve understanding of the species and infections it causes, but now typing facilitates outbreak investigations and various research studies (Gibreel *et al*. 2012; Gordon *et al*. 2008; Johnson *et al.* 2012b; Ochman *et al*. 1983).

1.8.1 Serotyping

Serotyping of *Escherichia coli* was first described by Kauffman, who classified this genus according to its O (somatic antigen, part of the surface lipopolysaccharide), H (flagellar) and K (capsular) antigens (Kauffmann, 1947). One hundred and eighty-one O-antigens, 80 K-antigens and 56 H-antigens have since been identified, creating thousands of possible unique O:H:K serotypes. Occasionally multiple genera of Enterobacteriaceae may share the same serogroup, such as *E. coli* O8 and *Klebsiella pneumoniae* O5 (Orskov and Orskov, 1992; Scheutz *et al*. 2004; Reeves and Wang, 2002).

Within virulent *E. coli* specific serogroups have been associated with intestinal and extra-intestinal infections. In 1978, serogroups O6 and O16 were associated with adult and neonatal infections, respectively, with O4, O6 and O18 particularly associated with septic shock and death (McCabe *et al.* 1978). Other important extra-intestinal serogroups included O1, O2, O7, O8, O11, O15, O22, O25, O75, O78, O83 and O86 (Ananias and Yano, 2008; Kanamuru *et al.* 2006; Korhonen *et al.* 1985; McCabe *et al.* 1978; Melzer *et al.* 2008; Orskov and Orskov, 1992). Multiple *E. coli* pathotypes (e.g. ExPEC, EAEC and ETEC) often share the same serogroups, probably due to genetic exchange in the intestine before transmission to other sites. These include O1, O2, O4, O6, O15, O18, O25 and O77 (Nataro and Kaper, 1998). ExPEC causing UTIs in companion animals, meanwhile, also share serogroups common to human strains: O4 and O6. Together these serogroups demonstrate the universal, cross-species virulence and success of *E. coli* (Orskov and Orskov, 1992; Wang *et al.* 2010).

1.8.2 Multi-locus enzyme electrophoresis

Multi-locus enzyme electrophoresis (MLEE) was first described in the 1980s to characterise *E. coli* by allelic variation of enzymes encoded at specific loci in the genome. Differences between alleles could be used to approximate the number of nucleotide differences or amino acid substitutions. Unlike serotyping, which often branded strains non-typeable; MLEE identified an allele every time. Analysis of *E. coli* allelic variation identified three distinct clusters - I, II and III - with groups II and III closely related (Ochman *et al.* 1983; Selander *et al*. 1986). The ECOR collection comprised 72 *E. coli* reference strains isolated from humans and animals, from around the world, which represented the genotypic diversity of *E. coli,* as determined by MLEE (Ochman and Selander, 1984). MLEE characterisation of these isolates using 38 enzyme loci, instead of the traditional 20, separated these isolates into four main phylogenetic groups (A, B1, B2 and D), which corresponded with Ochman's groups: I (A), II (B) and III (D). Originally there was also a phylogenetic group designated C, but MLEE analysis led to the redistribution of these strains among the four other phylogenetic groups (Herzer *et al*. 1990). However, phylogenetic group C has since been re-established as a sister group to B1, while phylogenetic group E contains EHEC O157:H7 strains and phylogenetic group F is a sister group to B2 strains (Clermont *et al*. 2011b; Croxen *et al.* 2013).

1.8.3 Phylogenetic Grouping

These studies and subsequent ones revealed phylogenetic groups (phylogroup) A and B1 to comprise principally commensal and intestinal pathogenic strains, while phylogroups B2 and D contained virulent, persistent strains causing extra-intestinal pathogenic infections. Occasionally there were strains that contradicted this general consensus, such as EAEC and EPEC isolates which have been classified as phylogroup D (Bingen *et al*. 1998; Croxen *et al.* 2013; Herzer *et al.* 1990; Ochman *et al.* 1983). Phylogroup distribution of *Shigella* species also varied, with most *Shigella dysenteriae* classified as phylogroup B1, *Shigella boydii* classified as either phylogroup B1 or D, while the remaining species were classified as phylogroup D. However, the closely related EIEC was classified

mostly within phylogroups A or B1 (Rolland *et al*. 1998). In addition, phylogroups B2 and D were reported to have much larger genomes, suggesting acquisition of virulence-associated DNA fragments and mobile elements from other strains or species (Boyd and Hartl, 1998).

To avoid the complex and time-consuming use of MLEE, a triplex PCR was developed, using the genes *chuA*, *yjaA* and the DNA fragment TspE4C2, to identify these four principal phylogenetic groups based on combinations of presence and absence of these three amplicons (Clermont *et al.* 2000). This simple, rapid procedure has since been used internationally to classify *E. coli* isolates (Bukh *et al*. 2009; Cagnacci *et al*. 2008; Johnson *et al.* 2009a). However, comparison of this triplex PCR with multi-locus sequence typing (MLST) schemes (see 1.1.2.4) identified some discrepancies, due to genetic variation in the PCR target genes (Gordon *et al.* 2008). MLST data suggested that this triplex PCR could not accurately assign 100% of *E. coli* isolates to the correct phylogroup. This report also assigned isolates to phylogroups C and E, according to MLST data, but the PCR assay reported these isolates as either phylogroup A, B1, B2 or D. It is possible that these strains represent hybrids of the main phylogroups or that the assay needs updating, as these rare phylogroups have been described previously (Clermont *et al.* 2011a; Escobar-Paramo *et al*. 2004; Gordon *et al.* 2008). Recently a novel phylogroup has been described, but it is unclear how these strains relate to the established phylogroups A - F (Mendonca *et al.* 2011; Molina-Lopez *et al*. 2011). To resolve this issue of the novel phylogroup, the triplex PCR was revisited and primers redesigned for optimal assignation of phylogroups (Doumith *et al*. 2012).

1.8.4 Multi-locus sequence typing (MLST)

Six years after the development of the phylogroup triplex PCR, MLST was developed to analyse and classify the *E. coli* population. There are two main MLST schemes used for *E. coli*: the Institut Pasteur scheme (www.pasteur.fr/mlst), which uses six gene targets, and the UK-based Achtman scheme, which uses seven alternative gene targets, described here (Wirth *et al.* 2006). Sequencing and concatenation of seven chromosomally conserved (house-keeping) genes interspersed around the *E.*

coli genome created a numeric profile or sequence type (ST): *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase, subunit B), *icd* (isocitrate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate synthetase) and *recA* (recombinase A). This scheme has identified between 292 and 533 alleles, based on sequence polymorphisms, for each of the seven target genes, creating thousands of hypothetical STs. However, only 600 STs and 54 sequence type complexes (STC) have been identified (http://mlst.warwick.ac.uk), demonstrating the conservative and clonal nature of *E. coli* (according to ST and STC), but also the diversity (according to alleles). Clustering of ST data was mostly in agreement with the four major phylogenetic groups. However, there were additional hybrid groups containing isolates belonging to two (AxB1) or three (ABD) of the phylogenetic groups, which appear to have resulted from homologous recombination. These virulent hybrids likely represent phylogroups C and E, as these clusters included *Shigella* species, diarrhoeagenic strains and some extra-intestinal strains (Wirth *et al.* 2006).

The Achtman MLST scheme has since become well established in the field of ExPEC (Dias *et al*. 2009; Manges *et al*. 2008; Nicolas-Chanoine *et al*. 2008). However, it should be noted that data generated by the Institut Pasteur scheme and Achtman scheme are not entirely comparable (Gordon *et al.* 2008) and major ExPEC clones have often been defined by the Achtman scheme only (Tartof *et al*. 2005).

With the advent of whole genome sequencing, MLST and phylogroup data can now be acquired simultaneously, along with the detection and identification of genes for virulence factors and those conferring antibiotic resistance or raised MICs. Isolates may also be compared by SNP analysis (McNally *et al.* 2013). In the future, this technique may supersede all those used in this study to characterise ExPEC.

1.8.5 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) has been used to analyse inter-strain, inter-species and intergenic variation in DNA. Restriction enzymes are used to cleave DNA, infrequently, at specific

restriction sites, producing DNA bands of various lengths according to the number of restriction sites and polymorphisms. Separation of these bands using an alternating electric current enables optimal resolution of large DNA fragments and separation of the smaller DNA fragments (Bingen *et al*. 1994). In reference facilities and research laboratories, PFGE compares epidemiologically linked isolates to determine their genetic relatedness and to identify outbreak strains (Gibreel *et al.* 2012; Manges *et al.* 2008). As with serotyping this method can be time-consuming, but PFGE provides a deeper analysis of *E. coli* populations, often sub-dividing different clones and lineages. While this can facilitate understanding of a particular lineage, it also adds another layer of complexity in understanding extra-intestinal *E. coli*. Therefore, it is important to use this method in the right setting, such as discriminating outbreak and non-outbreak strains.

1.9 Lineages of extra-intestinal pathogenic *Escherichia coli*

1.9.1 Major lineages

Monitoring of antibiotic resistance and molecular typing has identified specific ExPEC lineages. They pose a significant public health threat due to their resistance and circulation in the community (Johnson *et al.* 2009a; Woodford *et al.* 2004). The five major lineages are ST131, ST95, ST73, ST69 and ST127 and belong principally to phylogroup B2, but also to group D (ST69). Particular serogroups associated with these lineages include O1 (ST95), O6 (ST73 and ST127), O17 (ST69) and O25 (ST131 and ST69), which are frequently identified along with O2, O4, O8, O9, O15, O17, O18 and O75 (Gibreel *et al.* 2012; Johnson and Stell, 2000; Johnson *et al*. 2013; Lau *et al*. 2008b; Manges *et al.* 2008; Weissman *et al*. 2012; Johnson and Russo, 2002).

1.9.2 ST127

ST127 (phylogroup B2) is associated with UTIs, serotype O6:H31 and IroN, S/F1C-fimbriae, HlyA and Cnf1 (Johnson *et al*. 2006; Lau *et al.* 2008b). An alternative study linked ST127 to canine infections and virulence factors IreA, K15 and F48, F536 and F16 fimbriae (Johnson *et al.* 2008b). Compared to

the other ExPEC lineages, ST127 reportedly has the highest number of virulence factors, but is relatively antibiotic susceptible (Gibreel *et al.* 2012). Despite this, ST127 comprised approximately 1%-6% ExPEC strains (Banerjee *et al*. 2013a; Gibreel *et al.* 2012; Horner *et al*. 2014; Lau *et al.* 2008b; Mahjoub-Messai *et al.* 2011).

1.9.3 ST73

ST73 (phylogroup B2) was associated with serogroup O6, specifically serotype O6:K2:H1, and causing UTIs. Additional associated serogroups included O2 and O18, plus the virulence factors P-fimbriae, F1C-fimbriae, IutA, Iha, Sat and HlyA (Johnson *et al.* 2006; Johnson *et al.* 2008b; Lau *et al.* 2008b; Mahjoub-Messai *et al.* 2011; Manges *et al.* 2008).

ST73 strains are often pan-susceptible (29%), with ampicillin, amoxicillin-clavulanate and trimethoprim resistance reported, very occasionally with ESBL expression (Dias *et al.* 2009; Horner *et al.* 2014; Kang *et al*. 2013; Manges *et al.* 2008). However, ST73 strains were more prevalent accounting for 7%-15% of ExPEC (Dias *et al.* 2009; Lau *et al.* 2008b; Mahjoub-Messai *et al.* 2011).

1.9.4 ST95

Human ST95 (phylogroup B2) strains are reported to have originated from APEC. Classified by serogroups O2 and O18, plus serotype O1:K1:H7/NM, isolates appear to have an antibiogram similar to ST73 (Manges *et al.* 2008; Mora *et al.* 2009). Associated virulence factors included type I fimbriae, P-fimbriae, Tsh, IroN, colicin V and Iss*,* although Tsh, IroN, Iss and colicin C were more common in APEC (Mora *et al.* 2009). Studies reveal ST95 to account for 7%-38% ExPEC strains causing UTI and bacteraemia (Lau *et al.* 2008b; Mahjoub-Messai *et al.* 2011).

1.9.5 ST69

ST69 (phylogroup D) was originally isolated from women with a UTI in North America, in 2001, and was associated with trimethoprim-sulfamethoxazole resistance (Manges *et al.* 2001). Also known as Clonal group A (CgA) the majority of isolates belong to serogroups O11, O15, O17, O25, O44, O73,

O77, O86 and O125ab. However, resistance to trimethoprim-sulfamethoxazole has varied by study (8%-93%), calling into question the validity of UTI treatment guidelines in eradicating this lineage (Blanco *et al*. 2011; Dias *et al.* 2009; Gupta *et al.* 2011; Lau *et al.* 2008b; Manges *et al.* 2008; SIGN, 2006; Skjot-Rasmussen *et al* .2012b; Tartof *et al.* 2005). Virulence factors are also varied, but include type I fimbriae, P-fimbriae, Sat, IutA, group II capsule, TraT, Iha, FyuA, Sat, OmpT and Ag43 (Blanco *et al.* 2011; Johnson *et al.* 2009a; Skjot-Rasmussen *et al.* 2012b). Prevalence of ST69 also varies by study and country (4%-15%), demonstrating that the significance of this particular lineage varies geographically (Blanco *et al.* 2011; Dias *et al.* 2009; Johnson *et al.* 2009a; Skjot-Rasmussen *et al.* 2012b).

1.9.6 ST131

The ST131 lineage contains an extensive range of strains, predominantly of phylogroup B2, but also group D, as well as ranging in antibiotic susceptibility. However, studies tend to focus on CTX-M-15 producing, ciprofloxacin resistant, O25b:H4 strains; using fluoroquinolone resistance as the definitive characteristic of this sub-clone (Clermont *et al*. 2008; Gibreel *et al.* 2012; Lau *et al*. 2008a; Nicolas-Chanoine *et al.* 2008; Peirano and Pitout, 2010). PFGE analysis also demonstrates great sub-clonal diversity (Lau *et al.* 2008a), but virulence factors are relatively conserved with most strains expressing type I fimbriae, Sat, FyuA, Usp, OmpT, TraT and *malX* (Blanco *et al.* 2011; Clermont *et al.* 2008; Johnson *et al.* 2009a; Nicolas-Chanoine *et al.* 2008). The prevalence of this clone ranges from 12% to 43%, as does resistance to extended-spectrum cephalosporins (16%-69%), fluoroquinolones (26%-67%), aminoglycosides (28%-55%) and trimethoprim-sulfamethoxazole (18%-31%) (Blanco *et al.* 2011; Johnson *et al.* 2009a; Johnson *et al*. 2010).

1.10 Present-day situation

E. coli is the predominant cause of UTIs and bacteraemia within the UK and worldwide (de Kraker *et al.* 2012; Den Heijer *et al.* 2010; Health Protection Agency, 2011; Koeijers *et al.* 2010).

Phylogroups B2 and D are most prevalent, but phylogroups A and B1 are also increasing (Abdallah *et al*. 2011; Clermont *et al*. 2013a; Lee *et al*. 2010), with a significant number of ESBL producers observed among phylogroup A (Lee *et al.* 2010; Pitout *et al.* 2005).

Increasing resistance to cephalosporins, fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole has been associated with these circulating lineages, particularly ST131 and ST69 (Colpan *et al*. 2013; Johnson *et al*. 2011). However, it is unclear how other sequence types may be contributing to antibiotic resistance in *E. coli*. In addition, UK resistance rates are routinely monitored for *E. coli* causing bacteraemia by the BSAC bacteraemia resistance surveillance programme, but little is known about resistance in other infections, such as urinary tract infections, with the exception of hospitals or research groups reporting results from their studies (Reynolds *et al*. 2008; Bean *et al.* 2008; Gibreel *et al.* 2012). Resistance surveillance is especially important in light of the rapid rise and spread of carbapenemase-producing Enterobacteriaceae across Europe, with 13% of those referred to Public Health England's Antimicrobial Resistance and Healthcare Associated Infections Reference Unit identified as *E. coli* (Woodford *et al.* 2013).

ExPEC isolates are reported to have an inverse relationship between antibiotic resistance and virulence (Cooke *et al.* 2010; Piatti *et al.* 2008). As the major ExPEC lineages vary in their virulence and resistance it is important to investigate this phenomenon, to achieve greater understanding of the true implication of these lineages (Gibreel *et al.* 2012; Johnson *et al.* 2009a).

In order to accomplish this the current ExPEC population needs to be defined and the future population structure predicted; the repercussions of ESBL-producing phylogroup A strains needs to be evaluated, as well as the likelihood of carbapenem-resistant and pan-resistant ExPEC emerging; and the heterogeneity of virulence factor possession within clonal strains and ExPEC as a whole needs to be investigated, as does the potential for novel MLST types or emerging clones.

2. Hypothesis and Project aims

Hypothesis 1: Urinary *E. coli* strains are predominantly isolated from female patients aged 18-60 years, while bacteraemia strains are frequently isolated from patients >60 years old. ExPEC strains in the UK and London are highly variable, but predominantly comprise specific lineages and serogroups, with the majority of isolates belonging to phylogroups B2 and D.

Aim 1: Define the patient population for ExPEC infections and the UK ExPEC population using patient data and molecular typing techniques. Compare isolates from London and the UK to identify similarities between these two populations.

Hypothesis 2: Urinary *E. coli* are more susceptible to antibiotics than bacteraemia strains, with resistance more significant in bacteraemia isolates. However, overall antibiotic resistance in *E. coli* is rising.

Aim 2: Determine the antibiograms for urinary and bacteraemia *E. coli* strains by agar dilution and perform PCRs to detect various resistance genes. Describe resistance rates according to patient and strain variables; compare with published reports; and interpret the results in light of current therapeutic guidelines.

Hypothesis 3: ST131-O25b is the most prevalent ExPEC lineage and the greatest threat to empirical antibiotic therapy in the UK.

Aim 3: Determine the prevalence of ST131 strains in the UK and by Public Health England region. Characterise the clone using established methods.

Hypothesis 4: Specific virulence factors are required to cause bacteraemia, which differ from those required for causing UTI. *E. coli* causing urosepsis are more resistant and virulent.

Aim 4: Define and compare the virulence factors required for causing UTI, bacteraemia and urosepsis. Use an infection model to determine the significance of virulence or resistance in urosepsis.

3. Materials and Methods

This chapter includes details of the isolate collections and the tests performed on these specimens prior to study collection. Generic methods used throughout the study are detailed here, while chapter-specific methods and amendments are included in the relevant sections of this thesis.

3.1 Study Definitions

E. coli Bacteraemia – *E. coli* isolated from a positive blood culture as determined using the BD BACTEC™ (Becton Dickinson, Maryland, USA). At the Royal London Hospital only isolates with ≥1 of the following signs or symptoms were collected during the study: fever, rigors, confusion, query sepsis, pyelonephritis, fall, diarrhoea and vomiting, query unwell or hypotension (O'Grady *et al.* 2008).

Asymptomatic Bacteruria - Isolation of $\geq 10^4$-10^5 CFU per ml of bacteria, in pure form, from the patient's urine (Kass, 1957), with no symptoms suggestive of UTI at the time of collection (Nicolle *et al.* 2005; SIGN, 2006) and no other signs or sources of infection detected. For pregnant women, isolates were collected from urine that was taken at the first antenatal appointment.

Uncomplicated Cystitis – Diagnosis was based on semi-quantitative culture, as described above, and the presence of ≥1 of the following symptoms: frequency, urgency, dysuria and/or suprapubic pain, pyuria and/or haematuria, with no signs suggestive of kidney infection (Nicolle *et al.* 2005; SIGN, 2006).

Complicated cystitis and/or pyelonephritis – Complicated cystitis was defined as a patient with lower UTI symptoms in the presence of a functional/structural abnormality or complication, including prostatitis, recurrent infection, immunocompromised (e.g. diabetes, multiple sclerosis), urinary tract abnormality or recent genito-urinary surgery. UTIs in men were considered complicated infections. Pyelonephritis was defined as bladder infection, accompanied by ≥1 symptoms suggestive of upper

UTI, including costovertebral angle (flank) pain and tenderness, loin pain, fever, chills, rigors or other signs suggestive of systemic inflammatory response (Nicolle *et al.* 2005; SIGN, 2006).

Note: there has been much debate about how to classify UTI in children due to difficulty diagnosing UTIs and the risk of renal scarring. As part of this study, children <18 years who presented with symptoms suggestive of lower urinary tract infection were classified as uncomplicated cystitis. Children <18 years presenting with a fever, loin pain and/or tenderness or other signs suggestive of upper urinary tract infection were classified as complicated UTI (Habib, 2012; NICE, 2007). As with adults, all males with a UTI were considered complicated infections.

Community-associated Infection (CAI) – Patient presenting with signs or symptoms of an infection to their General Practitioner (GP), community clinic or out-patient department (OPD), including Accident and Emergency. CAI also included hospitalised patients who presented with signs or symptoms of infection within 48 hours of admission.

Hospital-associated Infection (HAI) – Hospitalised patient who presented with signs or symptoms of infection more than 48 hours after admission. Infections may also be defined as healthcare-associated, such as those in nursing homes or those that have been hospitalized within the last 30 days but have since been discharged (Cooke *et al.* 2010). Due to the lack of patient information it was not possible to identify isolates that fit this definition. Therefore, this study defines isolates as CAI and HAI only.

Non-susceptible – In this study non-susceptible referred to isolates considered as completely resistant or with intermediate resistance to an antibiotic, as determined by comparing the minimum inhibitory concentration (MIC) with the antibiotic specific breakpoint (Andrews, 2001; The European Committee on Antimicrobial Susceptibility Testing, 2014).

3.2 *Escherichia coli* Isolates

3.2.1 Local Collection

In total 556 (n=508 urinary & n=48 bacteraemia) *E. coli* isolates were collected from 540 patients at the Clinical Microbiology Laboratory at the Royal London Hospital, between October 2011 and February 2012. Demographical data collected alongside each isolate included patient age, sex and healthcare setting (e.g. GP, Accident & emergency, Hospital name, etc). The Clinical Microbiology laboratory serves GPs, clinics and hospitals of three London boroughs; Hackney, Tower Hamlets, Newham and now Waltham Forest, although Waltham Forest was included after all study isolates were collected.

Patients were classed as having either a community-associated infection (CAI) or a hospital-associated (HAI); urinary tract infections were defined as asymptomatic bacteruria (ABU), uncomplicated cystitis (UC) or complicated cystitis and/or pyelonephritis (COMP) based on clinical details; and source of infection was identified by a positive culture of the same organism, collected within 24 hours of the blood culture, from urine, stool, site-specific swab, site-specific fluid, cerebral spinal fluid (CSF), tips or sputum specimen.

Only one isolate was collected per patient, except for 17 patients where *E. coli* isolates were collected from both their urine and their blood (paired isolates), for comparison. Paired isolates were only collected from clinical specimens that were taken within 24 hours of each other.

3.2.2 BSAC Collection

In total 521 *E. coli* isolates were collected as part of the BSAC Bacteraemia Resistance Surveillance Programme by Public Health England (PHE, Colindale, UK), from 38 laboratories around the UK and Republic of Ireland, between 1st January and 31st December 2011. Each laboratory sent up to 14 consecutive *E. coli* isolates from separate patients. Demographical data received with the isolates included patient age, sex, source of bacteraemia and healthcare setting. From here on these isolates will be known as the BSAC (2011) collection. Only isolates accompanied by a full set of demographical data were included in the study.

3.2.3 Identification

Local collection: Pink colonies grown on Brilliance™ UTI Agar (Oxoid, Basingstoke, UK) were provisionally identified as urinary *E. coli*. Blood culture isolates were sub-cultured on to blood agar (Oxoid) after a Gram stain of the positive bottle revealed Gram-negative rods. All urinary and blood culture isolates were identified as *E. coli* using MALDI-Tof MS (Bruker Daltonik GmbH, Bremen, Germany), according to the manufacturer's instructions. Briefly, individual colonies were lightly smeared on to the target plate and overlaid with matrix (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid). Once dried, the plates are loaded into the mass spectrometer, where each sample is vaporised (desorption) by the laser and ionised. The mass analyser detects these ions, creating a spectral image unique to bacterial and fungal genera and most species (Clark *et al*. 2013).

BSAC collection: Isolates were sub-cultured on to MacConkey agar (PHE) to check for pure growth. Pure cultures were then sub-cultured on to nutrient agar (PHE) before their identity was confirmed using MALDI-Tof MS (Bruker Daltonik), as above.

3.3 DNA extraction

DNA lysates were prepared from pure cultures using a simple boil extraction. A 10 µl loop of overnight culture was suspended in 200 µl sterile distilled water and incubated at 95°C for 10 minutes. The suspensions were cooled to room temperature and centrifuged for 1 minute at 13,000 rpm in a Heraeus™ Pico™ microcentrifuge (Thermo Scientific, MA, USA) to pellet the cell debris. Supernatant containing the DNA was transferred to a new eppendorf and stored at -20°C, for approximately one month, until required for PCR assays and/or transformation.

3.4 Polymerase chain reaction (PCR)

Throughout this project multiple genes were sought to facilitate characterisation and identification of the study isolates.

Assays performed at the Blizard Institute (Queen Mary's) used 12.5 µl ReddyMix™ PCR Master Mix (Thermo Scientific) as the main component for all PCR reactions. While assays performed at PHE used 12.5 µl MyTaq™ Red Mix (Bioline, London, UK) as the main component for all PCR reactions. Only the phylogrouping assay (section 4.2.2) was performed at both sites, using both master mixes. Positive controls were used to validate the assay at each site and master mixes were found to be comparable.

Primers were used at a concentration of 0.5 µM and reactions made up to 20 µl with sterile-filtered water (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK), unless otherwise stated.

Amplification typically consisted of an initial hold at 94°C for 3 minutes; followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 1 minute; with a final extension at 72°C for 5 minutes. Alternative cycling conditions are included in the relevant sections and all primers are listed in appendix C.

Amplified DNA was separated by 2% gel electrophoresis (section 3.5), alongside an Invitrogen™ 100 base-pair (bp) or 123 bp DNA ladder (Life Technologies, Paisley, UK).

3.5 Gel electrophoresis

A 2% agarose gel was prepared using 2 g UltraPure™ agarose (Invitrogen™, Life Technologies) dissolved in 100 ml 1X Tris-Borate-EDTA (TBE) buffer (Promega, Southampton, UK). DNA samples were electrophoresed in Bionic™ 1X buffer (Sigma) or TBE buffer at 130 volts for 90 minutes. Gels were stained for at least 30 minutes in ethidium bromide solution or Gelred™ (Biotium, CA, USA) and excess removed by rinsing in distilled water. The banding patterns were visualised using a G:Box imaging camera (IMGEN technologies, Alexandria, VA, USA) and software.

3.6 PCR product purification

Purification of CMY PCR products, at QMUL, (see section 5.2.8) was performed using the MinElute® PCR purification kit (Qiagen, Crawley, UK). The in-house method was used to purify all other PCR

products. Purified DNA was stored at 4°C to -20°C until required for DNA sequencing (maximum one week).

For the MinElute® PCR purification PCR products were diluted 5-fold in binding buffer and transferred to a spin column. Centrifugation for 1 minute at 13,000 rpm transferred DNA onto the column membrane. The DNA was washed with 750 µl of wash buffer and centrifuged again. DNA was then eluted in 20 µl elution buffer (10mM Tris-chloride, pH 8.5) or water, by centrifuging again for one minute.

For the in-house method 2 µl reaction solution was mixed with 10 µl PCR product and incubated at 37°C for 45 minutes (enzyme activation), followed by 80°C for 20 minutes (enzyme inactivation). The final purified product was diluted 1:2 prior to sequencing. The reaction solution was made up in 10 µl aliquots consisting of 1 µl exonuclease I reaction buffer (New England BioLabs© Inc., MA, USA), 1 µl rAPid alkaline phosphatase reaction buffer (Roche Diagnostics Ltd., Burgess Hill, UK), 1 µl exonuclease I enzyme (New England BioLabs©), 1 µl rAPid alkaline phosphatase enzyme (Roche Diagnostics Ltd.) and 6 µl sterile-filtered water.

3.7 Sanger DNA sequencing

PCR amplicons were sequenced using the dideoxy Sanger sequencing method (Shendure et al. 2004) and DNA sequences determined using an ABI Genetic Analyser Capillary Platform (Life technologies). CMY plasmids were sequenced by Source Bioscience (London) and all other purified PCR products were sequenced by the Genomic Services Unit at PHE.

3.8 Statistics

Statistical analysis was performed using STATA®, version 12 (StataCorp LP, TX, USA). For tests used and set significance level please refer to each chapter.

GraphPad Prism version 5.04 (GraphPad Software Inc., CA, USA) was used for analysis of data generated using the *Galleria mellonella* assay (chapter 8).

3.9 Ethics

This research project was logged with the Queen Mary Research Office, the Barts Health NHS Trust Joint Research and Development Office and Public Health England's Research and Development Office (Appendix A).

NHS research ethics approval was not required for this study, as this study used sub-cultures of bacteria isolated from patient's specimens and patient identifiable information was not used. Please see a quote from the Barts Health NHS Trust website below:

> "We often receive queries asking if it is necessary to seek ethics approval before commencing research using collections of micro organisms, where the organisms were derived from human samples. Professor Terry Stacey, Director of the National Research Ethics Service, has advised that there is no need to seek approval from an NHS research ethics committee for such projects.
>
> Guidance from the Department of Health's inspector of microbiology confirmed this, and in addition stated that "any bacterial sub-cultures and viruses isolates, made from clinical specimens, are not part of the specimen themselves, they do not belong to the patient from whom they were obtained".
>
> However, if you are at all in doubt about your study, it is best to check before going ahead, to confirm that no ethics approval is needed."

(http://www.bartshealth.nhs.uk/research/governance/administrative-processes/ethical-approval/ Accessed 8th October 2011).

4. The UK ExPEC population

4.1 Introduction

Escherichia coli is the most common cause of urinary tract infection (UTI) in the UK and Europe (Chakupurakal *et al.* 2010; Denes *et al*. 2012; Kahlmeter, 2003). Predominantly an infection of women, the majority of symptomatic patients (men and women) present to their GP or community clinic, with fewer healthcare-associated UTIs reported (Bean *et al.* 2008; Hryniewicz *et al.* 2001). Although, the World Health Organisation ranks UTIs as the most common hospital-associated infection (HAI), in the UK UTI is secondary to respiratory infections among hospitalised patients, while across Europe UTIs are the third most frequently reported HAI (World Health Organisation, 2002; Health Protection Agency, 2012c; European Centre for Disease Prevention and Control, 2013a). Importantly, the urinary tract is reported as the most frequent source of bacteraemia and *E. coli* the most common pathogen (Bukh *et al.* 2009; de Kraker *et al.* 2012; Horner *et al.* 2014; Hounsom *et al.* 2011). Unlike UTI, bacteraemia is more frequently diagnosed in men and is predominantly an infection of those aged >64 years. However, as with UTIs, bacteraemia is more frequently a community-associated infection (CAI) (Health Protection Agency, 2011; Health Protection Agency, 2014; Hounsom *et al.* 2011; Laupland *et al.* 2008).

With these slight variations in patient demographics, it is important to define the UK patient population at risk of *E. coli* UTI and/or bacteraemia. This will provide insight into how these two infections are linked or whether they are primarily independent infections that occasionally occur together.

Over the years extra-intestinal pathogenic *E. coli* (ExPEC) have been characterised by source of infection (extra-intestinal site), phylogroup (chiefly B2 and D), virulence factors and antibiotic resistance (Clermont *et al.* 2000; Johnson *et al.* 2009a; Johnson and Stell, 2000; Russo and Johnson, 2000). However, studies of ExPEC often focus on restricted geographical areas (e.g. North-West England), particular infection syndromes (e.g. urosepsis) or strain characteristics (e.g. serogroup O6),

rather than the ExPEC population as a whole in a particular region or country (Johnson *et al.* 2008b; Lau *et al.* 2008a; Lau *et al.* 2008b). In the UK the BSAC Bacteraemia Resistance Surveillance Programme routinely monitors the antibiogram of *E. coli* isolates collected from around the UK (Reynolds *et al.* 2008), but does not analyse the genotypic characteristics that comprise this *E. coli* population. In addition, there is no UK-wide study of urinary *E. coli* and it was impractical to collect urinary *E. coli* from around the UK for this study. However, as *E. coli* bacteraemia is primarily caused by urinary *E. coli*, it was decided that urinary *E. coli* isolates collected in East London could be directly compared to the UK-derived bloodstream isolates.

Therefore, this study characterised 521 bloodstream isolates collected from across the UK, as well as 556 urinary and bloodstream isolates collected in London, with the aim of defining the UK ExPEC population, the patients frequently affected by these strains and to determine whether these populations have changed over time.

4.2 Methods

4.2.1 Isolates

Details of the Local (n=556; 508 urinary, 48 bloodstream) and BSAC (n=521) collections of *E. coli* isolates can be found in section 3.2. It was originally planned to collect urinary and bloodstream isolates over a similar time period so that they could be directly comparable. Unfortunately it was not possible to collect urinary isolates retrospectively from January 2011 through to October 2011 when this project started. Therefore, urinary isolates were collected between October 2011 and March 2012. A similar number of urinary and BSAC isolates were collected to facilitate fair comparisons between specimen collections, although the bacteraemia isolates were collected from a widespread geographical region compared to the urinary isolates which were restricted to East London.

Only BSAC isolates with a full complement of patient data (sex, age, location) were analysed, regardless of the source of infection. The strategy for collecting the urinary isolates was different and focused on the type of UTI, with relatively equal proportions of uncomplicated (UC) and complicated (COMP) cystitis/pyelonephritis isolates and a smaller proportion of asymptomatic bacteruria (ABU) isolates. This was to enable large-scale characterisation of *E. coli* isolates causing uncomplicated cystitis, complicated cystitis/pyelonephritis and asymptomatic bacteruria, but also considered the difficulty in collecting an equally large proportion of asymptomatic bacteruria isolates. The lack of clinical details accompanying urine specimens and available information on the hospital information systems led to development of the study definitions (section 2.1). These definitions ensured urinary isolates were collected that categorically matched each UTI type. Local bloodstream isolates were collected similarly to the BSAC isolates, with complete patient demographics. However, the focus on collecting paired isolates, from both the urine and blood of a patient, led to a much smaller collection of Local bloodstream isolates.

4.2.2 Phylogenetic Grouping

All 1077 isolates underwent phylogenetic grouping using a modified multiplex PCR assay (Doumith *et al.* 2012) based on the PCR previously described by Clermont *et al* (2000). This method enabled distinction between phylogroup A isolates that lacked all three ExPEC genetic markers and PCR-negative isolates, by inclusion of a universal *E. coli* (*gadA*) marker.

PCR reaction and amplification conditions were as described in section 3.4, with an abbreviated extension step of 30 seconds per cycle. Primers are listed in appendix C. The banding pattern generated by gel electrophoresis (section 3.5) was used to identify the phylogroup (Table 5).

Table 5 Multiplex PCR profiles for the four principal *Escherichia coli* phylogenetic groups

All possible phylogroup A, B1, B2 and D profiles are included, along with an unknown profile reported by several authors and seen in this study (Clermont, Bonacorsi, & Bingen 2000;Doumith, Day, Hope, Wain, & Woodford 2012;Mendonca, Calhau, Lin, Boaventura, Ribeiro, & Da Silva 2011;Skjot-Rasmussen et al. 2013). Targets included the *E. coli* specific target, glutamate decarboxylase alpha gene (*gadA*), an outer membrane heme receptor (*chuA*), a hypothetical protein (*yjaA*) and a DNA fragment from a lipase esterase gene (TspE4.C2).

Targets	A	A	B1	B2	B2	D	D	Unknown
gadA	X	X	X	X	X	X	X	X
chuA				X	X	X	X	
yjaA		X		X	X			X
TspE4.C2			X		X		X	X

4.2.3 Sequence type PCR

The five major ExPEC lineages are ST131, ST127, ST95, ST73 and ST69 (Gibreel *et al.* 2012; Lau *et al.* 2008b; Manges *et al.* 2008). All isolates were screened for these lineages by multiplex PCR (Doumith *et al*. 2014). Primers are listed in appendix C. PCR reactions were prepared according to section 3.4, with 12.5 µl PCR buffer. Amplification conditions were as described in section 3.4, with an annealing

temperature of 60°C and an extension step of 30 seconds, followed by a final hold at 72°C for 3 minutes. Products were separated by gel electrophoresis (section 3.5) and band size used to identify the lineage: ST131 (310bp), ST127 (404bp), ST95 (200bp), ST73 (490bp) and ST69 (100bp).

4.2.4 Multi-Locus Sequence Typing (MLST)

Paired isolates that were PCR-negative for the five major ExPEC lineages underwent MLST, using the Achtman scheme (http://mlst.warwick.ac.uk/mlst/) and a modified version of the protocol described by Wirth *et al* (Wirth *et al.* 2006). Primers and corresponding annealing temperatures can be found in appendix C. The isolate with the unusual phylogroup profile also underwent MLST, as did an ST131 isolate with a serogroup novel to this clone, to confirm the sequence type PCR result.

PCR reactions were as described in section 3.4, using 15 µl PCR buffer. Cycling conditions consisted of an initial hold at 94°C for 2 minutes; followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute and extension at 72°C for 1 minute; completed by a final hold at 72°C for 5 minutes. Products were separated by gel electrophoresis (section 3.5) to confirm gene presence; amplicons purified (section 3.6) and sequenced (section 3.7). Sequences were aligned in BioNumerics version 6.1 (Applied Maths NV, Sint-Martens-Latem, Belgium) and entered into the Achtman scheme database to identify the specific gene alleles and composite sequence type.

4.2.5 Serogrouping

Six hundred and fifty-nine of 1077 (61%) sequential *E. coli* isolates (n=380 urinary and n=279 bloodstream) were serogrouped and this was done according to the method by Gross and Rowe (Gross and Rowe, 1985), as adopted by the Gastrointestinal Bacterial Reference Unit, Public Health England.

Isolates were grown overnight at 37°C in Hedley Wright broth, then steamed at 100°C for 40 minutes using an SBB14 boiling bath (Grant Instruments Ltd, Cambridge, UK). Cultures were cooled and preserved in 0.3% (v/v) formal saline at a ratio of 1:2.

Serogrouping was performed in three stages. 1) Equal volume of culture (40 µl) was added to the screening plate, containing all 191 O-antisera (PHE), and incubated at 50°C overnight. Positive agglutinations were recorded and these O-antisera used in the second stage. 2) Titrations of the positive sera were prepared (doubling dilutions in saline from 1/100 to 1/6400), equal volumes of culture were added (50 µl) and the plate incubated as previous. Positive agglutinations and titres were recorded and compared with titres of the relevant O-antigen control strain. Titres equal to that of the control strain, or within two doubling dilutions, were titrated against pure absorbed antisera. 3) Pure monoclonal antisera were diluted 1:5 then titrated in a 96-well microtitre plate (Thermo Scientific) as before (doubling dilutions in saline). Equal volumes of culture were added (50 µl) and suspensions were incubated overnight as previous. If the test isolate generated a titre equal to, or within one doubling dilution, of the O-antigen control strain the serogroup was recorded and identified.

All bloodstream isolates and urinary isolates that were negative at the initial screen (191 O-antisera) were autoclaved at 121°C for 15 minutes to remove possible capsular material. Isolates that agglutinated with all O-antisera at the initial screen were sub-cultured into a Thiotone Craigie broth for 1-5 days at 37°C to remove the rough coat and serogrouping repeated.

Isolates that were positive for one of the five major ExPEC lineages (section 4.2.3) were screened for all serogroups previously identified in each corresponding ST (Table 6) (Johnson *et al.* 2008b; Manges *et al.* 2008; Platell *et al*. 2011). If negative for these serogroups, the complete serogrouping procedure was performed. All other isolates underwent the complete serogrouping procedure. Isolates that did not generate significant agglutination titres or did not agglutinate with any of the initial antiserum were classified as O-non-typeable (NT). Isolates which agglutinated with more than 40 antisera were classified as rough/NT.

Table 6 The five major ExPEC lineages and their corresponding serogroups as reported in studies conducted in the UK, North America and Australia (Johnson *et al.* 2008b; Lau *et al.* 2008b; Manges *et al.* 2008; Platell *et al.* 2011).

Sequence type	Phylogroup	Corresponding serogroups
ST131	B2	O16 O25
ST127	B2	O6
ST95	B2	O1 O2 O18
ST73	B2	O6 O18
ST69	D	O11 O15 O17 O25 O73 O77

4.2.6 Enteroaggregative *E. coli* (EAEC) PCR

A selection of urinary isolates, with serogroups frequently detected in EAEC (O3, O15, O44, O62, O77, O78 and O86), were selected for *aggR* screening (Olesen *et al.* 2012; Smith *et al*. 1994; Wallace-Gadsden *et al.* 2007). The *aggR* gene regulates expression of aggregative adherence fimbriae I (AAF/I) and is restricted to EAEC, making it an ideal target for differentiating between EAEC and other diarrheal pathotypes (Nataro *et al*. 1994), plus extra-intestinal *E. coli*. This assay was performed by colleagues in the Gastrointestinal Bacterial Reference Unit, PHE, using the method published by Chattaway and Jenkins (2014) (Chattaway *et al*. 2014).

Each PCR reaction contained 12.5 µl FAST BLUE qPCR MasterMix (Eurogentec, Southampton, UK), 10 µM of each primer, 1 mM of probe and 1 µl of DNA lysate made up to 25 µl with sterile-filtered water (Sigma). Primers are listed in appendix C. Amplification consisted of an initial hold at 95°C for 5 minutes; followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1 minute; and a final extensions at 72°C for 10 minutes on a Rotor-Gene Q (Qiagen GmbH, Hilden, Germany).

4.2.7 Pulsed-field Gel Electrophoresis (PFGE)

Paired isolates, isolate 3837 (unknown phylogroup) and a random, diverse selection of isolates (n=22) representing the major ExPEC lineages and phylogroups were analysed by PFGE. This was performed to facilitate identification (unknown phylogroup), to determine if urosepsis is caused by

one or multiple strains (paired isolates) and to determine the general relatedness of ExPEC strains (lineage isolates). PFGE was performed using the method described by Turton *et al* (Turton *et al*. 2004), as adopted by the Antimicrobial Resistance and Healthcare Associated Infections Reference Unit (AMRHAI), PHE.

Isolates were grown on nutrient agar overnight at 37°C and colonies suspended in 1 ml suspension (SE) buffer to a density of 2.3-2.7 McFarland. A 1:1 suspension of culture and 2% agarose (Sigma) was prepared and aliquotted into gel blocks. Blocks were set at 4°C for <1 hour, then suspended in 3 ml first lysis buffer with 0.5 mg/ml lysozyme (Sigma), overnight at 37°C, with shaking at 550 rpm on an Innova® 2100 platform shaker (New Brunswick Scientific, Stevenage, UK). Lysis buffer was aspirated and replaced with 3 ml alkaline phosphate buffer and 3.6 µl proteinase K, then incubated in an OLS200 shaking waterbath (Grant instruments Ltd) overnight at 56°C and 550 rpm. Buffer was aspirated and blocks washed 3 times with 1X Tris-EDTA (TE) buffer at 4°C for at least 30 minutes. After the final wash blocks were suspended in 2 ml 1X TE buffer until digestion.

1X FastDigest buffer (Thermo Scientific, MA, USA) was aliquotted (100 µl) into 0.5 ml eppendorf tubes and a 1-2 mm strip from each agarose block was suspended in the digest buffer. Blocks were incubated overnight at 4°C. Digest buffer was aspirated and replaced with 100 µl fresh 1X FastDigest buffer, plus 2 µl (2U/100µl) *Xba*I FastDigest enzyme (Thermo Scientific), before incubation at 37°C for at least 30 minutes. Isolate blocks were loaded into the wells of a 1.25% (approximately) agarose gel (MacroSieve low melt agarose; SLS Ltd, Hessle, UK), interspersed every 6-8 lanes with a concatenated lambda ladder (New England BioLabs©). The gel was run for 30 hours, at 6 volts and at 12°C, with an initial switch of 5 seconds and a final switch of 35 seconds, using a CHEF-DR II chiller system (Bio-rad, Hemel Hempstead, UK). After electrophoresis gels were stained with Gelred™ (Biotium, CA, USA; section 3.5) and analysed using BioNumerics, version 6.1 (Applied Maths NV). Isolate similarity was calculated using dice coefficient and isolates clustered using unweighted pair group method with arithmetic averages (UPGMA) with a band tolerance of 1%.

4.2.8 Statistical analysis

Associations between isolate and patient variables were calculated using the chi^2 test, with Stata, version 12 (StataCorp LP). A *P-value* of ≤0.05 was considered significant, unless stated otherwise.

4.3 Results

Patient demographics

Patient demographics for the urinary isolates can be found in table 7. Urinary isolates were primarily from female patients (n=426, 84%), aged 21-50 years (n=250, 59%; Figure 3) and were mostly diagnosed in the community (n=453, 89%). Due to the lack of clinical details accompanying urine specimens, all ABU isolates, except one (64 year old female), were collected from pregnant women aged 18-41 years at their first antenatal appointment. UC isolates were also collected from women only, as men were considered to have a complicated UTI. COMP isolates were collected from significantly more HAIs and from more patients >50 years of age (*P*<0.0001), than the remaining urinary isolates.

Table 7 Patient demographics for the urinary isolates

The number of isolates is listed for each UTI type, with the percentage listed in parenthesis.

Demographic	Asymptomatic bacteruria (ABU)	Uncomplicated cystitis (UC)	Complicated cystitis /pyelonephritis (COMP)
Total:			
	102	198	208
Healthcare setting:			
CAI	101 (99)	190 (96)	162 (78)
HAI	1 (1)	8 (4)	46 (22)
Sex:			
Male	-	-	82 (39)
Female	102 (100)	198 (100)	126 (61)
Age:			
Range	18-64	2-99	1-94
Mean	28	31	54
Median	29	32	58

Forty (8%) urinary isolates were collected from children (<18 years old). Overall these accounted for 2% (n=2) and 9% (n=38, *P*=0.04) of urinary isolates from male and female patients, respectively. Including the two isolates from boys, 35% were classified as COMP and the remaining 65% as UC.

Co-morbidities and complicating factors accompanying urinary isolates from children included fever, convulsions, recurrent infection and pyelonephritis. For adult patients these factors were also listed, along with urological instrumentation/surgery, diabetes, systemic symptoms or sepsis, catheter, renal disease/failure/transplant, HIV, lupus, liver disease, cancer/receiving chemotherapy, prostatitis, spinal compression and recent ESBL. The most frequently reported complicating factors, accounting for 42% (n=87) of all COMP isolates, were diabetes, recurrent infection, pyelonephritis and systemic signs.

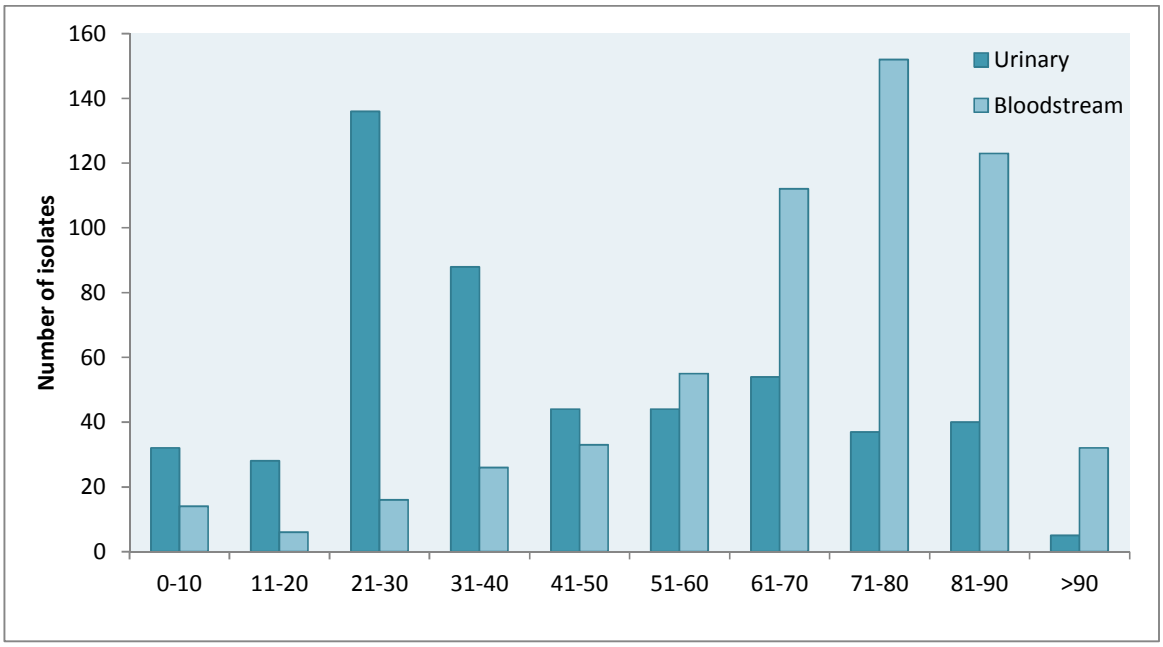


Figure 3 Age range of patients comprising the urinary and bloodstream isolates

Patient demographics for the bloodstream isolates can be found in table 8. As with urinary isolates, the majority of bloodstream isolates were from CAIs, but at a lower proportion than seen with UTIs (67% bloodstream vs. 89% urinary). A similar proportion of male and female patients contributed to the bloodstream isolates, despite the low number of local bloodstream isolates collected, which made drawing conclusions difficult. For example, the number of bloodstream isolates collected as part of the BSAC collection, increased with age regardless of sex, but within the local bloodstream

isolates this pattern was only noted for male patients, as there was no discernible relationship between age and the female sex.

Bloodstream isolates were also collected from children (<18 years old), accounting for 4% (n=11) and 1% (n=4) of male and female patients with bacteraemia, respectively.

Table 8 Patient demographics for the bloodstream isolates

Demographic	Local No. Isolates (%)	BSAC No. Isolates (%)
Total:		
	48	521
Healthcare setting:		
CAI	30 (63)	353 (68)
HAI	18 (37)	168 (32)
Sex:		
Male	23 (48)	260 (50)
Female	25 (52)	261 (50)
Age:		
Range	11-90	0–99
Mean	55	68
Median	64	73

The genitourinary (GU) tract was the most common source of bacteraemia, accounting for 56% and 38% of the local and BSAC isolates, respectively (Figure 4). The gastrointestinal tract (GIT) was the second most common source, followed by the respiratory tract (Chest). Skin and soft tissue infections (SSTI), intravenous lines (Line) and cerebral spinal fluid (CSF) comprised the remaining bloodstream isolates in the BSAC collection, but these sources were not reported in the local bloodstream isolates. Unknown source was recorded for 31% and 43% of local and BSAC bloodstream isolates, respectively, with ascites, diabetic foot ulcers, trauma, cancer and surgery listed as potential foci of infection.

Notably, all local bloodstream isolates from female patients were recorded as having a urinary focus of infection and in the BSAC collection GU-source isolates were more frequently collected from female patients than male patients. In contrast, respiratory-source isolates were more frequently collected from male patients (15 male, 5 female).

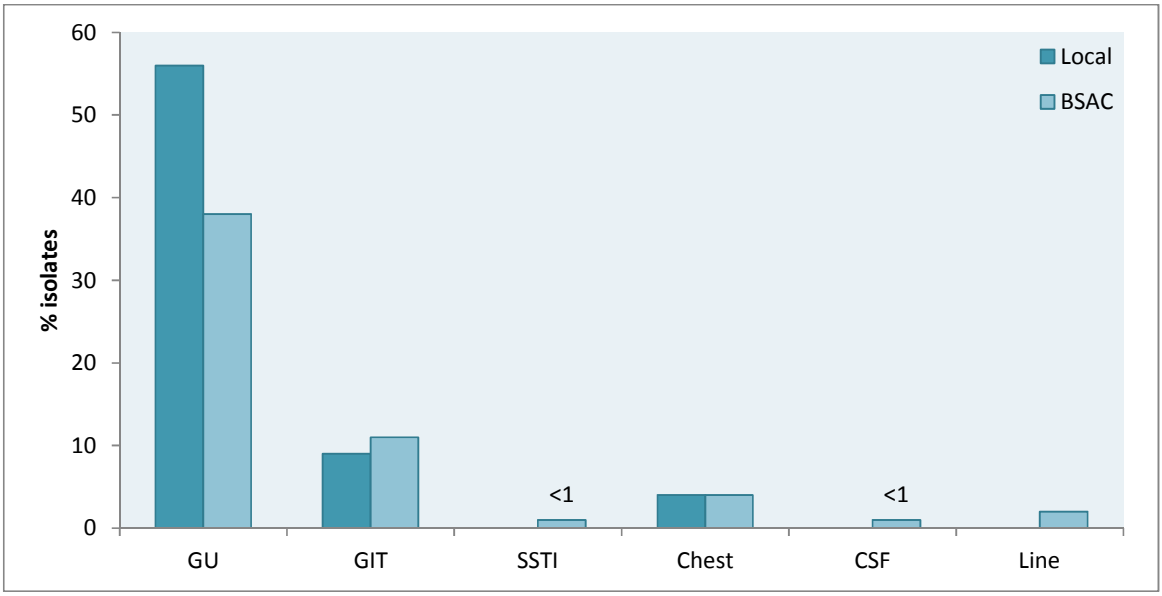


Figure 4 Source of bacteraemia for both Local and BSAC collections Sources included the genitourinary (GU) tract, gastrointestinal tract (GIT), skin and soft tissue infections (SSTI), respiratory (Chest) infection, cerebral spinal fluid (CSF) and intravenous (Line) line infections.

Strain characteristics

Phylogenetic grouping

Phylogroup distribution according to infection type (UTI or bacteraemia) is displayed in figure 5. Phylogroup B2 comprised 64% of all study isolates, followed by phylogroup D (22%). Phylogroup A isolates consisted mostly of urinary strains (13% vs. 7% bloodstream), while phylogroup B1 strains were relatively evenly distributed between the urinary (5%) and bloodstream (3%) isolates. Generally, phylogroups A, B1 and D were more frequently detected in urinary isolates, but B2 isolates were more frequent in bloodstream isolates. Local urinary and bloodstream isolates followed a similar trend in phylogroup.

Overall phylogroup B2 was more common in male patients (74% vs. 59%) and phylogroup D was more common in female patients (26% vs. 13%), except for the local bloodstream isolates, where phylogroups A (26% vs. 0%) and B1 (9% vs. 4%) were more prevalent in male patients and

phylogroups B2 (60% vs. 43%) and D (36% vs. 22%) were more prevalent in female patients (Figure 6).

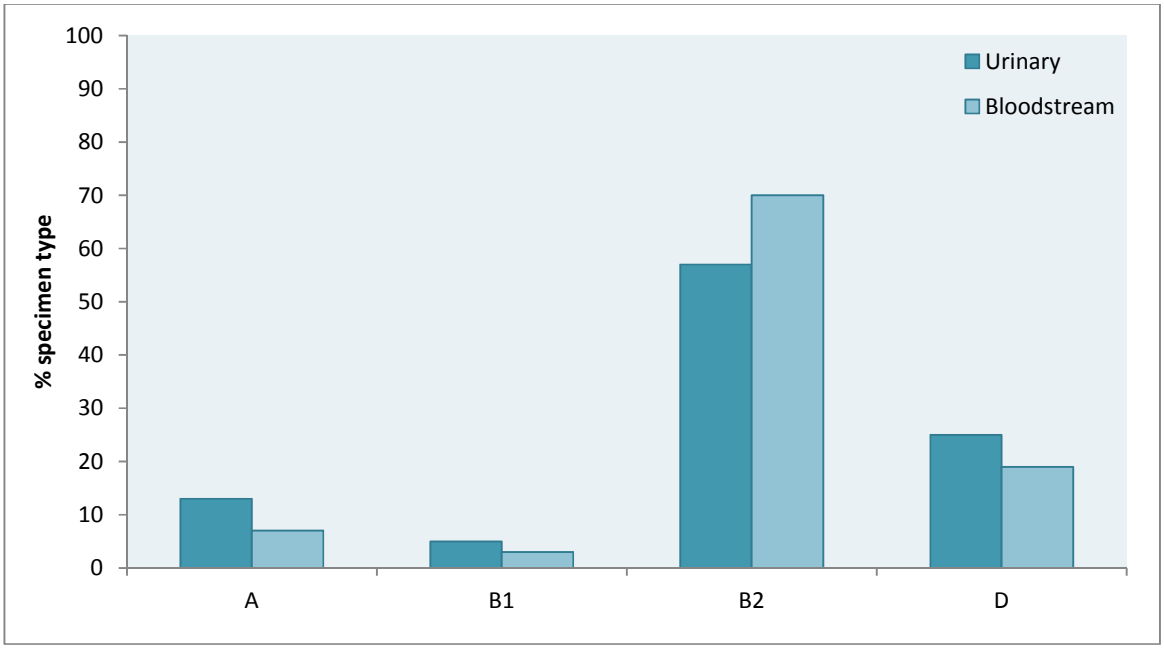


Figure 5 Phylogroup distributions within the urinary and bloodstream isolates

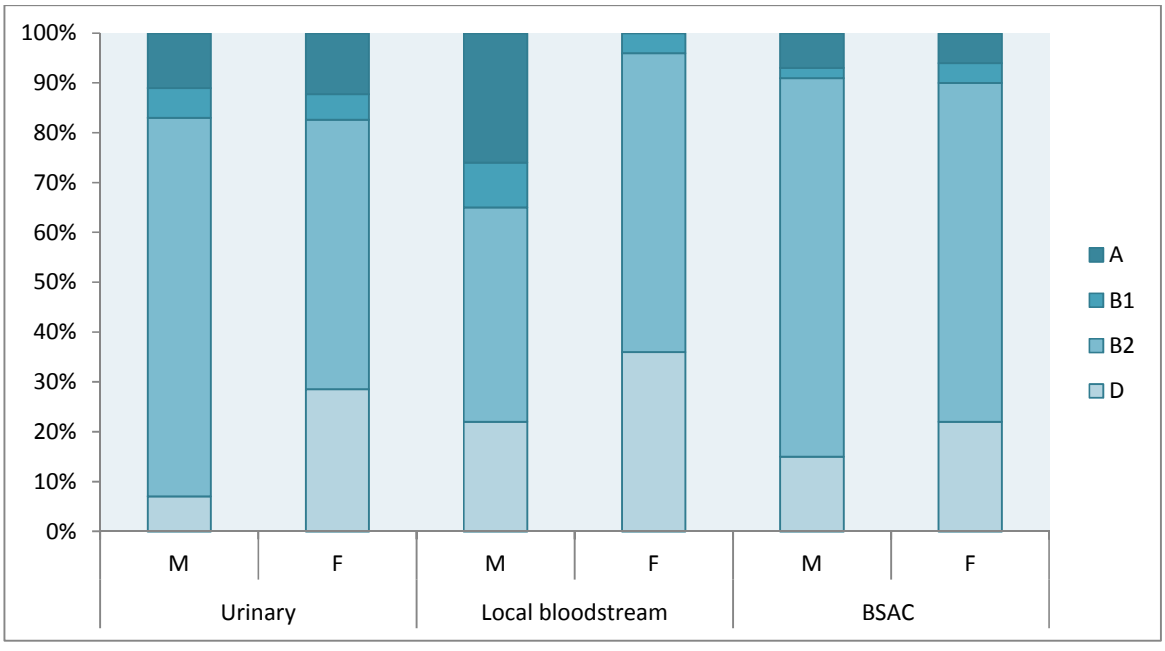


Figure 6 Phylogroup distributions according to patient sex and ExPEC collection

In the urinary isolates the proportion of phylogroup B2 isolates increased from ABU<UC<COMP, while phylogroup A and D isolates decreased and phylogroup B1 remained constant (Figure 7). In the bloodstream isolates phylogroup B2 dominated all sources of bacteraemia, followed, in decreasing order, by groups D, A and B1. In addition, phylogroups B1 was not detected in bloodstream isolates with a SSTI or line-source, group A was lacking from the respiratory-source isolates and the one CSF-source isolate belonged to group B2 (Figure 8).

The other exception was a bloodstream isolate, of unknown source, which generated an unusual phylogroup profile (*gadA*$^{+}$, *chuA*$^{-}$, *yjaA*$^{+}$, TspE4.C2^{+}).

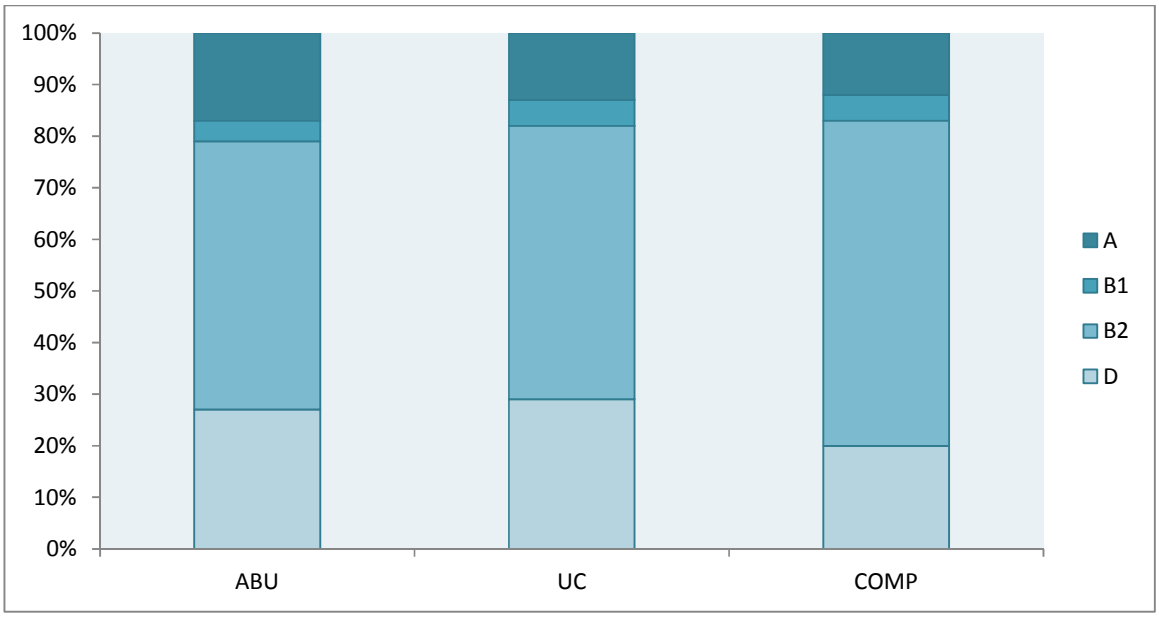


Figure 7 Phylogroup distributions according to UTI type

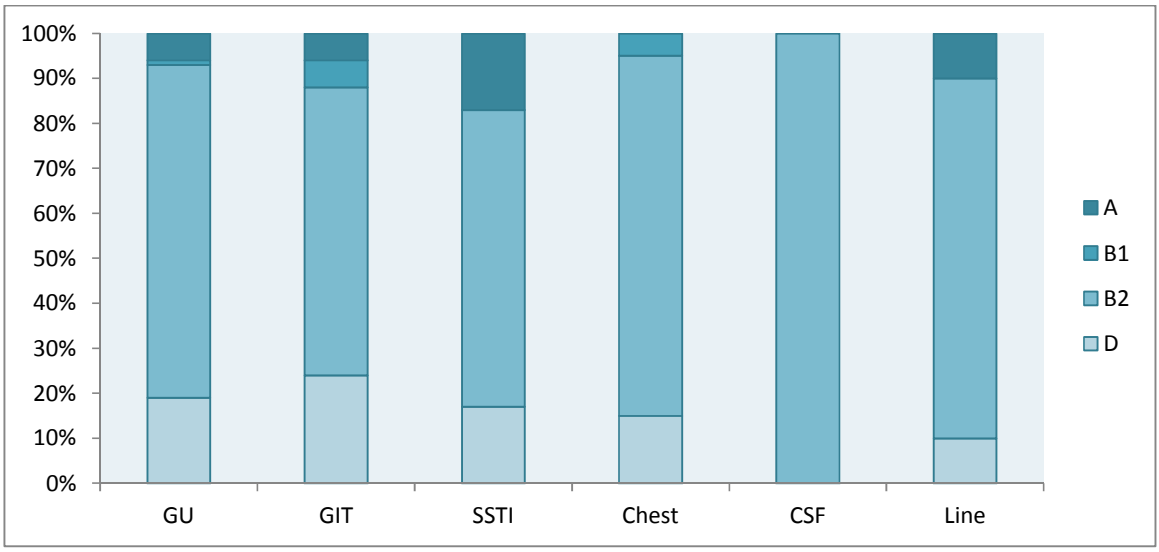


Figure 8 Phylogroup distributions according to source of bacteraemia. Number of isolates per source is as follows: GU (n=224), GIT (n=63), SSTI (n=6), Chest (n=20), CSF (n=1) and Line (n=10).

Sequence type

The major ExPEC lineages (ST131, ST127, ST95, ST73 and ST69) accounted for 40% and 44% of urinary and bloodstream isolates, respectively, with a slightly larger proportion detected in HAIs (53%).

ST95, followed by ST69, was the most frequently detected lineage in urinary isolates. In the bloodstream isolates ST131 was the most common lineage, followed by ST73 (BSAC) or ST69 (Local), as displayed in figure 9.

The major lineages predominantly comprised phylogroup B2 strains, except for ST69, which was mostly comprised of phylogroup D strains. Exceptions to this included an ST131 isolate (phylogroup D), five ST127 isolates (phylogroup A), three ST95 isolates (phylogroup D) and three ST69 isolates (2 phylogroup B2, 1 phylogroup B1).

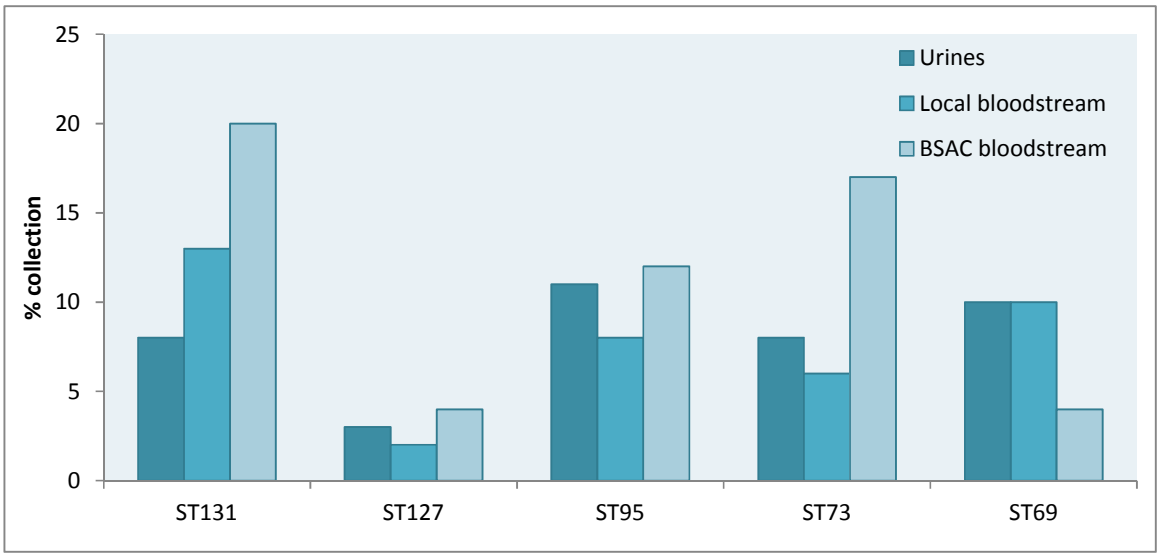


Figure 9 Sequence type distributions according to ExPEC collection and specimen type. These five lineages accounted for 40% of the urinary isolates, 39% of the local bloodstream isolates and 57% of the BSAC bloodstream isolates.

As expected, ST95 and ST69 comprised a large cluster of urinary isolates from female patients aged 21-40 years (Figure 10), while ST131 was more prevalent in patients >50 years from which bloodstream isolates were collected (Figure 11). These clusters correlate with the patients most frequently affected by urinary tract and bloodstream infections. In addition, ST95 clustered predominantly in patients aged 21-50 years, ST73 increased in prevalence in patients aged >70 years, ST69 peaked in patients aged 31-40 years, while ST127 was lacking in patients ≤30 years. Regarding patient sex, both ST131 (P=0.04) and ST127 (P=0.02) were more frequently detected in male patients (38% and 13%, respectively) rather than female patients (23% and 5%, respectively).

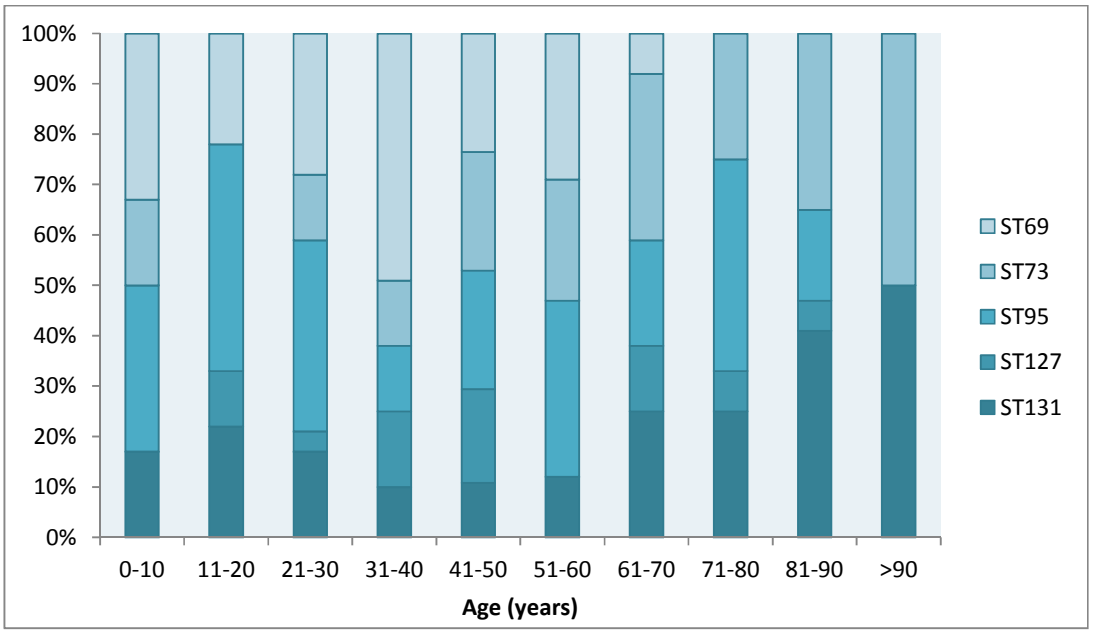


Figure 10 Sequence type distributions in urinary isolates according to patient age

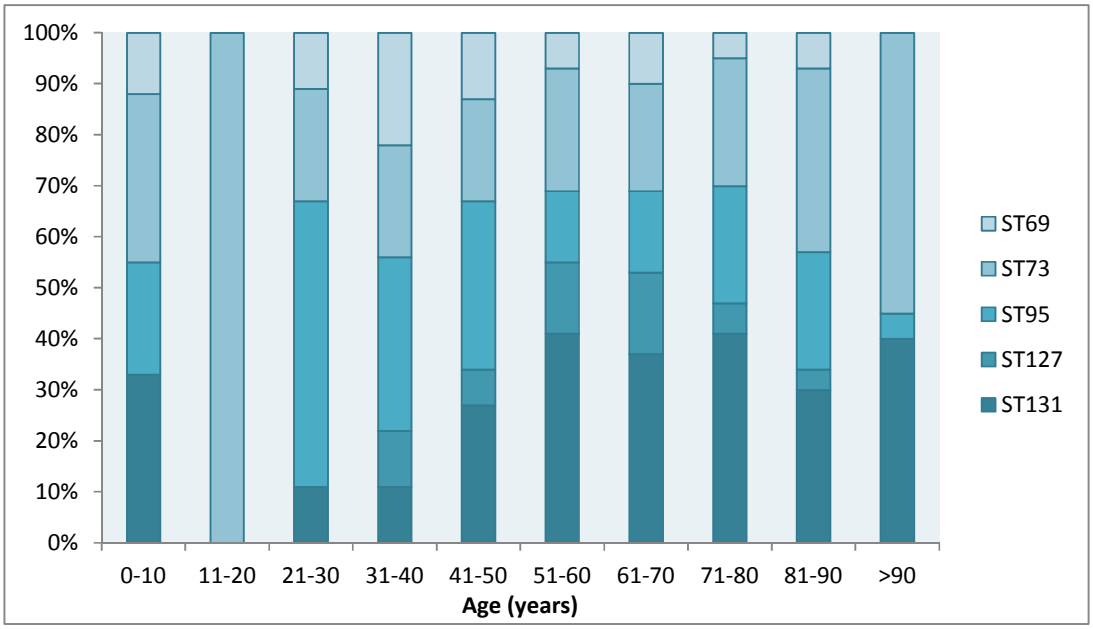


Figure 11 Sequence type distributions in bloodstream isolates according to patient age

Among the urinary isolates ST69 (8%) was the most common lineage in ABU infection, followed by ST95 (6%). ST95 was also the most common lineage in COMP isolates (13%), followed by ST131 (12%) and ST73 (11%). While in UC isolates ST95 (12%) was second to ST69 (14%).

For the GU-derived bloodstream isolates ST131 (21%) was the most common lineage, closely followed by ST73 (20%). This pattern was seen for respiratory-source isolates also (3% and 2%, respectively). While GIT strains were mostly ST131 (21%) and ST95 (13%) and the sole CSF strain also belonged to ST95. The line-source isolates demonstrated no obvious trend and all five lineages were absent from the SSTI strains.

As the BSAC isolates were collected from around the UK and Republic of Ireland, the distribution of each of the major lineages by country (Table 9) and region (Table 10) was reviewed. Of note, ST131 was almost twice as prevalent in Welsh isolates ($P \leq 0.008$), than isolates from England, Scotland and the Republic of Ireland. ST127 and ST95 were more common in English isolates than all other countries, while ST73 was more prevalent in Irish isolates. Interestingly, ST69 was only detected in isolates collected from England and the Republic of Ireland ($P \leq 0.04$).

Table 9 Distributions of the major ExPEC lineages in bloodstream isolates according to country of origin. Number of isolates is shown, plus the proportion of isolates encompassing that lineage per country, in parenthesis.

Country	ST131 N (%)	ST127 N (%)	ST95 N (%)	ST73 N (%)	ST69 N (%)
England	75 (20)	22 (6)	53 (14)	62 (17)	14 (4)
Ireland	12 (17)	3 (4)	7 (10)	15 (21)	8 (11)
Scotland	7 (17)	1 (2)	3 (7)	6 (15)	0
Wales	14 (37)	1 (3)	5 (13)	6 (16)	0

Public Health England has defined nine regions of England, as follows: South-East, South-West, London, East Midlands, West Midlands, East of England, North-East, North-West and Yorkshire and Humber (Figure 12). Within these regions ST131 was most prevalent in London and the South-West,

compared with all other regions ($P \leq 0.02$). ST69 was also more prevalent in London, along with the East and West Midlands. In addition to ST69, the East Midlands also had the highest proportion of ST127 isolates ($P \leq 0.01$), compared to all other regions, and ST95 ($P \leq 0.02$). However, ST73 was most prevalent in Yorkshire and Humber isolates ($P \leq 0.03$), whereas ST127 could not be detected in isolates from here or in South-East isolates either.

Table 10 Distributions of the major ExPEC lineages in bloodstream isolates according to region of origin in England. Number of isolates is shown, plus the proportion of isolates encompassing that lineage per region, in parenthesis.

Region	ST131 N (%)	ST127 N (%)	ST95 N (%)	ST73 N (%)	ST69 N (%)
South-East	7 (10)	0	9 (13)	14 (13)	1 (1)
South-West	15 (28)	4 (7)	5 (9)	6 (11)	3 (6)
London	16 (29)	2 (4)	11 (20)	8 (14)	4 (7)
East Midlands	2 (14)	4 (29)	5 (36)	0	1 (7)
West Midlands	9 (21)	6 (14)	5 (12)	8 (19)	3 (7)
East England	9 (22)	3 (7)	8 (20)	4 (10)	1 (2)
North-East	4 (14)	1 (4)	2 (7)	6 (21)	0
North-West	4 (14)	2 (7)	6 (21)	5 (18)	0
Y. & Humber	9 (21)	0	2 (5)	11 (26)	1 (2)

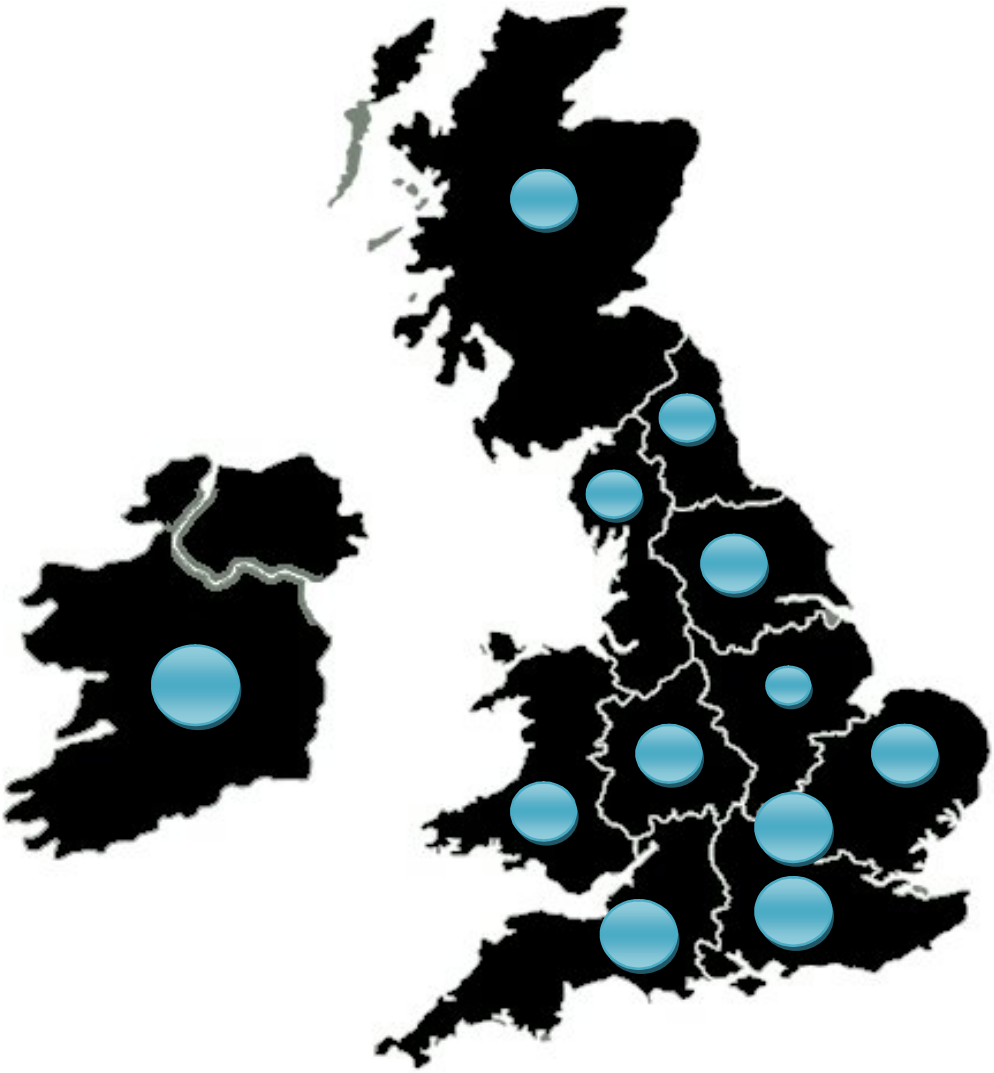


Figure 12 Map displaying the countries and English regions from which the BSAC bacteraemia resistance surveillance study (2011) isolates were collected, as defined by Public Health England. The number of centres is represented by proportionally sized circles, increasing from one (small) to five (large).

Serogroup

Six hundred and fifty-nine isolates were serogrouped, including 380 urinary and 279 bloodstream isolates. Sixty serogroups were identified, of which 29 were only identified in urinary isolates, 6 were only identified in bacteraemia isolates and 25 were identified in both (Figure 13). In 21% (n=139) a serogroup could not be identified and isolates were recorded as non-typeable.

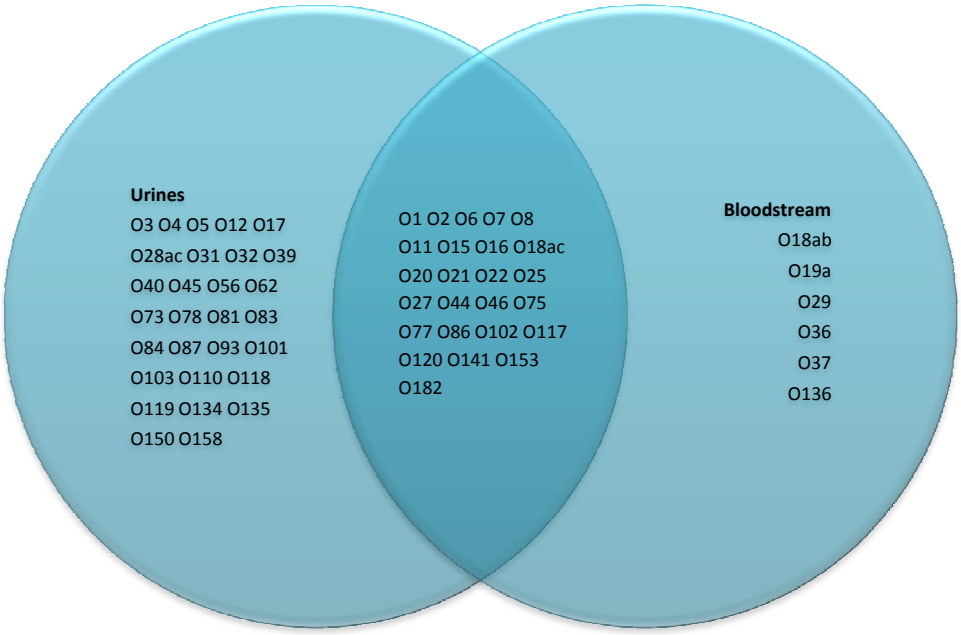


Figure 13 Serogroups identified in either urinary or bloodstream isolates or both

Sixty-three percent (n=413/659) of isolates comprised the 15 most common serogroups (table 11), of which O2, O6 and O25 were the three most frequently identified, comprising a higher proportion of the BSAC isolates (46%) than the urinary (29%) and local bloodstream isolates (24%).

Within the urinary isolates, serogroups O2, O6 and O25 were more frequently detected in COMP (n=58, 15%) isolates rather than UC (n=39, 10%) or ABU (n=11, 3%) isolates. While in the BSAC isolates these serogroups were more prevalent in GU-source isolates (n=52, 22%), compared to all other bloodstream isolates (n=2-13, ≤5%). These findings were not significant (P>0.05).

Table 11 the most common serogroups according to each of the ExPEC collections. Number of isolates is listed in the table, with the percentage in parenthesis

Serogroup	Urinary	Local bloodstream	BSAC bloodstream	Total
O1	14 (4)	0	13 (5)	27 (4)
O2	21 (6)	2 (5)	21 (9)	44 (7)
O4	7 (2)	2 (5)	0	9 (1)
O6	45 (12)	4 (11)	29 (12)	78 (12)
O7	10 (3)	1 (3)	2 (1)	13 (2)
O8	10 (3)	1 (3)	5 (2)	16 (2)
O15	6 (2)	0	4 (2)	10 (2)
O16	6 (2)	2 (5)	10 (4)	18 (3)
O18ac	9 (2)	1 (3)	3 (1)	13 (2)
O22	5 (1)	1 (3)	2 (1)	8 (1)
O25	43 (11)	3 (8)	61 (25)	107 (16)
O44	7 (2)	2 (5)	2 (1)	11 (2)
O75	11 (3)	2 (5)	10 (4)	23 (3)
O77	17 (4)	0	6 (2)	23 (3)
O86	5 (1)	1 (3)	7 (3)	13 (2)

Four serogroups were only detected in urinary and local bloodstream isolates from female patients: O7, O44, O75 and O77 (P>0.05). All of the O44 and O77 isolates belonged to phylogroup D (n=24/24) and most to ST69 (n=20/24). The O75 isolates belonged to phylogroup B2 and the O7 strains to a combination of phylogroups, all of unknown sequence type, and both serogroups were lacking from ABU isolates. However, O75 along with O77 were prevalent among UC isolates, in particular, while O7 and O44 isolates were equally prevalent among UC and COMP isolates.

As well as infection-specific patterns in serogroup, a limited serogroup profile was also observed in each of the major ExPEC lineages (Table 12), although some serogroups were found in multiple lineages, such as O6 in ST73 and ST127.

Table 12 Serogroups detected in each of the major ExPEC lineages in order of frequency

For serogroups comprising more than 10% of isolates, the proportion is listed in parenthesis

Lineage	Serogroup
ST131	O25 (77%), O16 (14%), O19a, O136, O153
ST127	O6 (75%), O27, O11, O16, O25
ST95	O2 (28%), O1 (21%), O4, O18ac, O25, O16, O31, O6, O110, O150, O153
ST73	O6 (57%), O2 (11%), O22, O25, O8, O4, O18ac, O27, O158
ST69	O77 (25%), O44 (14%), O15, O25, O11, O17, O27, O45, O73, O86, O102, O117, O125ab, O150, O153

In addition to the frequently identified O-antigens, several serogroups that have not been reported in ExPEC strains were identified. These included O12, O31, O32, O36, O37, O39, O40, O46, O56, O62, O81, O83, O87, O93, O110, O118, O135, O150, O158, O162 and O182, of which most were detected in urinary isolates, except O36 and O37 (bloodstream isolates) and O162 and O182 (urinary and bloodstream isolates). Together these serogroups encompassed 6% of the ExPEC collections.

Enteroaggregative *E. coli*

Thirty-one urinary isolates, encompassing three phylogroups (A, B1 and D), possessed serogroups associated with EAEC: O3 (n=1), O15 (n=5), O44 (n=7), O62 (n=1), O77 (n=11), O78 (n=1) and O86 (n=5). However, the *aggR* gene was not detected in any of these isolates, indicating they all lacked EAEC traits.

Paired isolates

Thirty-four paired (urinary and bacteraemia) isolates were collected from seventeen patients with urosepsis. Patient demographics and strain characteristics are listed in table 13. Data reveals that 16/17 of these patients were infected with a single *E. coli* strain, while patient 16 had two *E. coli* strains simultaneously causing infection (ST14/B2-O18ac and ST354/D-O153). The majority of paired isolates belonged to phylogroups B2 and D (16/17), with 10/17 encompassing the five major ExPEC lineages. Other sequence types detected included ST14, ST62, ST354, ST404, ST405, ST617 and

ST1405. Eight of the common serogroups (O2, O4, O6, O7, O18ac, O25, O44 and O75) were identified in 21/34 of the paired isolates. The remaining isolates were non-typeable (n=8) or were phylogroup D isolates with serogroups O11, O125ab and O153.

PFGE

PFGE of the paired isolates revealed those of the same phylogroup and/or sequence type to cluster in groups and confirmed that UTI and bacteraemia in patient 16 was caused by two separate isolates (Figure 14). Percentage relatedness was much higher between isolates of the same sequence type than those of the same phylogroup. For example, phylogroup D isolates demonstrated approximately 40% DNA relatedness, but ST69 isolates demonstrated approximately 60% relatedness.

While PFGE of isolate 3837 (unusual phylogroup profile), along with a selection of ExPEC isolates, revealed approximately 40% relatedness to all other ExPEC strains analysed, including ST127 isolates (Figure 15). These ExPEC isolates also appeared less related, especially within the different sequence types, to the paired urosepsis isolates.

Table 13 Patient and strain characteristics for the thirty-four paired urosepsis isolates

Isolate pair	Sample	Age (years)	Sex	CAI/HAI	Phylogroup	Serogroup	MLST	PFGE relatedness
1	Blood Urine	22	F	CAI	D	O44	69	92.7%
2	Blood Urine	88	F	CAI	D	NT	1405	97.3%
3	Blood Urine	87	F	CAI	D	O7	62	95%
4	Blood Urine	52	F	CAI	B2	NT	405	100%
5	Blood Urine	81	M	CAI	B2	O2	95	86.7%
6	Blood Urine	90	F	CAI	B2	O25	131	100%
7	Blood Urine	83	M	CAI	A	NT	617	97.3%
8	Blood Urine	69	F	HAI	B2	O2	73	95%
9	Blood Urine	44	F	CAI	D	NT	405	94.4%
10	Blood Urine	88	M	CAI	B2	O25	131	92.3%
11	Blood Urine	32	F	CAI	B2	O75	404	95.2%
12	Blood Urine	63	M	HAI	B2	O6	127	100%
13	Blood Urine	88	F	CAI	B2	O6	73	95%
14	Blood Urine	31	F	CAI	D	O125ab	69	98%
15	Blood Urine	31	F	HAI	D	O11	69	100%
16	Blood Urine	27	F	CAI	B2 D	O18ac O153	14 354	38.1%
17	Blood Urine	26	F	CAI	B2	O4	95	100%

F – female, M – male, CAI – community-associated infection, HAI – hospital-associated infection, NT – non-typeable, PFGE relatedness used a cut-off of 85% to identify *E. coli* isolates with the same PFGE profile (Gibreel, Dodgson, Cheesbrough, Fox, Bolton, & Upton 2012b).

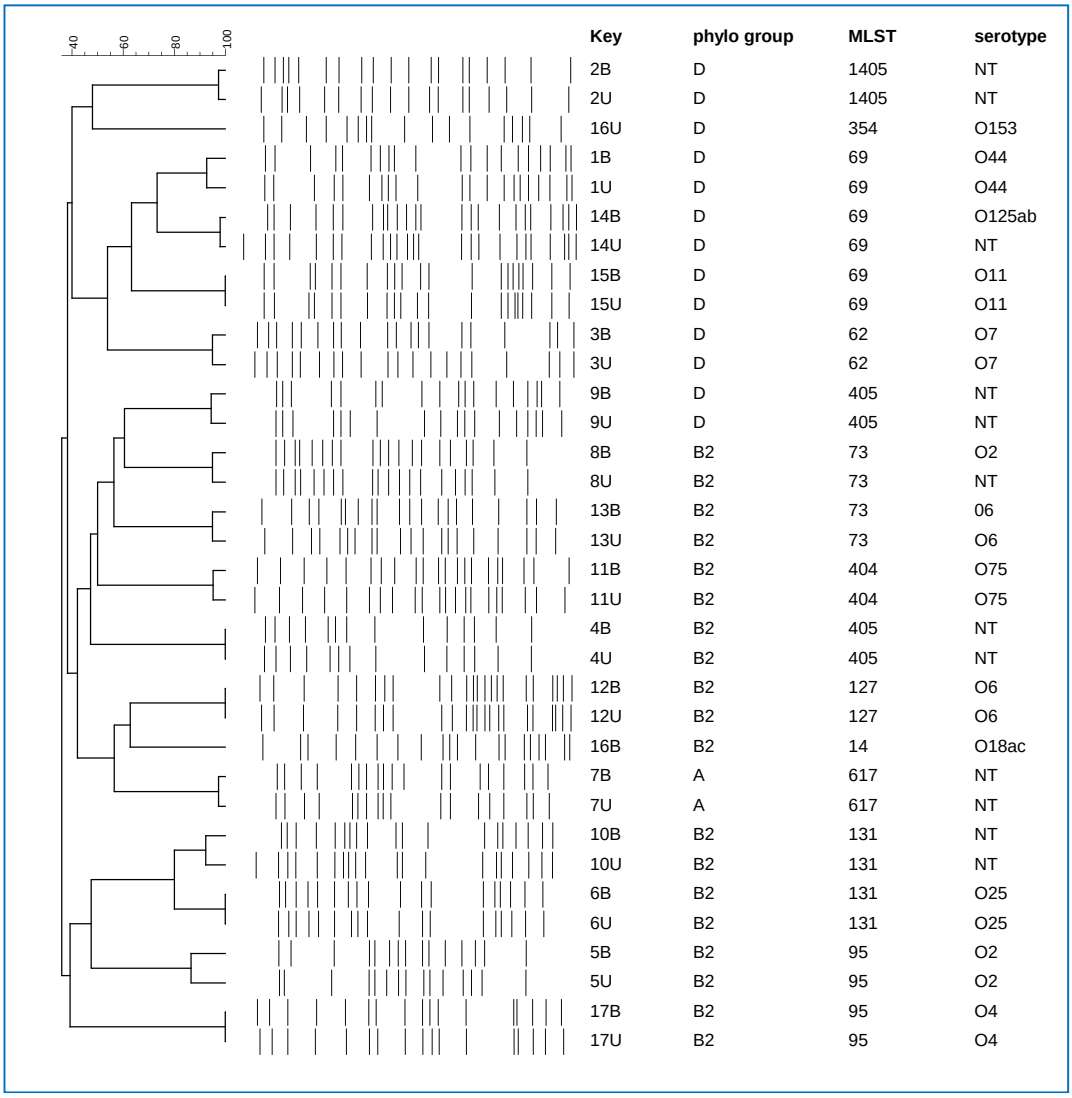


Figure 14 Dendrogram showing *Xba*I PFGE profiles for the 34 paired ExPEC isolates representing the principal phylogroups and lineages. The key identifies the paired isolates by number and source of isolation. For example, 2B is the bloodstream isolate from pair 2. Percentage band relatedness is detailed on the left hand side.

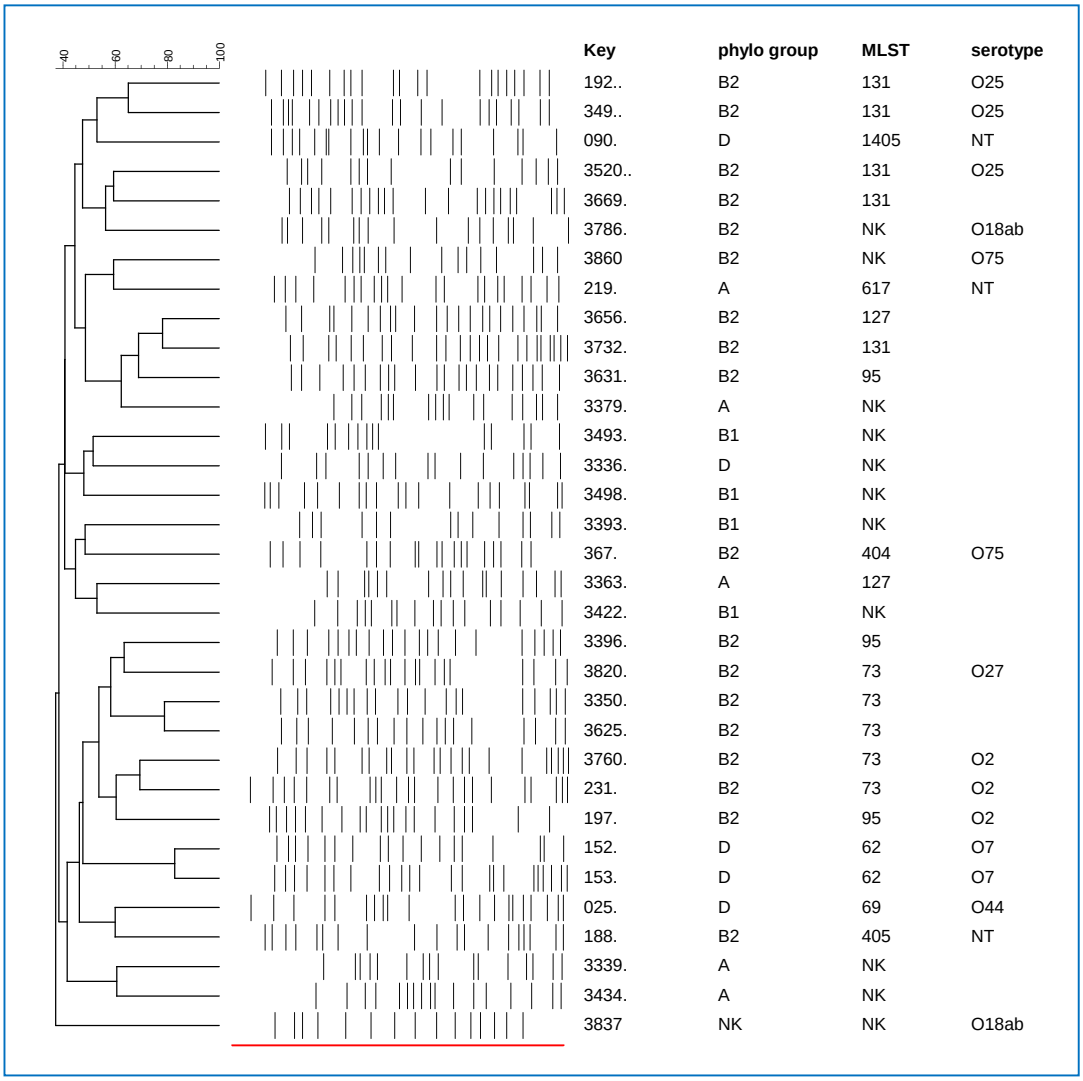


Figure 15 Dendrogram showing *XbaI* PFGE profiles for a selection of ExPEC isolates representing various sequence types and phylogroups, including the unusual phylogroup (underlined in red). Percentage band relatedness is detailed on the left hand side.

4.4 Discussion points

UTIs were predominantly a community-associated infection (87%) of women (84%) aged approximately 40 years, while bacteraemia was less frequently community-associated (68%) and equally affected men and women with an average age of 68 years. However, UTIs (8%) and bacteraemia (3%) also affected paediatric patients (<18 years old).

Collection of urinary isolates was heavily influenced by the specimen collection criteria. In contrast, the random specimen collection of the BSAC bloodstream isolates suggests accurate conclusions can be drawn regarding the typical patient presenting with bacteraemia, including the urinary tract as the number one source of infection.

ExPEC strains causing UTI and bacteraemia predominantly belonged to phylogroups B2 (64%) and D (22%), with the five major ExPEC lineages (ST131, ST127, ST95, ST73 and ST69) comprising a significant proportion of the isolates collected locally (40%) and nationally (57%). In addition, distribution of these lineages, within the BSAC collection, varied by country and region.

Despite the range of serogroups (n=60) identified in this study, isolates encompassing the major ExPEC lineages also possessed the most frequently identified serogroups. Other reported ExPEC clones identified in this study included ST62-O7 and ST404-O75. PFGE revealed isolates of these lineages and isolates of the same phylogroup to cluster in groups, but also demonstrated the great diversity of ExPEC strains.

Urosepsis was typically associated with a single strain, although one patient suffered simultaneous infection with 2 different *E. coli* strains.

5. Antibiotic resistance in *Escherichia coli* causing urinary tract infections and bacteraemia, in East London and across the United Kingdom

5.1 Introduction

The previous chapter demonstrated that patient demographics have remained relatively unchanged for those with *E. coli* causing UTIs and bacteraemia, compared to recent UK and international studies. However, ExPEC strains are highly varied, encompassing many serogroups, sequence types and lineages, as demonstrated in chapter 4. With this heterogeneity it is important to review and understand the current antibiotic resistance profiles and to determine if they have changed and whether therapeutic guidelines are still applicable or need updating.

Increasing antibiotic resistance in *E. coli* has led to changes in the treatment of UTIs, with current guidelines recommending trimethoprim or nitrofurantoin for uncomplicated UTIs and ciprofloxacin or amoxicillin-clavulanate with amikacin for complicated UTIs (Cormican *et al*. 1998; Dyer *et al.* 1998; Gupta *et al.* 2011; Huovinen and Toivanen, 1980; SIGN, 2006; Barts and the London Trust Antimicrobial Review Group, 2011). However, the most recent UK studies of urinary *E. coli* in paediatric patients (<16 years) reported resistance rates of 3.8%-7% to nitrofurantoin, 5.9% to ciprofloxacin, 3.6% to gentamicin, 8.2%-11% to cefalexin, 34%-46.3% to trimethoprim and 12.9%-48% to amoxicillin-clavulanate (augmentin) (Bean *et al.* 2008; Chakupurakal *et al.* 2010). While in adults with urinary *E. coli*, 13.7% were resistant to amoxicillin-clavulanate, 10.6% to cefalexin, 6.1% to nitrofurantoin, 39.4% to trimethoprim, 12.7% to ciprofloxacin and 6.6% to gentamicin (Bean *et al.* 2008). In comparison, European studies typically reported lower resistance rates of 2.5%-8.9% amoxicillin-clavulanate, 15.8%-19.1% trimethoprim, 0%-1.4% nitrofurantoin, 2.5%-7.6% ciprofloxacin, 1%-2.8% gentamicin and 0%-2.7% ceftazidime (Den Heijer *et al.* 2010; Kahlmeter and Poulsen, 2012).

E. coli causing bacteraemia are reported to have higher rates of antibiotic resistance than urinary isolates (Kahlmeter, 2003; Livermore *et al.* 2008; Martin *et al*. 2012). There are no specific guidelines for treating bacteraemia due to the multiple sources of infection and causative organisms. Therefore, empirical treatment is often broad-spectrum (Joint Formulary Committee, 2013). At the Royal London Hospital, *E. coli* bacteraemia is typically treated with amoxicillin-clavulanate with or without an aminoglycoside, which may be changed to ciprofloxacin (osteomyelitis), meropenem (meningitis) or piperacillin-tazobactam (cholecystitis) depending on the source of bacteraemia (Phee, 2013).

HAIs often demonstrate higher rates of resistance than CAIs, as these patients have likely received antibiotics previously and are more likely to be colonised with a multi-drug resistant nosocomial strain (Bean *et al.* 2008). In the UK, 15% of Enterobacteriaceae causing HAIs are reported to involve an ESBL (Health Protection Agency, 2012c), with CTX-M-15 the most frequently detected enzyme in *Klebsiella* and *E. coli* (Woodford *et al.* 2004; Younes *et al*. 2011). Multiple studies have linked circulation of these enzymes and particular resistance phenotypes to lineages such as ST131, ST405 and CgA (ST69) (Johnson *et al.* 2009a; Matsumura *et al*. 2012; Peirano and Pitout, 2010; Wallace-Gadsden *et al.* 2007).

Data on *E. coli* causing UTIs are often restricted to studies of healthy women with uncomplicated cystitis and test against few antibiotics (De Backer *et al.* 2008; Kahlmeter, 2003; Schito *et al.* 2009), while data on *E. coli* causing bacteraemia in the UK is limited to the BSAC Bacteraemia Resistance Surveillance Programme (Reynolds *et al.* 2008) and reports collected from UK hospitals via Public Health England's labBase2 system. As patients live longer and receive more antibiotics, it is important to continually review antibiotic resistance rates, especially with the rapid spread of β-lactamases and resistance determinants further complicating antibiotic treatment.

5.2 Methods

5.2.1 Antibiotic susceptibility testing (AST)

5.2.1.1 Local Antibiotic susceptibility testing

At the Royal London Hospital all urinary isolates underwent susceptibility testing with ciprofloxacin (1 µg), gentamicin (10 µg), cefalexin (30 µg), amoxicillin (25 µg), amoxicillin-clavulanate (30 µg), cefpodoxime (10 µg), trimethoprim (2.5 µg) and nitrofurantoin (200 µg) impregnated discs (Oxoid, UK), using the BSAC disc diffusion method (BSAC, 2011). Resistance to cefpodoxime prompted further AST using the MicroScan WalkAway 96 plus (Siemens) Gram-negative panel to identify potential ESBL-producers and determine susceptibilities against a larger panel of antibiotics. All isolates from blood cultures (bacteraemia) were subjected to AST using the MicroScan system.

5.2.1.2 Minimum Inhibitory Concentration (MIC)

MICs were determined for all 1077 isolates to ensure uniform testing against the same antibiotic panel. MICs were determined using the BSAC agar dilution method (Andrews 2001) and considered the gold standard for determining antibiotic susceptibilities. EUCAST breakpoints were used to determine whether an isolate was susceptible, had reduced susceptibility (intermediate) or was resistant to the antibiotics tested. As there is no EUCAST breakpoint for cefoxitin the Clinical Laboratory Standards Institute (CLSI) breakpoint was used. MICs were determined for the following antibiotics on ISO-Sensitest (ISO) or Mueller-Hinton (MH) agars for the carbapenems: amikacin (AMK), amoxicillin-clavulanate (AUG), ampicillin (AMP), aztreonam (AZT), cefotaxime (CTX) with and without clavulanate, cefoxitin (FOX), cefpirome (CPR) with and without clavulanate, ceftazidime (CAZ) with and without clavulanate, ciprofloxacin (CIP), chloramphenicol (CHL), ertapenem (ERP), gentamicin (GEN), imipenem (IM), meropenem (MEM), minocycline (MIN), nitrofurantoin (NIT), piperacillin-tazobactam (PZT), temocillin (TEM), tigecycline (TIG), trimethoprim (TRIM) with and without sulfamethoxazole (SXT), and tobramycin (TOB). Antibiotic suppliers are listed in appendix B. *E. coli* control strains ATCC 25922 and NCTC 10418 were included in each MIC run to validate the

MIC results and to check for any anomalies between the initial disc diffusion/ MicroScan® AST results and the MIC results. ESBL possession was inferred if the MIC of the cephalosporin compared to the MIC of the cephalosporin with clavulanate was ≥8-fold higher (Livermore and Brown, 2001). Isolates resistant to ≥1 cephalosporin were analysed by PCR for β-lactamase enzymes (see section 5.2.2). MICs were compared to MIC data reported by EUCAST (http://www.eucast.org/).

5.2.2 CTX-M group detection and identification

Genes for CTX-M enzyme groups 1 and 9 were detected using the PCR assay described by Woodford (2010) (Woodford 2010). PCR reactions and amplification conditions are as described in section 3.4, but with annealing step of 60°C for 40 seconds.

PCR products were separated by agarose gel electrophoresis and product size (appendix C) was used to identify the CTX-M group. CTX-M group 1 PCR products were purified (section 3.6) and sequenced (section 3.7). DNA sequences were aligned in BioNumerics version 6.1 (Applied Maths NV) and compared with sequences in the GenBank database (National Institute of Health, MD, USA) to identify the specific CTX-M allele.

Isolates that were positive for CTX-M group 9 genes were investigated using the primers described by Girlich *et al* (Girlich *et al*. 2009), which amplified the entire open reading frame of the CTX-M gene. PCR amplicons were generated and identified as described above. All primers are listed in appendix C.

5.2.3 TEM and SHV Detection

All CTX-M-positive strains and isolates with an antibiogram suggestive of cephalosporinase activity (cephalosporin resistant) were analysed for TEM and SHV genes using the primers described previously (Livermore *et al*. 2001; M'Zali *et al*. 1996). PCR reactions, amplification conditions and enzyme identification were as described in section 5.2.2. Primers are listed in appendix C.

5.2.4 OXA Detection

All CTX-M-positive strains and isolates with an antibiogram suggestive of cephalosporinase activity (cephalosporin resistant) were analysed for OXA-1-like genes using the primers described previously (Karisik *et al*. 2006). PCR reactions, amplification conditions and enzyme identification were as described in section 5.2.2. Primers are listed in appendix C.

5.2.5 AmpC β-lactamase detection and identification

Isolates that were cefoxitin-resistant and/or negative for CTX-M, TEM, SHV or OXA-1 enzymes (sections 5.2.2 – 5.2.4), were investigated for acquired AmpC enzymes using the PCR described by Dallenne *et al* (Dallenne *et al*. 2010). PCR reactions, amplification conditions and enzyme identification were as described in section 5.2.2. Primers are listed in appendix C.

Isolates that were positive for the CIT family of acquired AmpC β-lactamases were subjected to a CMY-specific PCR, as CMY is the most commonly detected CIT AmpC β-lactamase in *E. coli* (Dierikx *et al*. 2012; Jacoby, 2009). PCR reaction and cycling conditions were as described above, using primers (appendix C) published previously (Ahmed and Shimamoto, 2008). CMY PCR products were purified and cloned into plasmids (section 5.2.7) prior to DNA sequencing. DNA sequences were aligned and identified, as described in section 5.2.2.

5.2.6 Aminoglycoside-modifying enzymes

All urinary isolates demonstrating reduced susceptibility to ≥1 aminoglycoside were screened for the common aminoglycoside-modifying enzymes (AME), using the PCRs developed by Noppe-Leclercq *et al* (1999) and Leelaporn *et al* (2008). PCR reactions and amplification conditions were as described in section 3.4, but with varying annealing temperatures, as listed in appendix C (Leelaporn *et al*. 2008; Noppe-Leclercq *et al*. 1999).

Isolates demonstrating pan-resistance to all three aminoglycosides were screened for genes encoding all 16S rRNA methyltransferases using the multiplex PCR developed by Fritsche *et al* (2008). PCR reactions were as described in section 3.4, but with 25 cycles of denaturation for 15

seconds at 94°C, annealing at 58°C for 30 seconds and extension at 72°C for 60 seconds. PCR amplicons were resolved and identified by gel electrophoresis (section 3.5). All primers are listed in appendix C.

5.2.7 Plasmid-mediated quinolone resistance (PMQR) determinants

All ciprofloxacin-resistant isolates were subjected to a multiplex PCR, developed as part of this project, targeting eight plasmid-mediated genes that confer reduced susceptibility to fluoroquinolones. The targets were *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *oqxAB*, *qepA* and the *aac (6')-Ib-cr* allele (appendix C). The Qiagen Multiplex PCR kit (Qiagen) was used for the PCR reactions, according to the manufacturer's instructions. Each PCR reaction contained 12.5 µl master mix, 10X primer mix (2 µM each primer), 2.5 µl Q solution and 5 µl DNA lysate made up to a final volume of 25 µl with sterile-filtered water. Amplification conditions were as recommended with an initial hold at 95°C for 15 minutes; followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 90 seconds and extension at 72°C for 90 seconds; with a final hold at 72°C for 10 minutes. PCR products were resolved by gel electrophoresis (section 3.5) for 120 minutes and product size used to identify the PMQR determinant (Figure 16). In order to determine the optimal annealing temperature a gradient PCR was performed, with a range of annealing temperatures from 56-64°C. Pooled (positive control) DNA was amplified at each annealing temperature and products resolved by gel electrophoresis. The temperature at which optimal resolution of all eight targets was achieved was selected as the chosen annealing temperature for the PMQR PCR.

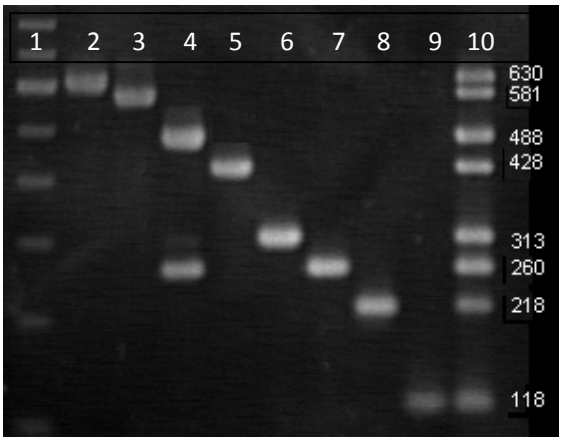


Figure 16 Gel electrophoresis of PMQR control strains and pooled control DNA: Lane 1) 100bp ladder; 2) *qnr*A; 3) *qnr*D; 4) *qnr*B and *aac(6')-Ib-cr*; 5) *qnr*S; 6) *oqx*A; 7) *aac(6')-Ib-cr*; 8) *qep*A; 9) *qnr*C; 10) pooled control DNA

5.2.8 PCR product cloning

5.2.8.1 CMY gene cloning

All purified CMY gene PCR amplicons were cloned using the TOPO TA cloning kit (Invitrogen). M13 PCR was performed to confirm presence of the CMY gene in the vector, before plasmid extraction using the QIAprep Spin miniprep kit (Qiagen). Plasmids were sent away for DNA sequencing (section 3.7).

The cloning reaction was prepared by combining 3 µl CMY PCR products with 1 µl each of salt solution, water and TOPO vector, before leaving the reaction on ice. A small aliquot of the cloning reaction (2 µl) was added to a vial of One Shot chemically competent DH5α *E. coli* cells and incubated on ice for a further 5 minutes. Cells were heat-shocked at 42°C for 30 seconds and immediately transferred back onto ice, before suspension in 250 µl S.O.C. medium, then incubation at 37°C for 1 hour on an Innova 4000 (New Brunswick Scientific, Stevenage, UK) at 220 rpm. Two volumes (10 µl and 100 µl) of transformed cells were spread on LB agar (Sigma) containing 50 µg/ml ampicillin (Sigma) and incubated overnight at 37°C. Colonies were analysed for transformed cells as described below.

5.2.8.2 M13 PCR

The M13 primers bind either side of the pCR4-TOPO vector insertion site. Therefore, positive PCR amplification products should contain the inserted CMY gene.

DNA lysates (section 3.3) were prepared from the transformed cells. PCR reactions are as described in section 3.4, but made up to a final volume of 25 µl with sterile-distilled water. Primers are listed in appendix C. Amplification was as follows: initial hold at 94°C for 4 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 90 seconds. PCR products were separated by gel electrophoresis (section 3.5) to identify the transformed isolates carrying the CMY gene.

5.2.9 Statistics

Associations between patient variables and antibiotic resistance were calculated using the chi^2 test, in Stata, version 12 (StataCorp LP). A *P-value* of ≤0.05 was considered significant, unless stated otherwise.

5.3 Results

Minimum Inhibitory Concentrations

Non-susceptibility (intermediate or resistant isolates) results for 22 antibiotics, as determined by agar dilution MICs, are displayed in figure 17, for all study isolates.

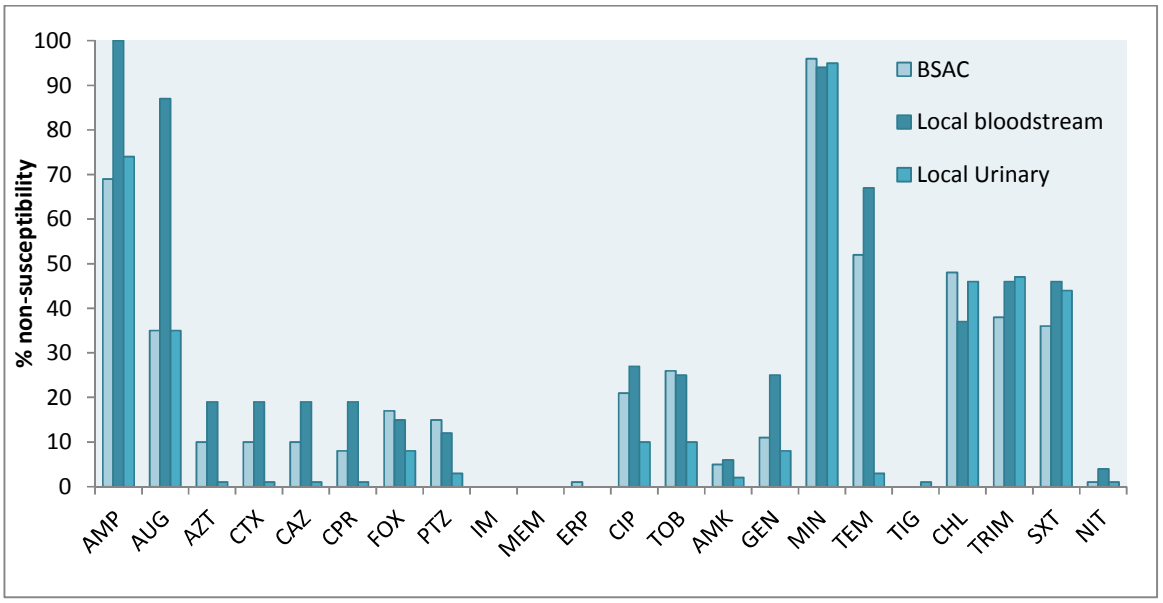


Figure 17 Percentage non-susceptibility to 22 antibiotics, as determined by MICs, for all ExPEC isolates Proportions are displayed for the local urinary isolates (n=508), local bloodstream isolates (n=48) and the BSAC bloodstream isolates (n=521).

Overall, the resistance rates in the local isolates were comparable to the prevalence seen in the national BSAC collection. The proportion of resistance in the local bloodstream isolates appear disproportionately higher due to the small sample size (n=48).

Excluding the local bacteraemia isolates, non-susceptibility among local urinary and BSAC bloodstream isolates to ampicillin and amoxicillin-clavulanate was 72% and 35%, respectively. Amoxicillin-clavulanate resistance was reduced even further in light of the amended EUCAST breakpoint for uncomplicated cystitis, as clavulanate was reported to accumulate in high

concentrations in the bladder (Alou *et al*. 2006). The breakpoint was increased to 32 mg/L, resulting in 0% of UC and ABU urinary isolates demonstrating amoxicillin-clavulanate resistance. Cephalosporin resistance was higher in bloodstream isolates versus urinary isolates (10% vs. 1%, *P*=0.01), as was resistance to cefoxitin (17% vs. 8%) and piperacillin-tazobactam (15% vs. 3%, *P*=0.01). Resistance to the carbapenems and tigecycline was negligible, with only one bloodstream isolate showing ertapenem resistance and two urinary isolates demonstrating reduced susceptibility to tigecycline. Ciprofloxacin resistance in BSAC isolates was double that of urinary isolates (21% vs. 10% NS, *P*=0.05), while gentamicin resistance was less different (11% vs. 8%). Temocillin resistance appeared higher in BSAC isolates than urinary isolates, due to the difference in breakpoint between these two infection types (8 mg/L vs. 32 mg/L), but analysis of the MIC distributions reveals a similar trend for urinary and bloodstream isolates (Figure 18). Chloramphenicol (48% vs. 46%) and nitrofurantoin (both 1%) resistance was also not significantly different between the BSAC bacteraemia and urinary isolates. However, the urinary isolates demonstrated slightly higher resistance rates to trimethoprim (47% vs. 38%) and trimethoprim-sulfamethoxazole (44% vs. 36%).

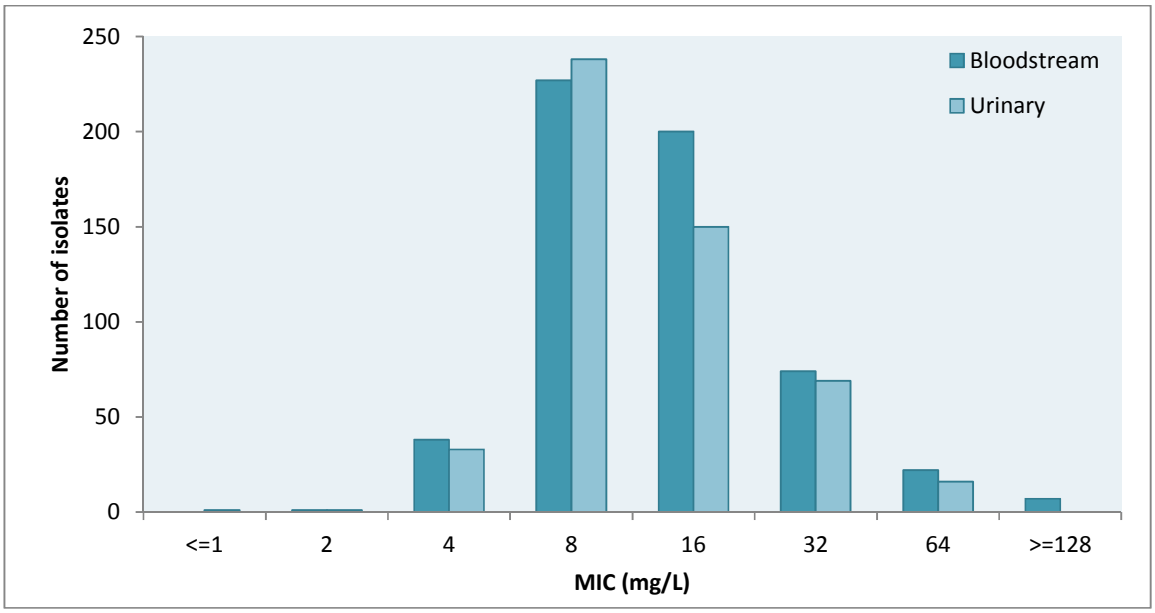


Figure 18 Temocillin MIC distributions for all urinary and bloodstream isolates. Dark blue bars represent bloodstream isolates (n=569) and light blue bars represents urinary isolates (n=508).

Comparison with the EUCAST wild type MICs (http://www.eucast.org/mic_distributions/ accessed 13th September 2014) revealed similar trends in MIC distribution for all antibiotics, except ampicillin, amoxicillin-clavulanate and cefoxitin (Figures 19 and 21). For these three antibiotics there was a shift towards higher MICs compared to the EUCAST wild type. There was also a slight variation in cefotaxime MICs where the EUCAST wild-type had two MIC peaks at <=0.125mg/L and 1mg/L. This second peak is missing in the study isolates, with most isolates generating an MIC <=0.125mg/L and a small peak of (ESBL-expressing) isolates with an MIC >=128mg/L. Unfortunately there were no wild-type data for temocillin MICs, but the urinary isolates and BSAC isolates demonstrated a normal MIC distribution, with a peak at 8 mg/L (Figure 18).

Disc diffusion versus MIC (urinary isolates)

The limited panel of antibiotics used to screen urinary isolates at the Royal London Hospital is designed to detect resistance against antibiotics recommended in the therapeutic guidelines, as well as ESBL production. MIC results suggested that antibiotic resistance, as determined by disc diffusion (DD), is greatly underestimated for amoxicillin, amoxicillin-clavulanate, chloramphenicol and

trimethoprim, while nitrofurantoin resistance is over-estimated (Figure 19). EUCAST data for *E. coli* also revealed resistance rates in London to be higher than Europe for all antibiotics, except ciprofloxacin, gentamicin and nitrofurantoin, which were similar (Figure 19).

According to MICs, isolates causing all three types of UTI (ABU, UC and COMP) displayed similar trends in resistance (>20% non-susceptible to ampicillin, amoxicillin-clavulanate, minocycline, chloramphenicol, trimethoprim and trimethoprim-sulfamethoxazole), but resistance to individual antibiotics was higher in COMP infections (Figure 20).

When comparing resistance rates between male and female patients, resistance was significantly higher in men for the cephalosporins, piperacillin-tazobactam, ciprofloxacin, tobramycin, gentamicin and temocillin. While in female patients resistance was higher against trimethoprim and trimethoprim-sulfamethoxazole, but not significantly so.

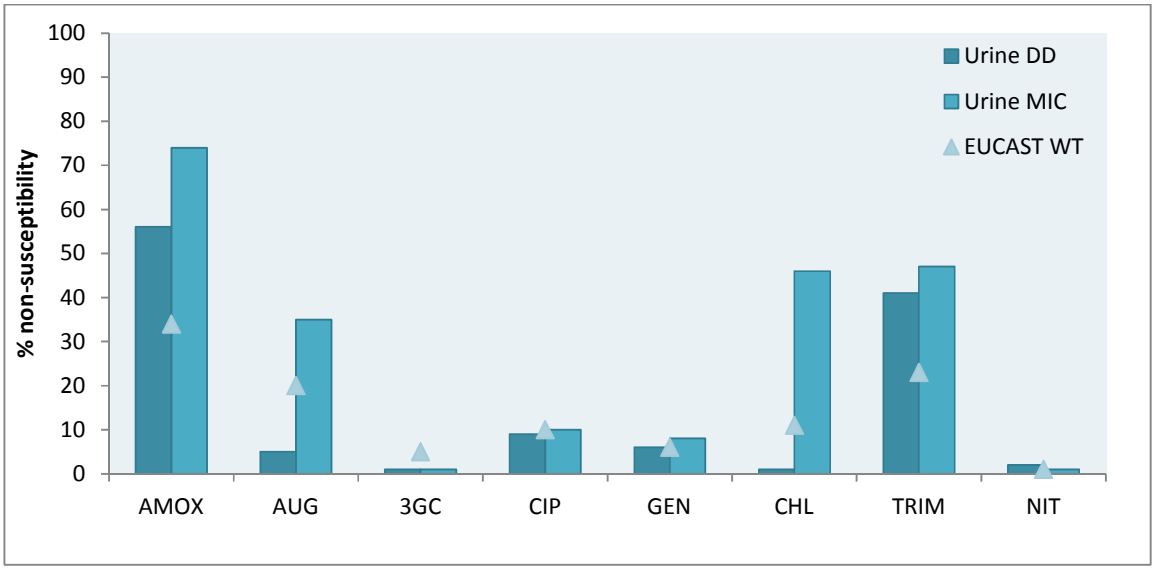


Figure 19 Percentage non-susceptibility of local urinary isolates (n=508), according to disc diffusion and MIC, overlaid with the European *E. coli* non-susceptibility rates as reported by EUCAST.

EUCAST data can be accessed here http://www.eucast.org/mic_distributions/).

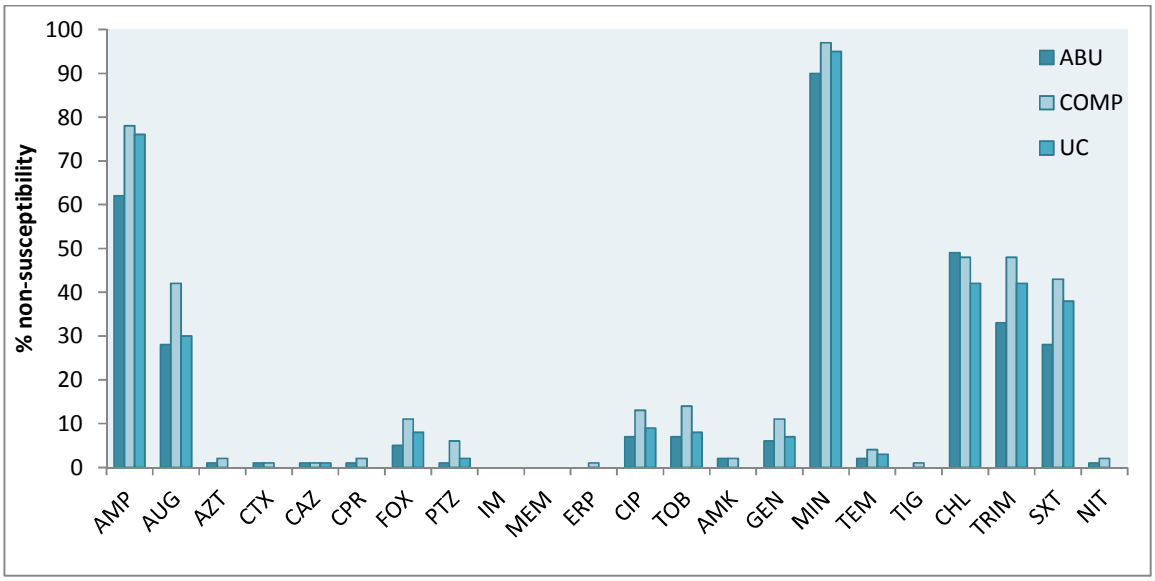


Figure 20 Percentage non-susceptibility according to the type of urinary tract infection: asymptomatic bacteruria (ABU, n=102), uncomplicated cystitis (UC, n=198), complicated cystitis/pyelonephritis (COMP, n=208).

MicroScan versus MIC

For the local bloodstream isolates, susceptibility results as determined by MicroScan and MIC were relatively comparable (within 1-5% of each other), with the exception of ampicillin, ampicillin-sulbactam, cefoxitin, minocycline and chloramphenicol (Figure 21). As with the urinary isolates, MICs for the local bloodstream isolates demonstrated higher resistance rates to ampicillin, ampicillin-sulbactam (compared to amoxicillin-clavulanate), cefoxitin, minocycline and chloramphenicol, with overall resistance higher than the rest of Europe (EUCAST data, figure 21). Resistance rates were also higher in isolates collected from male patients, compared to female patients: amoxicillin-clavulanate 74% vs. 60%, cefotaxime 35% vs. 4%, ciprofloxacin 48% vs. 8%, gentamicin 35% vs. 12%; except for trimethoprim resistance which was slightly higher in female patients (48% vs. 43%).

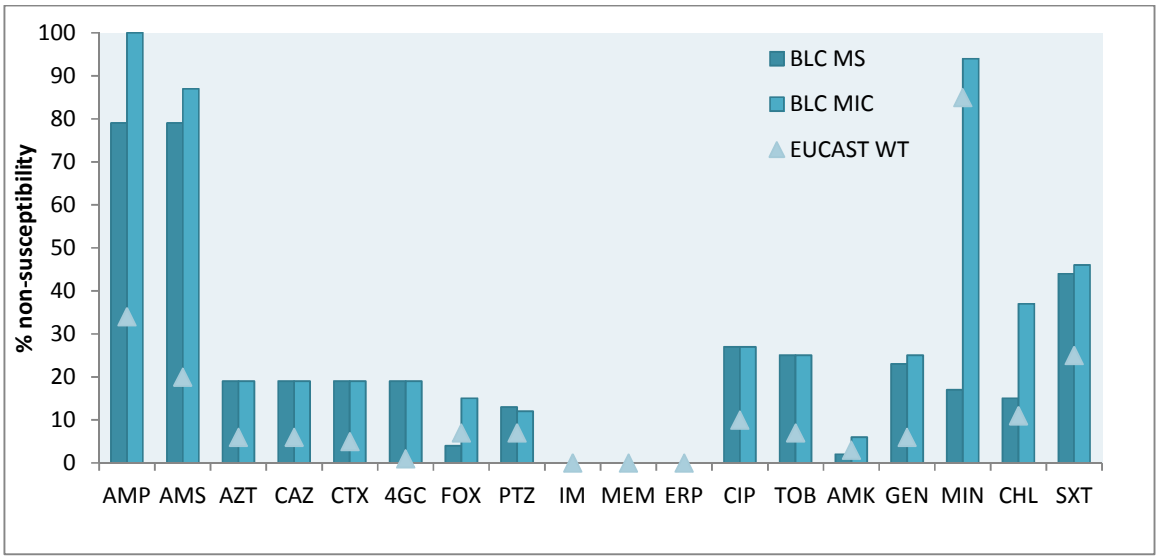


Figure 21 Percentage non-susceptibility of local bloodstream isolates (n=48), according to MicroScan and MIC, overlaid with the European *E. coli* non-susceptibility rates as reported by EUCAST. Additional antibiotics tested by MicroScan included ampicillin-sulbactam (AMS) and cefepime (4GC, compared to cefpirome MIC). EUCAST data can be accessed here http://www.eucast.org/mic_distributions/).

Within the BSAC collection, breakdown of the MICs by bacteraemia source revealed the CSF-source isolate to be relatively susceptible; resistant only to ampicillin, minocycline, trimethoprim and trimethoprim-sulfamethoxazole. Among the remaining sources of bacteraemia, resistance to individual antibiotics varied (Figure 22). For example, trimethoprim-sulfamethoxazole resistance was highest in GU isolates (42% vs. 25-41%).

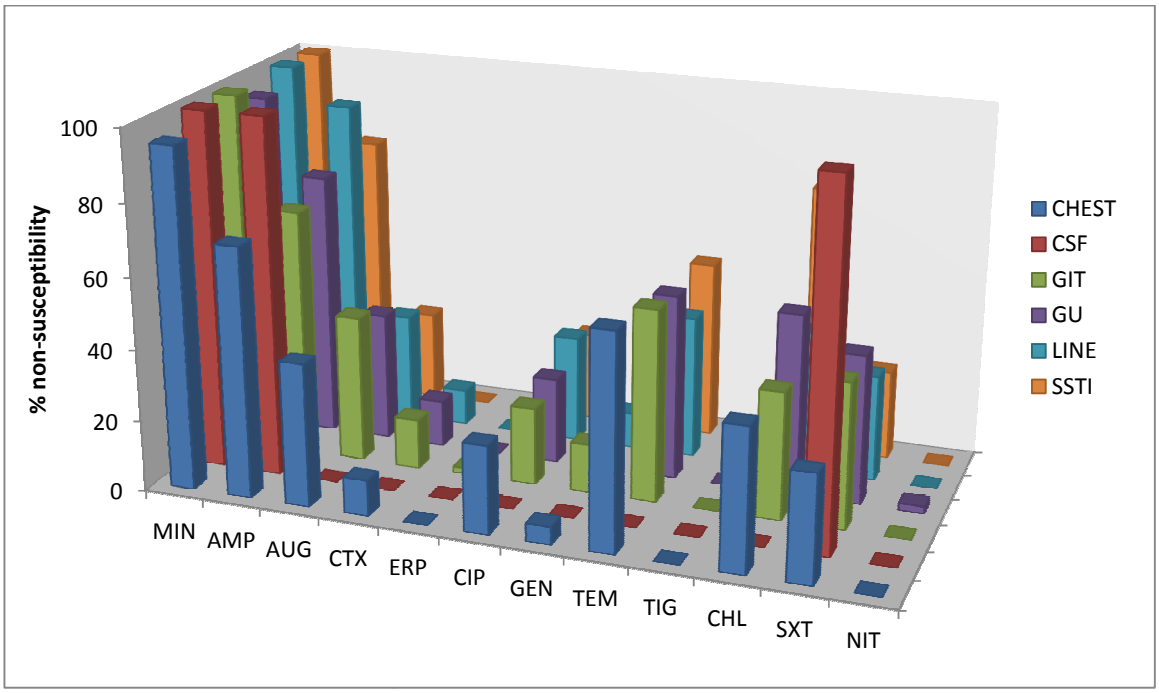


Figure 22 Percentage non-susceptibility according to source of bacteraemia: Twelve of the 22 antibiotics used in the MIC testing, representing all included antibiotic classes, were chosen to display resistance rates according to each source of bacteraemia: skin and soft tissue infection (SSTI), intravenous lines (Line), genitourinary infection (GU), gastrointestinal infection (GIT), respiratory tract infection (Chest) and cerebral spinal fluid (CSF).

Healthcare Setting

Comparison of MICs between isolates causing community-associated infections (CAI) and hospital-associated infections (HAI) revealed higher rates of resistance for HAI isolates, for nearly all antibiotics (Table 14). In particular, resistance to the cephalosporins, the aminoglycosides and ciprofloxacin was ≥5% higher in HAI isolates than CAI isolates, with temocillin resistance almost double that of the CAI isolates (P<0.01).

Table 14 Percentage non-susceptibility in ExPEC isolates from community-associated infections and hospital-associated infections

Antibiotic	Community (n=836, %)	Hospital (n=241, %)
Ampicillin	70	80
Amoxicillin-clavulanate	34	44
Cefotaxime	5	10
Ciprofloxacin	15	20
Gentamicin	9	15
Temocillin*	25	47
Chloramphenicol	46	49
Trimethoprim	40	41
Nitrofurantoin	1	2

Non-susceptibility, as defined by the breakpoint, for each antibiotic is as follows: ampicillin, amoxicillin-clavulanate, temocillin (bloodstream isolates) and chloramphenicol >8 mg/L; cefotaxime >2 mg/L; ciprofloxacin >1 mg/L; gentamicin and trimethoprim ≥4 mg/L; temocillin (urinary isolates) >32 mg/L; nitrofurantoin >64 mg/L. Significant differences in resistance rates are indicated with an asterisk.

Regional Setting

MICs of the BSAC isolates were analysed according to country and, for England, regions.

Overall resistance rates were similar for all four countries (Figure 23), but Welsh isolates demonstrated higher rates of resistance to amoxicillin-clavulanate, aztreonam, cephalosporins and aminoglycosides. Scottish isolates had the highest rates of chloramphenicol resistance and Irish isolates had the highest percentage cefoxitin resistance.

Major variation in regional resistance rates was observed for specific antibiotics (Figure 24). Of note, amoxicillin-clavulanate, trimethoprim and trimethoprim-sulfamethoxazole resistance was highest in York and Humber isolates; resistance to cephalosporins and ciprofloxacin was highest in London and the South-West; and amikacin and chloramphenicol resistance was highest in the East Midlands, with amikacin resistance two to four times higher than any other region.

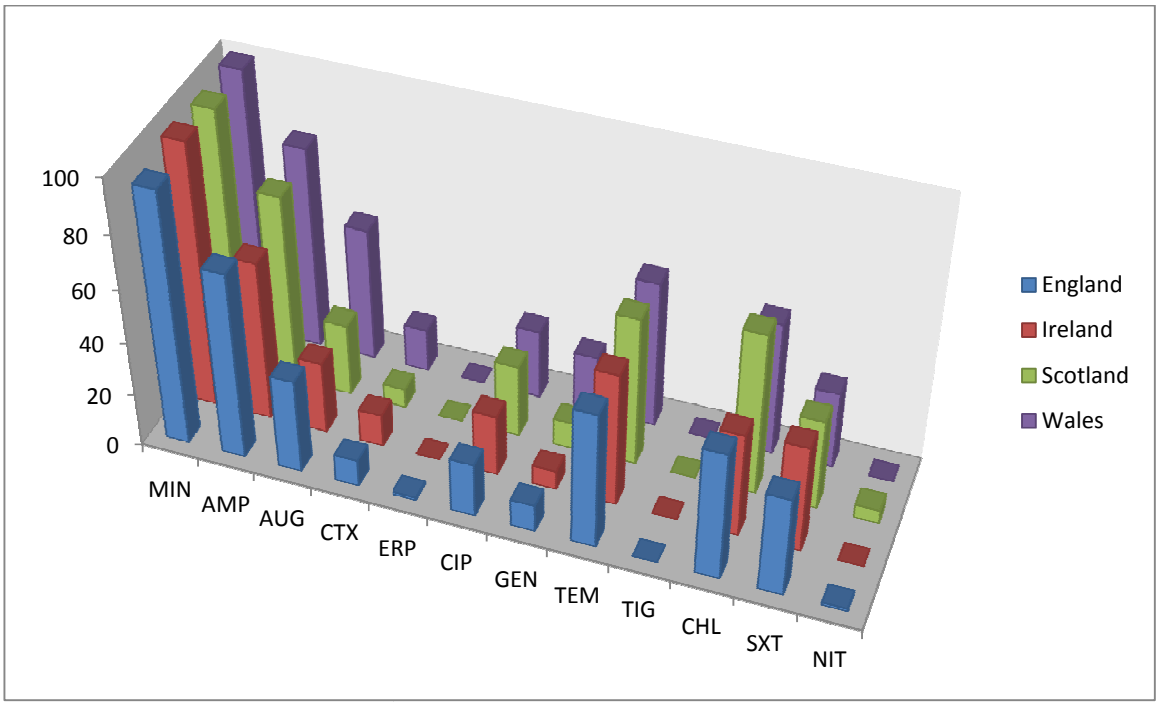


Figure 23 Percentage non-susceptibility according to country of origin: Twelve of the 22 antibiotics used in the MIC testing, representing all included antibiotic classes, were chosen to display resistance rates for BSAC isolates collected from England, the Republic of Ireland, Scotland and Wales.

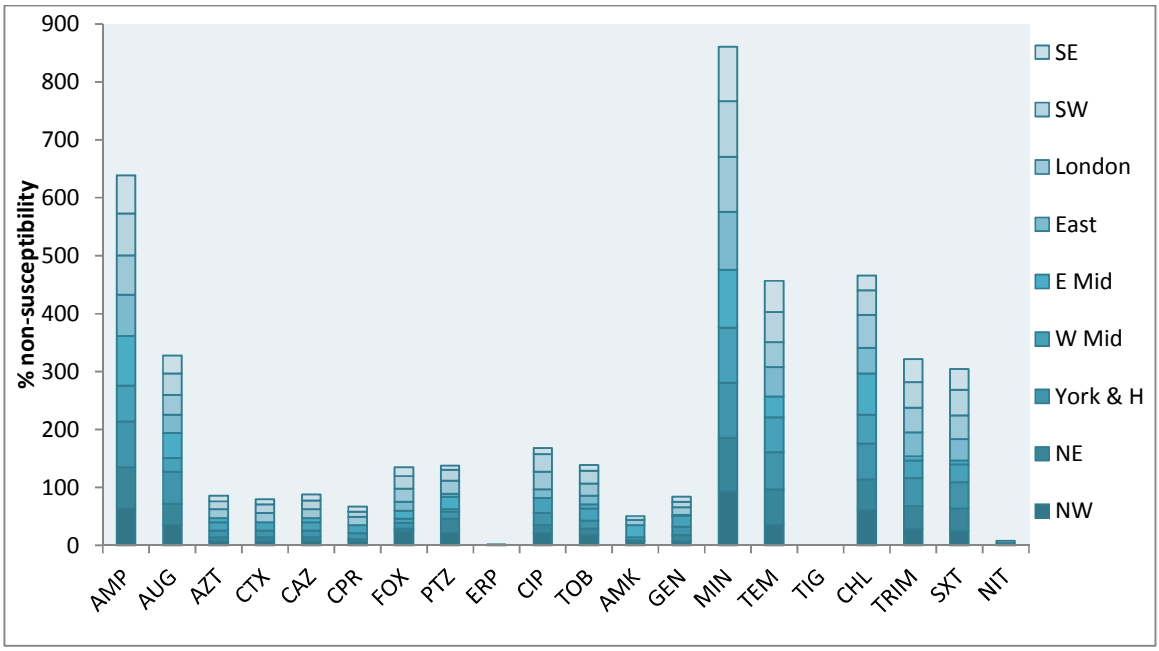


Figure 24 Percentage non-susceptibility according to region of England. Regions include the South-East (SE), South-West (SW), London, East of England (East), East Midlands (E Mid), West Midlands (W Mid), Yorkshire and Humber (York & H), the North-East (NE) and North-West (NW). Imipenem and meropenem are excluded as all isolates were 100% susceptible. Cumulative % resistance for all regions is plotted against the y-axis.

Strain characteristics

Phylogroup A and B1 isolates demonstrated the highest rates of resistance of all four phylogroups (Table 15). Comparison of the two principal virulent ExPEC phylogroups (B2 and D) revealed higher rates of resistance to the cephalosporins, ciprofloxacin and the aminoglycosides in B2 strains, while phylogroup D strains were more resistant to temocillin, chloramphenicol, trimethoprim and trimethoprim-sulfamethoxazole.

Table 15 Percentage non-susceptibility according to phylogenetic group

Antibiotic	A (n=109, %)	B1 (n=43, %)	B2 (n=690, %)	D (n=234, %)
Ampicillin	66	82	75	81
Amoxicillin-clavulanate	32	54	35	42
Cefotaxime	8	0	2	1
Ciprofloxacin	19	21	11	7
Gentamicin	14	4	11	6
Temocillin	41	7	24	36
Chloramphenicol	54	57	39	51
Trimethoprim	46	64	33	57
Nitrofurantoin	5	0	1	1

Breakpoints defining non-susceptibility are as per table 14.

Of the five major ExPEC lineages it was evident that ST131 strains demonstrated the highest rates of resistance to several antibiotics, including amoxicillin-clavulanate, cefotaxime, cefoxitin, piperacillin-tazobactam, ciprofloxacin and gentamicin (Figure 25).

Trimethoprim resistance was similar in ST131 (62%) and ST69 (58%) isolates, while temocillin resistance was almost equal in ST131 and ST73 isolates (52% and 53%, respectively). Overall, all non-ST131 lineages (included in the study) demonstrated a similar antibiotic resistance profile of 74% ampicillin, 30% amoxicillin-clavulanate, 8% cefoxitin, 6% piperacillin-tazobactam, 4% ciprofloxacin, 6% gentamicin, 95% minocycline, 40% chloramphenicol and 27% trimethoprim resistance.

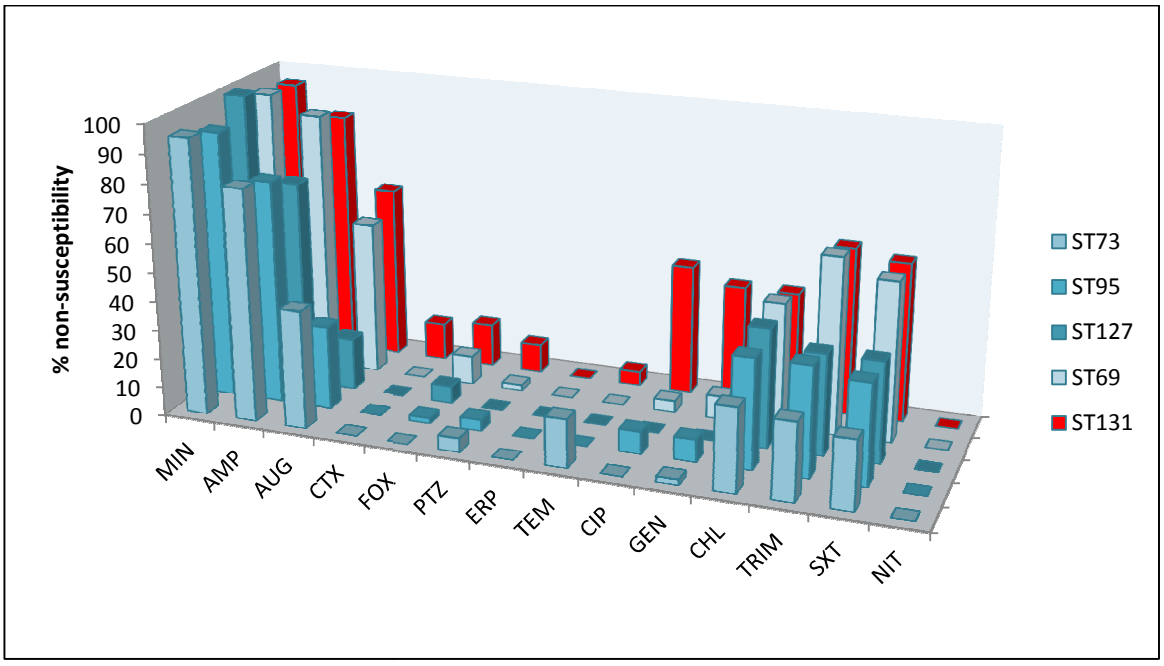


Figure 25 Percentage non-susceptibility for the five major ExPEC lineages. Non-susceptibility is displayed for minocycline (MIN), ampicillin (AMP), amoxicillin-clavulanate (AUG), cefotaxime (CTX), cefoxitin (FOX), piperacillin-tazobactam (PTZ), ertapenem (ERP), temocillin (TEM), ciprofloxacin (CIP), gentamicin (GEN), chloramphenicol (CHL), trimethoprim (TRIM), trimethoprim-sulfamethoxazole (SXT) and nitrofurantoin (NIT).

Penicillin, fluoroquinolone and aminoglycoside resistance determinants

Only isolates demonstrating resistance to one or more cephalosporins were tested for ESBL possession. In total, three urinary and 50 bloodstream *E. coli* strains encoded at least one ESBL enzyme, accounting for 0.6% and 8.8% of all urinary and bloodstream isolates, respectively. Separation of the local and BSAC bloodstream isolates revealed a β-lactamase prevalence of 18.8% and 9.6%, respectively. The specific enzymes detected and identified are listed in table 16. The most frequently detected enzyme was CTX-M-15, followed by OXA-1 and TEM-1. Of note, one local bloodstream isolate encoded both CTX-M-15 and CTX-M-27. Two CMY-42 enzymes were also detected in local bloodstream isolates, both of which encoded CTX-M-15 and OXA-1, with just one encoding TEM-1. The two common enzyme profiles detected were CTX-M-15/OXA-1/TEM-1 (n=18) and CTX-M-15/OXA-1 (n=20). In addition, isolates from male patients possessed a higher proportion

of CTX-M type enzymes for bloodstream isolates (11% vs. 7%) and for urinary isolates (3% vs. 1%, *P*<0.05).

These ESBLs were detected in 11, 2, 45 and 8 phylogroup A, B1, B2 and D isolates, respectively. Forty-one of the 53 ESBL isolates belonged to a major ExPEC lineage: ST131 (n=40) and ST69 (TEM-1 only). All but one (OXA-1 only) of the ST131 isolates expressed CTX-M-15, CTX-M-14 or CTX-M-27. The ST131 isolates are discussed in more detail in chapter 6.

Table 16 Beta-lactamases detected in cephalosporin-resistant isolates according to specimen type

B-lactamase	Urinary isolates (n=4)	Bloodstream isolates (n=55)	Total (n=59)
CTX-M:			
CTX-M-1	0	2	2
CTX-M-14	0	5	5
CTX-M-15	3	41	44
CTX-M-27	0	4	4
OXA:			
OXA-1	3	37	40
TEM:			
TEM-1	3	26	29
SHV:			
SHV-12	0	2	2
CMY:			
CMY-42	0	2	2

All ciprofloxacin-resistant isolates (n=170) were screened for PMQR determinants, but only *qnrS*, *qepA*, *oqxAB* and *aac(6')-Ib-cr* were detected, as detailed in table 17. The gene for *aac(6')-Ib-cr* was the most frequently detected PMQR determinant. In 38/59 *aac(6')-Ib-cr* positive isolates a β-lactamase was also detected. The remaining PMQR-positive isolates (n=26, 41%) were β-lactamase negative. According to phylogroup/lineage combinations, the PMQR determinants were identified within the following: *aac(6')-Ib-cr* (10 A/-; 1 B2/-; 1 B2/ST73; 1 B2/ST127; 45 B2/ST131; 3 D/-), *qnrS* (1 B1/-, 1 B2/-), *qepA* (1 B1/-, 1 D/-) and *oqxAB* (1 B2/ST95).

Table 17 Plasmid-mediated quinolone resistance determinants detected in ciprofloxacin-resistant isolates according to specimen type Ciprofloxacin resistance was determined according to the EUCAST breakpoint (>1 mg/L). Forty-seven urinary isolates and 123 bloodstream isolates were resistant and analysed for PMQR genes.

PMQR	Urinary isolates	Bloodstream isolates
aac(6')-Ib-cr **(n=59)**	10	49
qnrS **(n=2)**	2	0
qepA **(n=2)**	1	1
oqxAB **(n=1)**	1	0

Seventy-seven of 556 (14%) local isolates demonstrated non-susceptibility to ≥1 aminoglycoside and at least one aminoglycoside-modifying enzyme (AME) was detected by PCR in 43/77 (56%) of these isolates. These isolates encompassed 32 (6%) urinary and 11 (23%) bloodstream strains. According to phylogroup, the majority of AME-positive isolates belonged to phylogroup B2 (n=25), followed by group A (n=10) and D (n=7), with just one phylogroup B1 isolate. Only two AMEs were detected in these resistant isolates: *aac(3')-IIa* (n=36) and *aac(6')-Ib* (n=21). All of the isolates, except one, encoding the acetyltransferase *aac(6')-Ib* encoded its fluoroquinolone-resistant conferring allele *aac(6')-Ib-cr*. Fourteen isolates encoded both *aac(3')-IIa* and *aac(6')-Ib-cr*, of which 11/14 also encoded CTX-M-15.

Within the BSAC collection a slightly higher proportion of isolates (n=92/521, 18%) demonstrated reduced susceptibility to ≥1 aminoglycoside and 62/92 (67%) encoded at least one AME. Thirty-eight BSAC isolates encoded *aac(3')-IIa*, 43 encoded *aac(6')-Ib* and 19 encoded both. All but two isolates encoding *aac(6')-Ib* possessed the fluoroquinolone-resistance conferring allele *aac(6')-Ib-cr.* As with the local isolates, the majority of AME genes were detected in phylogroup B2 (n=50) isolates,

followed by groups D (n=8) and A (n=4). Interestingly, *aac(6')-Ib* and its allele were not detected in phylogroup D isolates.

Comparison of phenotypic resistance with AME production revealed the majority (n=63/74) of *aac(3')-IIa* isolates and (n=25/61) *aac(6')-Ib-cr* isolates to have the phenotype RSR (TOB/AMK/GEN). All three aminoglycoside pan-resistant (RRR) isolates, with MICs of >32, 16 and >32 mg/L, expressed both enzymes and were negative for any of the 16S methyltransferases. Analysis of MICs in conjunction with AME genes revealed an MIC of ≥16 for gentamicin and tobramycin to be linked to possession of *aac(3')-IIa* and *aac(6')-Ib*, respectively.

5.4 Discussion points

Within ExPEC strains antibiotic resistance is high (>20%) for many of the antibiotics used to treat UTIs and bacteraemia empirically. In particular, antibiotic resistance appears greater in bloodstream isolates, HAI isolates and those collected from male patients. Comparison of the three different AST methods revealed inconsistencies, especially when comparing disc diffusion to agar dilution.

Within the BSAC bacteraemia collection, isolates from Wales had the highest rates of resistance to the cephalosporins and aminoglycosides, Irish isolates had the highest rates of cefoxitin resistance and Scottish isolates had the highest rates of chloramphenicol resistance. Within England, resistance to trimethoprim +/- sulfamethoxazole was highest in strains from York and Humber; cephalosporin and fluoroquinolone resistance was highest in strains from London and the South-West; with amikacin and chloramphenicol resistance highest in strains from the East Midlands.

Despite the prevalence of phylogroup B2 and D isolates, strains belonging to phylogroups A and B1 demonstrated the highest rates of resistance to individual antibiotics. Within the five major ExPEC lineages, ST131 isolates were significantly more resistant than isolates belonging to the other four sequence types. However, trimethoprim +/- sulfamethoxazole resistance was similar in ST131 and ST69 isolates.

ESBL genes accounted for 0.6% of urinary and 8.8% of bloodstream isolates, with CTX-M-15 the most frequently detected ESBL and OXA-1 the most common β-lactamase in cephalosporin-resistant isolates.

PMQR genes were rarely detected within these ExPEC isolates, with the exception of *aac(6')-Ib-cr*, which was often detected alongside the aminoglycoside-modifying enzyme *aac(3')-IIa*.

Overall, MIC data and resistance genes detected in this study suggest that nitrofurantoin would be a more appropriate first-line antibiotic for uncomplicated cystitis, even in the presence of ESBLs, with trimethoprim-sulfamethoxazole no longer prescribed empirically, due to the high resistance rates.

Ideally patients with pyelonephritis and/or complicated UTIs should have urine culture and antibiotic sensitivities reviewed prior to commencing treatment, to prevent unnecessary use of ciprofloxacin and increasing resistance to this antibiotic. However, this delay in treatment would prolong symptoms and increase the risk of bacteraemia developing.

For *E. coli* causing bacteraemia use of cephalosporins as first-line therapy would be more effective than amoxicillin-clavulanate, due to the lower rates of resistance against this class of antibiotics. However, national data reveals reductions in cephalosporin prescriptions, to avoid selection of ESBL-positive isolates and reduce the risk of *Clostridium difficile*-associated diarrhoea. Therefore, alternative treatments such as piperacillin-tazobactam, tigecycline or a carbapenem may be more appropriate.

6. The UK ST131-O25b Clone

6.1 Introduction

ST131-O25b strains have dominated extra-intestinal infections since the early 2000s (Nicolas-Chanoine *et al.* 2008). Often cited as the most common sequence type causing multi-drug resistant UTIs and bacteraemia since 2000, this clone is associated with fluoroquinolone resistance and CTX-M-15 ESBL expression (Blanco *et al.* 2011; Johnson *et al.* 2010; Peirano and Pitout, 2010; Smet *et al*. 2010).

Additional resistances reported in this clone include non-susceptibility to chloramphenicol, tetracyclines, macrolides, sulphonamides and aminoglycosides, which adds to the cumulative resistance burden posed by this particular ExPEC clone. Genes conferring these resistances are usually located on IncF or IncI1 plasmids, alongside the genes for CTX-M, SHV, TEM, OXA and *aac(6')-Ib-cr*, although IncK/B and IncN plasmids have also been reported in this clone (Carattoli *et al*. 2005; Ho *et al*. 2013; Huang *et al.* 2012; Matsumura *et al.* 2012; Matsumura *et al*. 2013; Wang *et al*. 2013; Woodford *et al*. 2009). More recently, AmpC beta-lactamase and carbapenemase genes have been identified in this clone, including CMY-2, KPC and NDM-1 (Bonnin *et al*. 2012; O'Hara *et al*. 2014; Peirano and Pitout, 2014).

In the UK, epidemic CTX-M-15-producing ExPEC strains, collected between 2004 and 2005, were identified as belonging to the ST131-O25b clone and this clone was the most frequently detected lineage in urinary and bloodstream isolates (Lau *et al.* 2008a; Lau *et al.* 2008b). However, more recent studies conducted in the North of England, which included all ExPEC regardless of antibiotic resistance, revealed ST131 was second to ST73 in frequency among ExPEC isolates (Gibreel *et al.* 2012; Horner *et al.* 2014). In addition, these later studies revealed <70% DNA relatedness among clonal isolates by pulsed-field gel electrophoresis, compared with earlier studies reporting >70% DNA relatedness (Gibreel *et al.* 2012b; Lau *et al.* 2008a). Another interesting observation was the identification of CTX-M-3 enzymes in ST131 isolates collected from Northern Ireland, which conflicts

with all other studies reporting CTX-M-15 as the most frequently identified CTX-M enzyme; highlighting the ability of this clone to acquire locally circulating genes and plasmids quite readily (Dhanji *et al*. 2011). Despite all these differences, each UK study has confirmed that the ST131 clone is multi-drug resistant, regardless of ESBL expression.

Many molecular methods have been developed to facilitate identification of ST131 strains which often divide this clone into various sub-clades, including repetitive element (rep) PCR, *pabB* allele PCR, *mdh* and *gyrB* single nucleotide polymorphism (SNP) PCR and a triplex PCR that targets the *afa* operon and CTX-M-15 gene (Peirano and Pitout, 2010). These methods are often employed alongside identification of the O25b serogroup, which was reported to be unique to this clone (Clermont *et al*. 2007). However, another O-antigen has recently been identified in ESBL-positive ST131 strains, O16 (Dahbi *et al*. 2013; Matsumura *et al.* 2012).

Virulence factors are also relatively conserved among ciprofloxacin-resistant ST131 strains, with >75% reported to possess *fimH*, *fyuA*, *usp*, *ompT*, *iutA*, *sat*, *pap*, *iha*, *traT* and the virulence marker, *malX* (Platell *et al.* 2011). Of importance is *fimH*, the type I fimbrial adhesin, which comprises a multitude of alleles (Weissman *et al.* 2012). Sequencing of the *fimH* gene has identified specific alleles associated with ST131-O25b strains, including H30, H22 and H35 (Johnson *et al.* 2013), with particular focus on the H30-Rx sub-clone, which has been linked with fluoroquinolone resistant, ESBL-positive isolates only (Price *et al*. 2013). Taken together these data suggest that the ST131 clone is relatively homogeneous, with the occasional new addition, such as O16.

ST131 was identified in 7% of urinary and 19% of bloodstream isolates collected as part of this study. As such a prolific clone in the UK and worldwide, it was important to determine whether these UK isolates reflect the results of previous studies. Therefore, all ST131 isolates from this study were fully characterised using established methods to define the ST131 clone within the UK.

6.2 Methods

The ST131 clone can be sub-divided according to the laboratory tests performed, as described in the relevant sections below. Figure 26 details the sub-division of ST131, in conjunction with the tests performed.

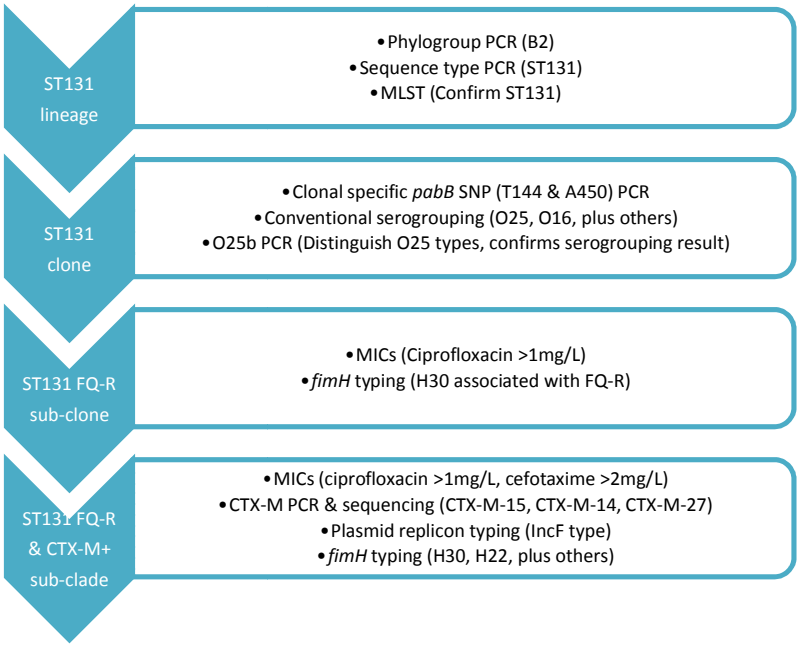


Figure 26 Sub-division of ST131 according to laboratory tests

6.2.1 ST131 identification

Thirty-eight urinary and 106 bloodstream (4 local, 102 BSAC) isolates were identified as phylogroup B2 (section 4.2.2) and ST131 (section 4.2.3), by PCR. A small random number of isolates (n=7), including paired ST131 strains (n=4) and isolates with novel serogroups (n=3), underwent MLST (section 4.2.4) to confirm their sequence type. These 144 ST131 strains were analysed, using the following methods, to assign them to either the resistant and virulent ST131-O25b clone or as non-clonal isolates.

6.2.2 ST131-O25b (*pabB*) real-time PCR

All 144 ST131 isolates were analysed for two ST131-O25b specific ('clonal') SNPs using the real-time assay described by Dhanji *et al* (2010).

Each PCR reaction contained 10 µl SensiFAST™ SYBR No-ROX buffer (Bioline), 0.4 µM each primer (appendix C) and 3 µl DNA lysate made up to a final volume of 20 µl with sterile-filtered water. The thymine-144 and adenine-450 SNP reactions were run separately on a Lightcycler® 2.0 (Roche, Burgess Hill, UK) according to the following cycling conditions: polymerase activation step at 95°C for 3 minutes; followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension for 10 seconds at 58°C, with a single fluorescence acquisition step after each cycle. After amplification the PCR reaction underwent high resolution melt, heating the PCR product to 95°C with a ramp rate of 0.05°C per second. *E. coli* ST131 epidemic strain A was used as a positive control and *E. coli* ATCC 25922 as a negative control to determine the positive and negative values for each of the SNPs. Isolates positive for the adenine and thymine ST131-O25b *pabB*-specific SNPs had a T_m value of 79.79 ± 0.29°C and 81.28 ± 0.17°C, respectively.

6.2.3 Serogroup O25b PCR

All 144 ST131 isolates underwent conventional serogrouping (section 4.2.5) and those that were identified as O25 were analysed using the O25b antigen-specific PCR described by Clermont *et al* (2008) (Clermont *et al.* 2008). Each reaction was prepared and amplified as described in section 3.4, using the primers listed in appendix C, with an annealing temperature of 60°C. The 300 bp product was resolved by gel electrophoresis (section 3.5).

6.2.4 *FimH* PCR

Isolates that possessed the clonal-specific SNPs (section 6.2.2) were analysed by PCR for the *fimH* gene, which was purified (section 3.6) and sequenced (section 3.7) if identified.

Each PCR reaction was prepared according to section 3.4, with 0.2 µM of each primer (appendix C) and an annealing step of 57°C lasting 15 seconds.

After DNA sequencing of the *fimH* amplicon, sequences were aligned using BioNumerics version 6.1 (Applied Maths NV). Aligned sequences were compared with the consensus (wild type) ECOR28 *fimH* sequence (GenBank ID: FJ865637) and the target region analysed for allele-specific SNPs (Weissman *et al.* 2012). The *fimH* target region and H30 allele-specific SNP are displayed in figure 27.

Statistical analysis of the relationship between *fimH* allele and ciprofloxacin resistance was performed using the chi^2 test, using Stata, version 12 (StataCorp LP). A *P-value* of ≤0.05 was considered significant, unless stated otherwise.

CCACTCAGGGAACCATTCAGGCAGTGATTANCATCACCTATACCTACAGCTGANCCCGAAGAGATAATTGTA**A**
TGAAACGAGTTATTACCCTGTTTGCTGTACTGCTGATGGGCTGGTCGGTAAATGCCTGGTCATTCGCCTGTAAA
ACCGCCAATGGTACCGCTATCCCTATTGGCGGTGGCAGCGCTAATGTTTATGTAAACCTTGCGCCTGCCGTGA
ATGTGGGGCAAAACCTGGTCGTAGATCTTTCGACGCAAATCTTTTGCCATAACGATTATCCGGAAACCATTACA
GACTATGTCACACTGCAACGAGGCTCGGCTTATGGCGGCGTGTTATCTAATTTTTCCGGGACCGTAAAATATA
GTGGCAGTAGCTATCCATTTCCGACCACCAGCGAAACGCCGCGGGTTGTTTATAATTCGAGAACGGATAAGCC
GTGGCCGGTGGCGCTTTATTTGACGCCTGTGAGCAGTGCGGGCGGGGTGGCGATTAAAGCTGGCTCATTAAT
TGCCGTGCTTATTTTGCGACAGACCAAAAACTATAACAGCGATGATTTCCAGTTTGTGTGGAATATTTACGCCA
ATAATGATGTGGTAGTGCCTACTGGCGGCTGCGATGTTTCTGCTCGTGATGTCACCGTTACTCTGCCGGACTA
CCCTGGTTCAGTGCCAATTCCTCTTACCGTTTATTGTGCGAAAAGCCAAAACCTGGGGTATTACCTCTCCGGCA
CAACCGCAGATGCGGGCAACTCGATTTTCACCAATACCGCGTCGTTTTCACCAGCGCAGGGCGTCGGCGTACA
GTTGACGCGCAACGGTACGATTATTCCAGCGAATAACACGGTATCGTTAGGAGCAGTAGGAACTTCGGCGGT
AAGTCTGGGATTAACGGCAAATTACGCACGTACCGGCGGGCAGGTGACTGCAGGGAATGTGCAATCGATTAT
TGGCGTGACTTTTGTTTATCAA**TAA**

Figure 27 *E. coli fimH* **target region for allele-specific polymorphisms.** Underlined sequences indicate the forward, middle and reverse primer-binding sites. The *fimH* start codon is indicated in bold text, while the codon underlined with an arrow represents (target) codon number 1, with the subsequent codons numbered sequentially. The stop codon is also highlighted in bold and comprises the last three bases of the reverse primer. Codon 166 (boxed) encodes the amino acid arginine (R). The H30 allele, associated with the ST131 clone, has a nonsynonymous substitution (R166H) (Weissman *et al.* 2012). This figure was adapted from the ECOR28 sequence in Genbank, accession number FJ865637.

6.2.5 Pulsed-field Gel Electrophoresis (PFGE)

All ST131 isolates were analysed by PFGE, as described in section 4.2.7, to determine the relatedness of the isolates and identify any specific clusters within the clone.

6.2.6 Plasmid extraction

6.2.6.1 Crude extraction

This method was performed on all 39 CTX-M-positive clinical isolates prior to transformation (section 6.2.6.3) and has been published elsewhere (Barton *et al*. 1995; Guerra *et al*. 2004).

Isolates were sub-cultured in 3 ml LB broth and incubated overnight at 37°C. Cultures were centrifuged for 5 minutes at 13,000 rpm and 4°C in an Eppendorf 5415 R bench top centrifuge (Eppendorf, Stevenage, UK). Broth was aspirated and the pellet was emulsified in 200 µl solution A (20mM EDTA and 400mM Tris, pH 8). Equal volumes of solutions B (0.4M sodium hydroxide) and C (400mM Tris and 4% sodium dodecyl sulphate, SDS) were mixed together and 400 µl aliquots were added to the emulsified pellet. The tube was then inverted 5-10 times and incubated at room temperature for 5 minutes. After cell lysis proteins were precipitated by adding 240 µl 5M sodium chloride. Tubes were inverted 5-10 times and incubated on ice for 5 minutes. After incubation, tubes were centrifuged for 20 minutes at 13,000 rpm and 4°C in the 5415 R centrifuge (Eppendorf). Protein-free supernatant was transferred to a new tube and DNA purified by adding 800 µl ice cold (stored at -20°C) absolute ethanol (VWR). Tubes were inverted 5-10 times and incubated at -20°C for one hour. Tubes were then centrifuged again for 20 minutes, as described previously. The supernatant was discarded, 1 ml 70% ethanol added to wash the DNA pellet and tubes centrifuged as described previously. After discarding the supernatant the DNA pellet was air dried for 10-15 minutes then re-suspended in 30 µl sterile-filtered water. Plasmid extracts were stored at -20°C until use (within one month).

6.2.6.2 Clean extraction

For a purer plasmid extract, an additional phenol-chloroform step was added. This method was performed on plasmid transformants, prior to S1 nuclease digestion (section 6.2.7).

Cells were lysed, as described above, using solutions A, B and C. In place of sodium chloride, 300 µl 3M sodium acetate (pH 5.5) was added to the cell lysis suspension. Tubes were inverted 5-10 times and incubated on ice for 5 minutes. Tubes were centrifuged for 20 minutes, as described previously (section 6.2.6.1), and the supernatant transferred to a new tube. Protein was precipitated by adding 500 µl phenol-chloroform and inverting the tubes 5-10 times, before centrifuging again for 5 minutes at 5,000 rpm and 4°C. The upper aqueous phase was transferred to a new tube and 500 µl chloroform was added, before repeating the 5 minute centrifugation step. The upper aqueous phase was transferred to a new tube and DNA purified using the two ethanol steps described previously (section 6.2.6.1). The pellet was re-suspended in 30 µl sterile-filtered water and stored at -20°C until use.

6.2.6.3 Plasmid Transformation

Seven isolates encoding the three CTX-M genes represented in this study (3 CTX-M-27, 2 CTX-M-14 and 2 CTX-M-15) underwent plasmid extraction and transformation into *E. coli* alpha cells (Bioline, London, UK) by electroporation. Results from these seven isolates were used to infer plasmid abundance in the remaining 32 CTX-M-15 isolates. Two microlitres of plasmid extract was mixed with 20 µl electrocompetent cells (defrosted on ice) and transferred to a pre-chilled GenePulser 0.1 cm cuvette (Bio-rad, Hemel Hempstead, UK). Cells were electroporated in a GenePulser Xcell (Bio-rad) set at 1.8 kV, 25 µF and 200 Ω. Cells were immediately recovered with 1 ml S.O.C. medium (Bioline) and transferred to a 15 ml polystyrene tube (Greiner Bio-one GmbH, Germany). Electroporated cells were incubated at 37°C, with rotation, for one hour. After incubation 100 µl cells were inoculated on to an LB agar plate containing 2 mg/L cefotaxime (Sigma), for selection. Plates were incubated overnight at 37°C. Cefotaxime-resistant colonies (transformed cells) were sub-cultured onto non-selective media (ISO) and incubated as above. Cultures of the transformants were

stored at 4°C. DNA lysates (section 3.3) and plasmid extraction (section 6.2.6.2) was performed on the transformants, as required.

6.2.7 REP typing

It was expected that the majority of CTX-M enzymes would be encoded on IncF plasmids of FIA:FIB type (Huang *et al.* 2012; Johnson *et al.* 2012b; Matsumura *et al.* 2013; Wang *et al.* 2013). Therefore, all CTX-M-positive isolates and the seven plasmid transformants were screened for IncF FIA and FIB alleles, using the PCR plasmid replicon-typing method described by Carattoli *et al* (2005). The PCR reactions and amplification conditions were as described in section 3.4, with 0.2 µM of each primer (appendix C), an annealing temperature of 60°C and a denaturation step lasting one minute. Products were resolved by gel electrophoresis (section 3.5). All FIA and FIB alleles were cleaned up (section 3.6) and sequenced (section 3.7). The generated sequences were aligned using BioNumerics version 6.1 (Applied Maths NV) and compared with known alleles in the plasmid MLST website (http://pubmlst.org/ plasmid/) to identify the allele combinations (Jolley and Maiden, 2010).

Isolates that did not carry an IncF plasmid were screened for all other replicon types, as described above, in simplex PCRs using the primers listed in appendix C. If isolates could not be typed using this PCR scheme they were screened using the PBRT kit (Diatheva, Italy), which contains updated primers. Each PCR reaction contained 23.8 µl PCR mix, 0.2 µl DNA polymerase and 1 µl DNA lysate, as per the manufacturer's instructions. There were eight PCR mixes together comprising primers for 25 replicon types. PCR amplification and product resolution was as described above.

6.2.8 S1 nuclease pulsed-field gel electrophoresis

Plasmid analysis by S1 nuclease PFGE was performed to identify the number of plasmids carried by each of the CTX-M-positive ST131 isolates; to confirm that the transformed plasmid was the same size as in the clinical isolate; to determine if plasmids of the same IncF type were the same size; and to determine the size of the plasmids that encoded CTX-M enzymes. This was performed using a modified version of the method described by Barton *et al* (1995) (Guerra *et al.* 2004).

Overnight cultures of all CTX-M-positive ST131 isolates and the seven transformants were used to prepare DNA blocks, as described in section 4.2.5. A 1-2 mm strip was cut from each block and incubated in 100 µl 1X S1 nuclease buffer (Promega), for 20 minutes at room temperature. The buffer was aspirated and replaced with 100 µl fresh buffer, plus 8 U S1 nuclease enzyme (Promega), for 45 minutes at 37°C. The enzyme reaction was stopped with 10 µl 0.5M EDTA (Sigma) and the enzyme-buffer reaction replaced with 100 µl 0.5X TBE (Invitrogen).

Each strip was loaded into an individual well of a 1.25% (approximately) agarose gel, as described in section 4.2.5. The gel was run for 17 hours, at 6 volts and at 12°C, with an initial switch of 1 second and a final switch of 25 seconds, using a CHEF-DR II chiller system (Bio-rad). After electrophoresis gels were stained with Gelred™ (section 3.5) and analysed using BioNumerics, version 6.1 (Applied Maths NV), to identify the bands and size the plasmids. Each defined band was considered to be a linearised plasmid (Barton *et al.* 1995; Wang *et al.* 2013). Isolates that failed to produce a band after initial digestion were repeated twice more.

6.3 Results

Clonal ST131 strains

All phylogroup B2, ST131 isolates (144/1077, 13%) were separated into two main clades, designated clonal and non-clonal, as determined by ST131-O25b specific SNPs detected by real-time PCR (section 6.2.2). The 'clonal' clade (*pabB* SNPs detected) was then further sub-divided according to CTX-M possession (Figure 28).

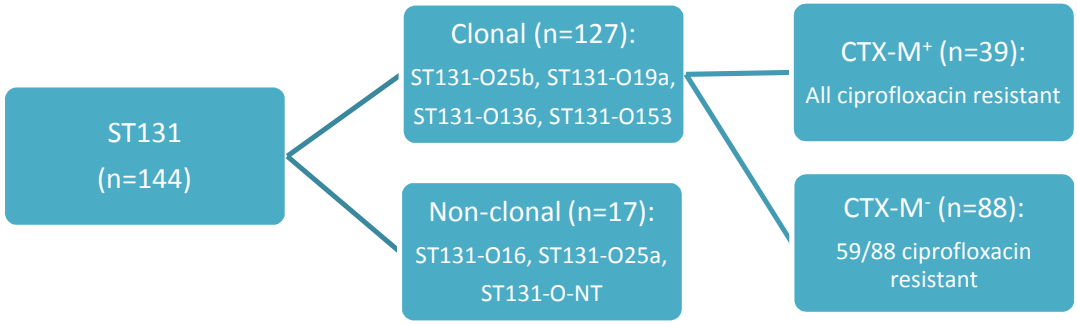


Figure 28 Sub-clades of *E. coli* ST131 isolates as defined by clonal specific single nucleotide polymorphisms, serogroup, CTX-M expression and ciprofloxacin resistance All non-clonal isolates were CTX-M negative and ciprofloxacin-susceptible, whereas clonal isolates included both ciprofloxacin-susceptible and resistant isolates, plus CTX-M positive isolates, all of which were ciprofloxacin-resistant.

Patient demographics

Of the clonal isolates, 27% belonged to the local *E. coli* collection (n=32 urinary, n=3 bloodstream, total n=35, 6%), but the majority belonged to the BSAC collection (n=92, 18%). Patients with ST131 isolates ranged from 8 to 96 years, with a modal age of 85 years and a mean of 43 years. The majority of clonal isolates (>70%) were isolated from patients aged >60 years, which corresponded with the high proportion of bloodstream isolates (75%, n=95) and the urinary tract (44%, n=42/95) as the most common source of these bloodstream isolates. Of the 32 urinary isolates, 19 (60%) were

classified as COMP, 11 (34%) as UC and two (6%) as ABU. The majority of these clonal isolates were classified as CAIs (59%, n=75/127).

Serogroup

Within the clonal isolates, 124 (98%) were identified as serogroup O25 by conventional serogrouping, which were confirmed as O25b by PCR (section 6.2.3). The three other clonal isolates were identified as serogroup O19a, O136 and O153 and were negative by the O25b PCR. Further characterisation, including *pabB* SNP detection, *fimH* typing and antibiogram, classified these three strains as clonal.

Within the non-clonal strains (n=17), one was identified as O25 by conventional serogrouping, but was negative by O25b PCR, identifying the allele as O25a. Twelve isolates belonged to serogroup O16 and the remaining four were non-typeable, but all were negative by the O25b PCR and for one or both of the ST131-specific SNPs.

CTX-M Extended-spectrum β-lactamases

Only 39 clonal ST131 isolates (31%) encoded a CTX-M enzyme, all of which were serogroup O25b. The CTX-M enzymes detected were CTX-M-15 (n=34), CTX-M-27 (n=3) and CTX-M-14 (n=2). In addition, 30 (24%) also expressed OXA-1 and 16 (13%) expressed TEM-1 (13 expressed both). None of the CTX-M-positive isolates expressed an SHV enzyme. Replicon typing of the 39 CTX-M-encoding isolates and the seven transformants revealed all, except two, to possess IncF type plasmids. CTX-M-15 enzymes were identified in isolates encoding FIA or FIA-FIB plasmid combinations, as displayed in figure 29. Both CTX-M-14 enzymes were encoded on approximately sized 100 Kb IncF A2:B- plasmids and the three CTX-M-27 enzymes were encoded on IncF A2:B20 plasmids of approximately 120-140 Kb. The two non-IncF plasmids were identified as IncU and assumed to encode the CTX-M-15 enzymes.

Only 35 of 39 CTX-M-positive isolates produced a linear plasmid band after S1 nuclease digestion and PFGE, despite repeat digestion. Plasmids of equal size were identified within the seven

transformants and their respective clinical isolates. The majority (n=25/35) possessed only an IncF plasmid, probably encoding the CTX-M enzyme, while the remaining (n=10) possessed an additional 1-2 plasmids. The four isolates that failed to generate a linear band on three separate occasions included the two IncU strains, the only A6:B26 IncF strain and one A3:B1 IncF strain.

The remaining 88 clonal ST131 isolates were negative for CTX-M, TEM, OXA and SHV enzymes, except for one isolate which encoded OXA-1. All non-clonal ST131 isolates were negative for these enzymes.

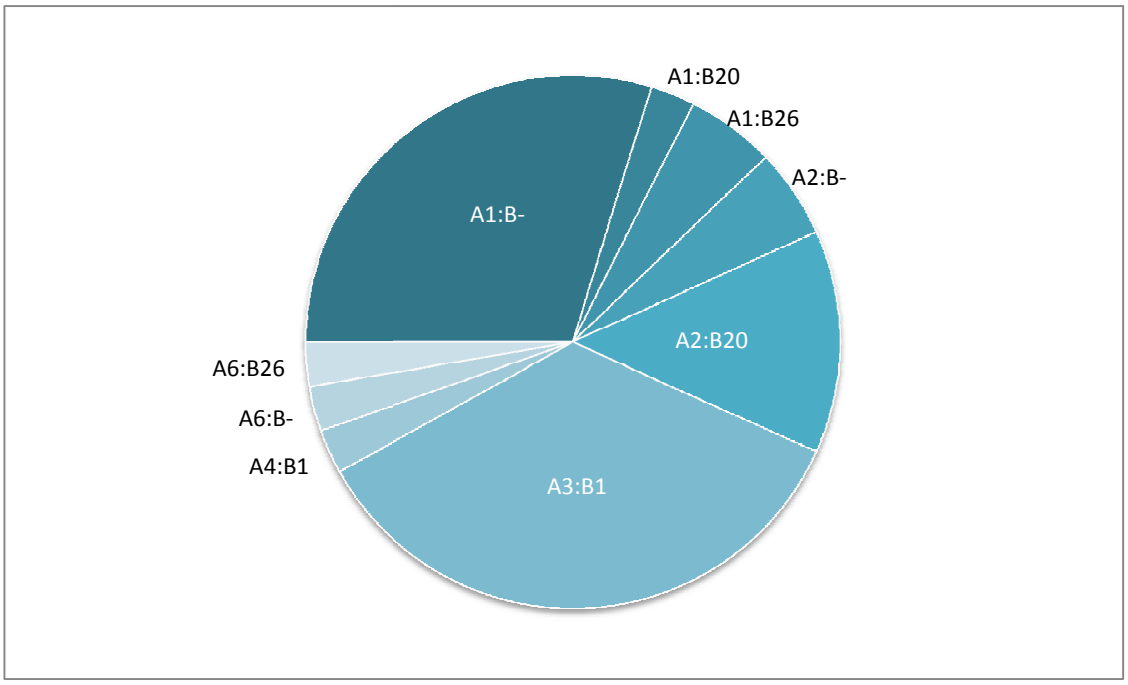


Figure 29 IncF plasmids identified in ST131 strains expressing CTX-M-15 enzymes. Plasmids ranged in size from 50Kb-165Kb. Alleles as defined by pubMLST database (http://pubmlst.org/plasmid/)

Ciprofloxacin resistance

All 39 CTX-M-positive isolates were ciprofloxacin-resistant according to MICs (section 5.2.1.2) and 59/88 (67%) CTX-M-negative isolates were also ciprofloxacin-resistant, including the O136 isolate, providing an overall resistance of 77%. All non-clonal isolates were susceptible to ciprofloxacin.

The *aac(6')-Ib-cr* enzyme was detected in 30/39 CTX-M positive isolates and 9/88 CTX-M negative isolates. This enzyme was not detected in the non-clonal ST131 isolates.

Antibiogram

As reported in chapter 5, antibiotic resistance in ST131 isolates was much higher than in the other lineages analysed in this study. Comparison of clonal versus non-clonal isolates and CTX-M positive versus CTX-M negative, revealed further differences within this clone (Table 18).

Table 18 Percentage non-susceptibility of non-clonal versus clonal ST131 isolates, including percentage resistance in CTX-M positive versus CTX-M negative strains. Breakpoints used to determine non-susceptibility were as follows: ≥1 (ciprofloxacin), ≥2 (cefotaxime), ≥4 mg/L (gentamicin, trimethoprim), >8 mg/L (ampicillin, chloramphenicol, temocillin for bloodstream isolates) and >32 mg/L (temocillin for urinary isolates). The two CTX-M-negative isolates that were cefotaxime resistant had MICs of 2 and 64, with the former encoding OXA-1, while the latter lacked genes for CTX-M groups, TEM, SHV and OXA enzymes, but may have lost porins and activated efflux pumps, conferring resistance (Perez-Moreno *et al*. 2004).

Antibiotic	Non-clonal (n=17)	Clonal (n=127)	CTX-M positive (n=39)	CTX-M negative (n=88)
Ampicillin	88	87	100	82
Cefotaxime	0	32	100	2
Ciprofloxacin	0	77	100	67
Gentamicin	24	34	49	27
Temocillin	53	53	85	39
Chloramphenicol	59	49	49	49
Trimethoprim	59	62	82	53

Between the clonal and non-clonal isolates similar resistance rates was observed for ampicillin, temocillin and trimethoprim. However, resistance was higher in clonal isolates for cefotaxime, ciprofloxacin and gentamicin, but lower for chloramphenicol. The higher rates of resistance in the clonal isolates is likely attributable to the CTX-M positive isolates, which demonstrated much greater levels of reduced susceptibility against all antibiotics tested, except chloramphenicol.

Alleles of type 1 fimbriae (*fimH*)

Alongside the consensus (wild-type) *fimH* DNA sequence, three different *fimH* alleles were identified within this ST131 collection; H30, H27 and H22. The proportion of each allele according to ciprofloxacin susceptibility is displayed in figure 30.

Greater diversity was seen within the susceptible isolates, while the ciprofloxacin-resistant isolates mostly possessed the H30 allele (94%, $P<0.01$). Despite being identified in both ciprofloxacin-resistant and -susceptible strains, the H22 allele was significantly associated with susceptible strains ($P<0.001$).

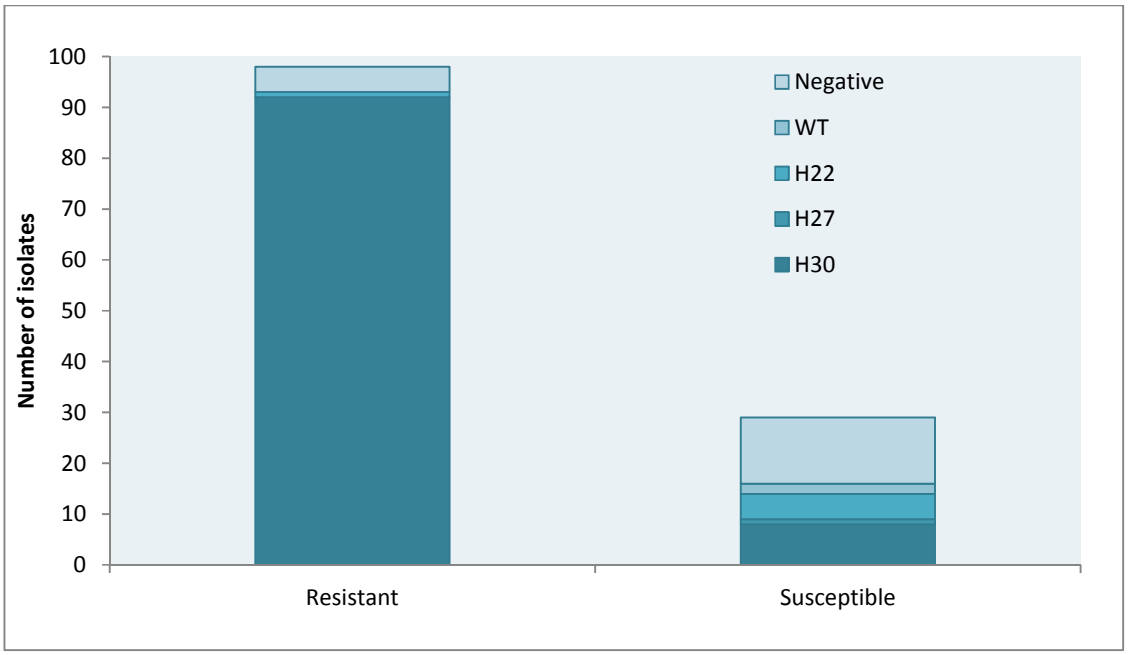


Figure 30 Alleles of the *fimH* target sequence according to ciprofloxacin susceptibility. Alleles detected included H30, H27, H22, wild type (WT) or isolates were negative for the *fimH* gene.

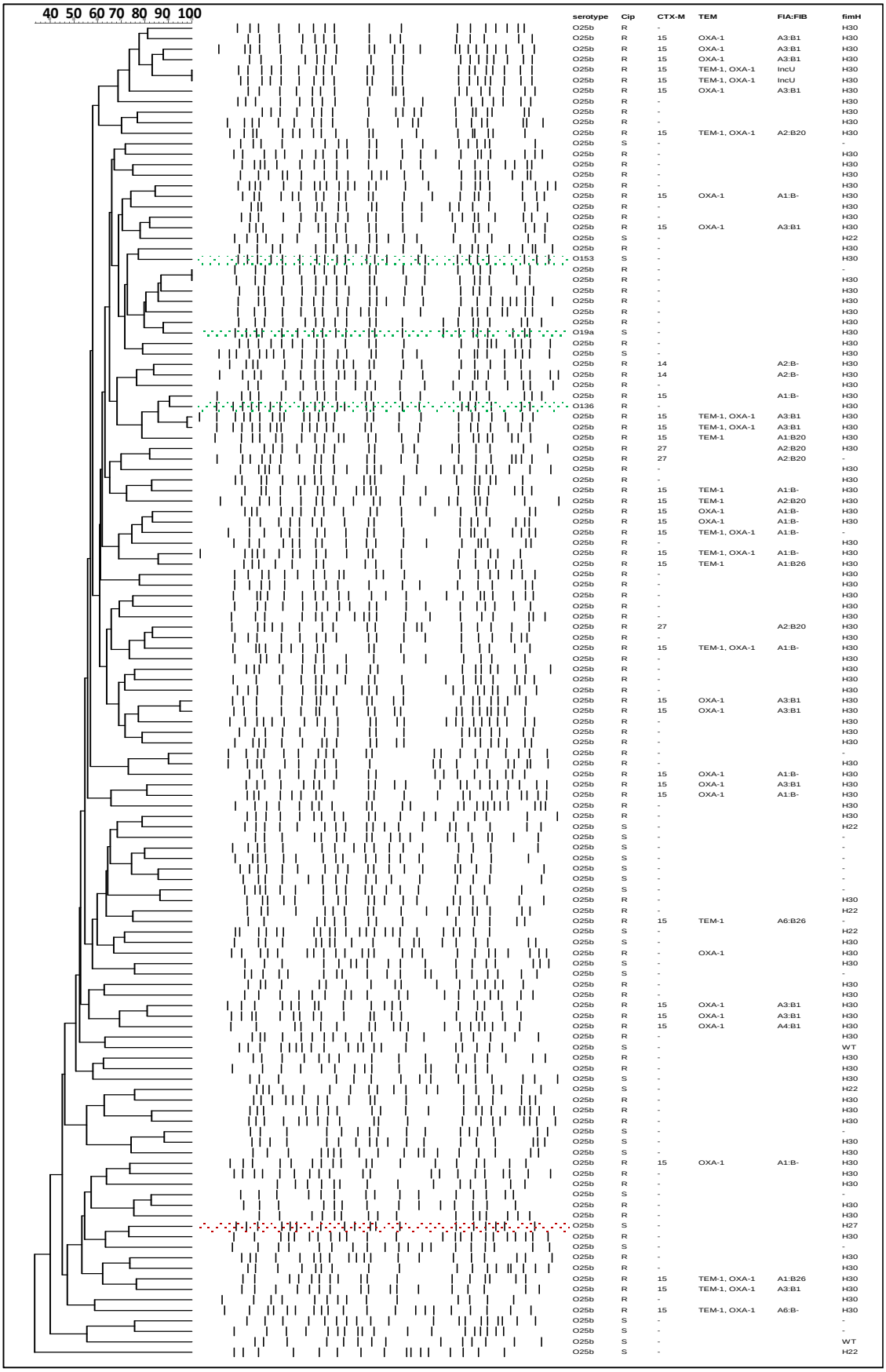
PFGE

Analysis of *Xba*I-digested DNA from all ST131 isolates revealed the clonal and non-clonal strains to share ≤40% similarity, with small isolate clusters of highly related PFGE profiles (>70%). Within the clonal isolates there was no clustering of strains according to *fimH* allele, CTX-M enzyme, IncF plasmid, ciprofloxacin resistance or serogroup; with random dispersion of these characteristics

within the clone (Figure 31). In particular, the H27 allele and serogroups that were atypical of ST131-O25b strains were all closely related (>70%) to ciprofloxacin-resistant ST131-O25b strains.

Figure 31 PFGE Dendrogram of *Xba*I-digested clonal ST131 strains. Isolate highlighted in red encoded the H27 *fimH* allele. Profiles highlighted in green identify the isolates of serogroups O19a, O136 and O153.

(Please turn the page for figure 31).



serotype	Cip	CTX-M	TEM	FIA:FIB	fimH
O25b	R	-			H30
O25b	R	15	OXA-1	A3:B1	H30
O25b	R	15	OXA-1	A3:B1	H30
O25b	R	15	OXA-1	A3:B1	H30
O25b	R	15	TEM-1, OXA-1	IncU	H30
O25b	R	15	TEM-1, OXA-1	IncU	H30
O25b	R	15	OXA-1	A3:B1	H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	15	TEM-1, OXA-1	A2:B20	H30
O25b	S	-			-
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	15	OXA-1	A1:B-	H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	15	OXA-1	A3:B1	H30
O25b	S	-			H22
O25b	R	-			H30
O153	S	-			H30
O25b	R	-			-
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O19a	S	-			H30
O25b	R	-			H30
O25b	S	-			H30
O25b	R	14		A2:B-	H30
O25b	R	14		A2:B-	H30
O25b	R	-			H30
O25b	R	15		A1:B-	H30
O136	R	-			H30
O25b	R	15	TEM-1, OXA-1	A3:B1	H30
O25b	R	15	TEM-1, OXA-1	A3:B1	H30
O25b	R	15	TEM-1	A1:B20	H30
O25b	R	27		A2:B20	H30
O25b	R	27		A2:B20	-
O25b	R	-			H30
O25b	R	-			H30
O25b	R	15	TEM-1	A1:B-	H30
O25b	R	15	TEM-1	A2:B20	H30
O25b	R	15	OXA-1	A1:B-	H30
O25b	R	15	OXA-1	A1:B-	H30
O25b	R	15	TEM-1, OXA-1	A1:B-	-
O25b	R	-			H30
O25b	R	15	TEM-1, OXA-1	A1:B-	H30
O25b	R	15	TEM-1	A1:B26	H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	27		A2:B20	H30
O25b	R	-			H30
O25b	R	15	TEM-1, OXA-1	A1:B-	H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	15	OXA-1	A3:B1	H30
O25b	R	15	OXA-1	A3:B1	H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			-
O25b	R	-			H30
O25b	R	15	OXA-1	A1:B-	H30
O25b	R	15	OXA-1	A3:B1	H30
O25b	R	15	OXA-1	A1:B-	H30
O25b	R	-			H30
O25b	R	-			H30
O25b	S	-			H22
O25b	S	-			-
O25b	S	-			-
O25b	S	-			-
O25b	S	-			-
O25b	S	-			-
O25b	S	-			-
O25b	R	-			H30
O25b	R	-			H22
O25b	R	15	TEM-1	A6:B26	-
O25b	S	-			H22
O25b	S	-			H30
O25b	R	-	OXA-1		H30
O25b	S	-			H30
O25b	S	-			-
O25b	R	-			H30
O25b	R	-			H30
O25b	R	15	OXA-1	A3:B1	H30
O25b	R	15	OXA-1	A3:B1	H30
O25b	R	15	OXA-1	A4:B1	H30
O25b	R	-			H30
O25b	S	-			WT
O25b	R	-			H30
O25b	R	-			H30
O25b	S	-			H30
O25b	S	-			H22
O25b	R	-			H30
O25b	R	-			H30
O25b	R	-			H30
O25b	S	-			-
O25b	S	-			H30
O25b	S	-			H30
O25b	R	15	OXA-1	A1:B-	H30
O25b	R	-			H30
O25b	R	-			H30
O25b	S	-			-
O25b	R	-			H30
O25b	R	-			H30
O25b	S	-			H27
O25b	R	-			H30
O25b	S	-			-
O25b	R	-			H30
O25b	R	-			H30
O25b	R	15	TEM-1, OXA-1	A1:B26	H30
O25b	R	15	TEM-1, OXA-1	A3:B1	H30
O25b	R	-			H30
O25b	R	15	TEM-1, OXA-1	A6:B-	H30
O25b	S	-			-
O25b	S	-			-
O25b	S	-			WT
O25b	S	-			H22

6.4 Discussion points

Peirano *et al* (2010) described ST131 as a successful, fluoroquinolone-resistant clone, whose rapid spread was intensified by the acquisition of a CTX-M-15 encoding IncF plasmid and the possession of the virulence factors *ompT*, *usp* and *malX*.

Within the UK the ST131 clone predominantly caused bloodstream infections, with a smaller proportion of urinary tract infections.

Thirty-one percent (n=39/127) of clonal isolates exhibited the traditional characteristics of ST131-O25b-CTX-M, but in-depth characterisation of ST131 isolates revealed new serogroups and plasmid replicon types associated with the UK clone.

Clonal ST131 strains were highly resistant, especially in conjunction with ESBL enzymes.

The ST131 clone within the UK appeared more diverse than strains analysed in other parts of the world.

Analysis of the virulence determinants expressed by these clonal isolates would reveal whether virulence of ST131 strains has changed and whether these factors can provide an alternative means to identify the clone.

7. ExPEC virulence determinants

7.1 Introduction

E. coli strains express a variety of virulence factors (VF) for attachment to host cells (*fimH, pap, hra, iha*), to facilitate transmission by damaging host cells (*sat, vat, cnf1*), to acquire iron (*hlyA, fyuA, iutA*) and to evade the host immune response (K1, *traT*) (Bhargava *et al.* 2009; Johnson, 1991; Johnson *et al.* 2005a; Waters and Crosa, 1991). In ExPEC, in particular, VF expression is reported to be highly varied.

Johnson and Stell (2000) originally defined ExPEC by the possession of ≥2 of the following: *pap*, *fimH*, *hlyA*, *fyuA*, *iutA*, *KpsII*, *traT* and *malX*. Specific VFs have also been linked with particular infections: *ibeA* and *sfa* have been associated with neonatal meningitis; *usp* was identified as UTI-specific; *pap* were discovered in *E. coli* causing pyelonephritis; plus *iss* and *traT* facilitate serum survival during bloodstream infections (Blum *et al.* 1991; Huang *et al.* 1995; Kurazono *et al.* 2000; Waters and Crosa, 1991).

Since then VFs have been used to characterise specific ExPEC clones and pathotypes, including ST131-O25b (*fimH, fyuA, iutA, traT, malX*), CgA (*pap, iutA, traT, kpsII*) and APEC (*iutA, cvaC, iss, traT, iroN, hlyF, fimH, ompT*) (Clark *et al*. 2012; Jeong *et al.* 2012; Johnson *et al.* 2012b; Manges *et al.* 2001; Platell *et al.* 2011). It has been reported that the expression of particular VFs (*pap*, *hlyA*, *cnf*1, *iutA*, *traT* and *ibeA*) is inversely related to antibiotic resistance (Cooke *et al.* 2010; Piatti *et al.* 2008). With the highly resistant ST131 clone and highly susceptible ST127 clone, it will be interesting to determine how relevant this observation is today.

Studies investigating the virulence of ExPEC have used multiple PCRs or traditional laboratory methods, such as latex agglutination and haemagglutination to identify few VFs (Blanco *et al.* 1997; Moreno *et al.* 2005; Venier *et al.* 2007). These types of investigation were improved by the development of six multiplex PCR assays, to screen for a multitude of *E. coli* virulence factors (Johnson and Stell, 2000), which have been used regularly since first reported (Czaja *et al.* 2009;

Karisik *et al.* 2008; Moreno *et al*. 2008; Pitout *et al.* 2005). However, multiplex PCRs are limited to approximately 4-8 targets per PCR. Microarrays provide an alternative to PCR, enabling simultaneous detection of hundreds of targets and can determine the expression level of specific targets. Many arrays have been developed for genus and species identification, typing data, antibiotic resistance and VFs (Hu *et al*. 2012; Platteel *et al*. 2011; Vanhomwegen *et al*. 2013).

Alere Technologies GmbH (Jena, Germany) has produced multiple arrays, including those for genotyping of common pathogens, antibiotic resistance and species identification (http://alere-technologies.com). For this project, an experimental *E. coli* combined genotyping array was evaluated alongside phenotypic (MIC determination, serogroup) and genotypic methods (multiplex PCR assays of Johnson and Stell (2000), as the 'gold standard'.

7.2 Methods

7.2.1 *E. coli* Genotyping Combined Array

The *E. coli* combined genotyping microarray (Alere Technologies GmbH, Jena, Germany) consisted of targets for 74 virulence determinants, 94 antibiotic resistance genes, 48 H-antigens and 23 O-antigens. Details of the targets can be found on the company website (http://alere-technologies.com) and appendix D. A schematic detailing the layout of the microarray and hybridisation process is detailed in figure 32.

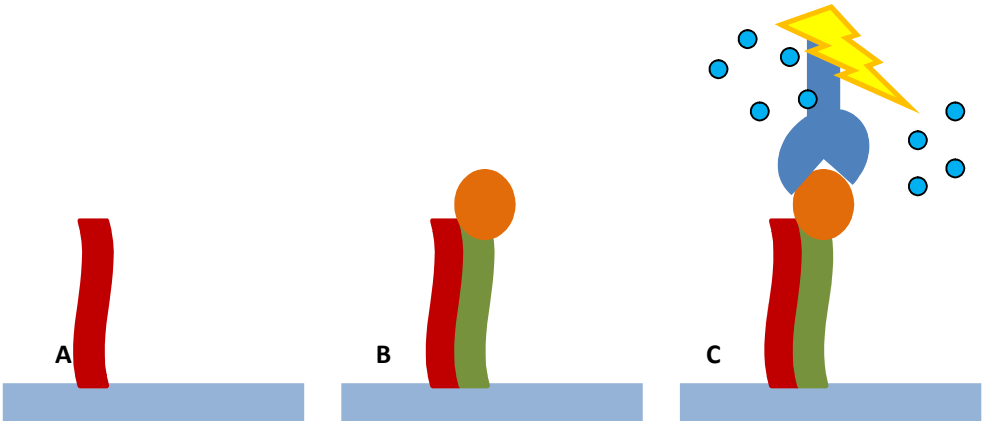


Figure 32 Schematic of the microarray including hybridisation and labelling steps

A) Target-specific probes are immobilised on the array surface. B) Biotin-labelled ssDNA, which has been amplified using one target-specific antisense primer, binds the complementary probe. C) During hybridisation streptavidin binds to the biotin label and horseradish peroxidase (HRP) subsequently binds to streptavidin. The conjugation of streptavidin with HRP causes seramun green dye to precipitate, which is detected by the ArrayMate reader.

7.2.1.1 Genotyping DNA Extraction

DNA was extracted from overnight cultures using a modified version of the Animal Tissue – spin column protocol of the DNeasy® Blood and Tissue Kit (Qiagen). Bacterial colonies were resuspended

in 180 µl buffer ATL and 20 µl proteinase K then incubated at 56°C for 10 minutes in an Eppendorf thermomixer comfort (Eppendorf, Hamburg, Germany). Four microlitres of RNase A (Qiagen) at 100 µg/ml was added to the suspension, vortexed briefly and incubated at room temperature for 2 minutes. Suspensions were vortexed again, and 200 µl buffer AL and 200 µl 95% ethanol added before vortexing for 10-15 seconds.

Suspensions were transferred to a spin column and processed according to the manufacturer's instructions. Columns were centrifuged for 1 minute at 8000 rpm in an IEC® MicroMax centrifuge (Thermo Scientific). The column was transferred to a new tube, 500 µl AW1 buffer added and centrifugation repeated as above. The column was transferred to a new tube, 500 µl AW2 buffer added and centrifuged for 3 minutes at 14000 rpm. The column was transferred to a new eppendorf tube, 200 µl AE buffer added and tubes incubated at room temperature for 1 minute. Tubes were centrifuged for 1 minute at 8000 rpm and eluted DNA stored at 4°C until required. Prior to analysis DNA was quantified using a ND-1000 NanoDrop (Thermo Scientific).

7.2.1.2 Genotyping PCR

Multiplex PCR was performed with primers and reagents provided by Alere Technologies (Germany), to label the DNA with a biotin marker for detection, according to the manufacturer's instructions. Each reaction contained 3.9 µl B1 buffer, 0.1 µl B2 (polymerase enzyme), 1 µl primer mix, 4 µl sterile-filtered water and 1 µg DNA. Alere Technologies recommended 0.5-2 µg, while Geue *et al* (2010), which have been referenced by several similar studies, recommended 1.0-1.5 µg (Monecke *et al*. 2011; Schilling *et al*. 2012; Wu *et al*. 2013). Therefore, a compromise of 1 µg was selected. Amplification consisted of an initial denaturation at 96°C for 5 minutes, followed by 45 cycles of denaturation at 96°C for 60 seconds, annealing at 60°C for 20 seconds and elongation at 72°C for 40 seconds, with a final 4°C hold. PCR products were stored at 4°C until required for hybridisation.

7.2.1.3 Genotyping Hybridisation

All reagents were supplied by Alere Technologies (Germany). Prior to hybridisation DNA samples were prepared by suspending 10 µl PCR product in 90 µl C1 buffer. Horse radish peroxidase (HRP)-streptavidin conjugate was also prepared by adding 1 µl C3 concentrated HRP to 99 µl C4 buffer, per sample.

The ArrayStrips™, which were spotted with probes for each of the targets, were washed with 200 µl sterile distilled water, per well, for 5 minutes at 56°C with shaking at 550 rpm in an OLS200 shaking waterbath (Grant Instruments). As with all subsequent washes and incubations, the water was removed using a pastette, at the edge of the well, so as not to scratch the array surface. The initial wash step was repeated with 200 µl C1 buffer and incubated as above. C1 buffer was aspirated and 100 µl DNA preparation was added to each well before re-incubation, as above, for 60 minutes. The wells were washed twice with 200 µl C2 buffer. C2 buffer was aspirated and 100 µl pre-prepared conjugate added to each well then incubated for 15 minutes at 30°C with shaking at 550 rpm. Wells were washed twice with 200 µl C5 buffer, before incubation with 100 µl D1 seramun green substrate at room temperature for 10 minutes. The substrate was aspirated before the target signals were read using an ArrayMate™ reader (Identibac, Alere Technologies GmbH, Cologne, Germany), using IconoClust software, version 3 (Alere Technologies, Germany).

7.2.1.4 Genotyping Analysis

The array signal value(s) was determined by subtracting the background signal from the target signal, using the manufacturer's software. The manufacturer recommends that all signal values of ≤0.1 should be considered negative (target absent), values of ≥0.3 should be considered positive (target present) and values between 0.1 – 0.3 as indeterminate. Array signals were considered positive if the signal value was above the cut-off level for both target spots. Four controls were included on the microarray: a biotin staining control and three *E. coli* controls; glutamate decarboxylase (*gad*), integration host factor subunit A (*ihfA*) and glyceraldehydes-3-phosphate (*gapA*).

Microarray results were compared with MICs (presence of certain antibiotic resistance genes should confer an MIC greater than the breakpoint), β-lactamase PCRs (specific β-lactamase gene should be detected by the microarray), PMQR determinant PCR (specific PMQR genes should be detected by the microarray) and serogrouping data. These tests were considered the gold standard for evaluating the microarray. As well as confirming and validating these results, the microarray would reveal the virulence factors encoded by these isolates.

Preliminary analysis resulted in the cut-off being revised. Several studies using this array, or alternative versions, used a positive cut-off of ≥0.4 (Charnock *et al*. 2014; Dierikx *et al.* 2012; Vogt *et al*. 2014; Wagner *et al*. 2014), but this did not improve the results here. While adjustment of the cut-off (≥0.86) according to the ATCC 25922 control results (as a fully susceptible type strain antibiotic resistance determinants conferring resistance should not be hybridized), did not improve the accuracy or reliability of the array either.

7.2.2 *E. coli* isolates

Ninety-five *E. coli* isolates (appendix E) and the ATCC 25922 control were analysed using the *E. coli* combined genotyping microarray (Alere Technologies), according to the manufacturer's instructions, as described above. Isolates encompassed a range of ages, sexes, infection types and sources of bacteraemia. The 34 paired isolates (section 3.2.1) were also included to determine whether they carried the same VFs and antibiotic resistance genes.

For the virulence determinant PCRs 154 newly selected isolates (appendix E) were investigated, including 53 of the microarray isolates. These included 30 asymptomatic bacteruria (ABU) isolates, 30 uncomplicated cystitis (UC) isolates, 30 complicated cystitis/pyelonephritis (COMP) isolates, 30 bloodstream isolates and the 34 paired urosepsis isolates.

7.2.3 Virulence determinant PCRs

E. coli isolates were investigated for *E. coli* virulence factors (VF), using an updated version (personal correspondence from Prof. James Johnson, 2013) of Johnson and Stell's PCR assays (Johnson and

Stell, 2000). The results of these PCRs, in conjunction with antibiotic resistance data and serogrouping data, were compared with the microarray results.

PCR reactions were prepared using the Qiagen® Multiplex PCR kit (Qiagen). Each PCR reaction contained 10 µl master mix, 2.5 µl Q solution, primer mix, and 5 µl DNA lysate made up to a final volume of 25 µl with sterile-filtered water. Primers were separated into 6 primer mixes (appendix C) at a working concentration of 0.6 µM per primer. Amplification consisted of an initial hold at 95°C for 15 minutes; followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 68°C for 30 seconds and extension at 72°C for 1 minute; with a final hold at 72°C for 5 minutes. Primer mix six was amplified with an annealing temperature of 58°C. Products were resolved by gel electrophoresis (section 3.5).

7.2.4 Cyclomodulin PCR

These 154 *E. coli* isolates were also analysed for cycle inhibiting factor (*cif*), cytotoxic necrotising factor – 1 (*cnf1*), cytolethal distending toxin (*cdtB*) and the colibactin-encoding *pks* island, which was detected by identification of two marker genes; *clbA* and *clbQ*. These cyclomodulins were detected using previously published PCRs (Dubois *et al.* 2010; Salvarani *et al*. 2011). PCR reactions and cycling conditions were as described in section 3.4, using two different annealing temperatures; 48°C (*cnf1*) and 55°C (all other targets) and the primers listed in appendix C. PCR products were resolved by gel electrophoresis (section 3.5).

7.2.5 Pathogenicity islands

In addition to VFs, the *Galleria* isolates were investigated for eight pathogenicity islands (PAI) that have been identified in ExPEC strains (Dobrindt *et al.* 2002; Sabate *et al*. 2006). Two multiplex PCR assays were used to screen for the PAIs. Multiplex 1 detected CFT073 II, 536 III and 536 IV, while multiplex 2 detected J96 I, J96 II, CFT073 I, 536 I and 536 II. PCR reactions were prepared as described in section 3.4, using the primers listed in appendix C, with 3 µl DNA lysate. Cycling conditions were also as described in section 3.4 with an annealing temperature of 55°C and all cycle

steps extended to 1 minute. PCR products were resolved by gel electrophoresis (section 3.5). CFT073 (Mobley *et al.* 1990), J96 (Minshew *et al*. 1978) and 536 (Blum *et al.* 1991) type strains were included as positive controls. These strains were kindly provided by Dr McNally (University of Nottingham, UK) and Professor Johnson (University of Minnesota, MN, USA). PAIs were included in the analysis of the *G. mellonella* model to identify any association with larvae mortality.

7.2.6 Rifamycin resistance PCR

Isolates identified by the microarray as encoding rifamycin resistance genes (*arr*) were screened by PCR for these targets (Hopkins *et al*. 2014), using the primers listed in appendix C. PCR reactions and cycling conditions were as described in section 3.4, with an annealing temperature of 55°C. PCR products were resolved by gel electrophoresis (section 3.5).

7.2.7 Statistics

All statistical analysis was performed using Stata®, version 12 (StataCorp LP, TX, USA).

Based on the VF determinant PCRs, isolates were given a virulence (VF) score, calculated as the sum of virulence factors (and cyclomodulins) identified in each isolate. Multiple targets for the same virulence factor were considered as one for scoring. Isolates were also given an antibiotic (Abx) resistance score, calculated as the sum of antibiotics demonstrating reduced susceptibility per isolate, according to MICs. VF and Abx scores were rounded to the nearest whole number and percentage positive calculated according to various strain characteristics and patient demographics. Associations between patient variables, strain characteristics and VFs were determined by correlation coefficient. Any significant associations were analysed using the Fishers exact test.

The association between VF and Abx score was calculated by linear regression. Comparisons of scores, according to various patient and strain variables, were calculated using the Mann-Whitney U test.

A *P-value* ≤0.05 was considered significant for all statistical tests, except for the correlation coefficient, which used a *P-value* ≤0.01.

Combined genotyping array

Sensitivity and specificity of specific targets on the microarray were calculated, by comparing to the gold standard tests (MICs and PCR).

Paired Isolates

A paired t-test was performed to calculate whether there was a significant difference in VF score and Abx resistance score between the urinary and blood culture isolate of each pair. Significance was set at a value $P<0.05$.

7.3 Results

Genotyping Microarray

Using the recommended cut-off of ≥0.3; 93 of the 95 isolates were positive for the biotin (staining control) and 54 were positive for the *gadA* (*E. coli* control), yet failed to hybridise with the *ihfA* and *gapA E. coli* control targets. All 95 isolates were positive for the *gadA* gene in the phylogrouping PCR (section 4.2.2), resulting in the array *gadA* target demonstrating a sensitivity of 57%. Employing the higher cut-off levels of 0.4 and 0.86, further reduced the sensitivity of *gadA* to 41% (39/95) and 0% (0/95), respectively. The ATCC 25922 control was negative for *gadA* (both target spots) at each cut-off. Although each of the four DNA controls should be positive for the array results to be accurately interpreted and validated, the data for the antibiotic resistance genes and O-antigens were analysed, as a second step in determining the reliability of this microarray, using the ≥0.3 cut-off.

Fifteen of the 95 isolates possessed either a CTX-M group 1 or group 9 gene, or both, as determined by PCR (section 5.2.5). However, only 2/15 of these genes (both CTX-M group 9) were detected using the microarray. An additional 27 ESBL-negative isolates also hybridized with these targets, resulting in a sensitivity and specificity of 13% and 66% respectively, for the microarray. Multiple AmpC/beta-lactamase targets (ACC-1, ACT-1, DHA, KHM, LEN-1, OXA, SPM, VIM) hybridized with 83 isolates that were negative for these genes by PCR and the microarray failed to detect 8/12 OXA and 10/10 TEM-1 genes. However, the two CMY-positive isolates were correctly identified using the microarray, although a further 44 also hybridized with the CMY target on the array, despite being negative by PCR.

PCR identified the PMQR genes *aac(6')-Ib-cr*, *qepA* and *qnrS* (section 5.2.7) in 19/25 of the ciprofloxacin-resistant isolates and 1/70 ciprofloxacin-susceptible isolates that were subsequently analysed using the microarray. The microarray successfully identified the *aac(6')-Ib* gene in 12 isolates, but also detected this target in 42 other ciprofloxacin-susceptible isolates, as well as *qnrB* and/or *qnrD* in 66 isolates (all false-positive as determined by PCR). In addition, the 16S rRNA methyltransferases *armA, rmtA, rmtB* and *rmtC*, which confer pan-resistance to the

aminoglycosides, were detected using the microarray in 85 isolates. However, none of these 16S methyltransferases were confirmed by PCR (section 5.2.6). Multiple aminoglycoside modifying enzymes (AME) were also detected by the array, including *aadA*, *aadB* and *aphD*, in isolates of varied aminoglycoside susceptibility, none of which were confirmed by PCR (section 5.2.6). The only AME detected by PCR was *aac(3')-IIa*, which was not detected by the array.

The *arr-1* and *arr-4* genes, that confer resistance to rifampicin (da Fonseca et al. 2008), were detected in 83 isolates. PCR (Hopkins *et al.* 2014) did not confirm the presence of these genes. The *tetA*, *tetB*, *tetC* and *tetD* genes were detected by microarray in 79 isolates, of which 73 (92%) were minocycline resistant. The *dfrA* and *sul2* genes confer trimethoprim and sulfamethoxazole resistance respectively, by encoding alternative alleles of the dihydrofolate reductase and dihydropteroate synthase enzymes (Skold, 2001). These targets were detected in 52 and 76 isolates, respectively. However, of these isolates 27/52 (52%) and 32/76 (42%) were resistant and 25/52 and 44/76 were susceptible to trimethoprim and sulfamethoxazole respectively. The chloramphenicol acetyltransferase, *catB*, was detected in 72 isolates, of which 25/72 (35%) were chloramphenicol resistant. The genes *floR* and *cmlA1* also confer florfenicol and chloramphenicol resistance and were detected in 49 and 26 isolates respectively, of which 19 were chloramphenicol resistant.

Eighty-two of 95 isolates analysed by the microarray underwent conventional serogrouping (section 4.2.5), of these 18/82 expressed an O-antigen that was included as a target on the microarray. However, the microarray failed to correctly identify the O-antigen in all isolates and often detected multiple (1 - 6) O-antigens per isolate.

Overall the microarray lacked both sensitivity and specificity when compared with PCR detection of antibiotic resistance targets and serogroup data. Therefore, the virulence factor data obtained from the microarray was also considered to be unreliable. Especially as the array failed to detect *pap*, *sfa*, *sat*, *tsh*, *iha* and *cnf1* in the majority of isolates, but hybridized with *cif*, *cdtB*, K88 fimbriae, *hlyE* and *vat* which were not confirmed by PCR. Other common virulence targets hybridized by the array included cloacin, microcins B, *ipaH*, *perA* and shiga-toxin.

Virulence factor PCR

The imprecision of the microarray (see section 7.3) led to 154 isolates (appendix E) being investigated for VF genes using the updated PCR protocol (2010) published by Johnson and Stell (2000). Fifty-three of the isolates analysed using the microarray were also included in this PCR analysis.

Multiple ExPEC and diarrhoeagenic *E. coli* (DEC) virulence factors were detected by PCR (Table 19), with a prevalence ranging from 1% (*gafD*, *clpG*, *pic*, and *kpsMTII*) to 81% (*fimH*). The determinants *fimH, pap, fyuA, iutA, kpsMTII, traT* and *usp* were all detected in >50% isolates.

The proportion of each detected VF according to infection (UTI or bacteraemia), UTI type (ABU, UC, COMP) and GIT versus GU source bacteraemia can be found in table 19.

Table 19 Proportion of virulence factors according to infection type

	UTI type			BLC source		Infection		
Characteristic	**ABU**	**UC**	**COMP**	**GU**	**GIT**	**UTI**	**BLC**	**Overall**
	(n=30)	(n=30)	(n=47)	(n=20)	(n=9)	(n=107)	(n=47)	(n=154)
Adhesins:								
fimH	97 **	93 *	79	45	78 *	88***	66	81
pap	53	67	70	80 **	44	64	62	64
iha	46	63 ***	43	60	40	50	49	49
sfa/foc	27	13	19	20	33	20	21	20
afa/dra	3 *	13	19	35 **	0	13	17	14
hra	3	3	6	-	-	5**	0	3
bmaE	3	0	6	-	-	4*	0	3
clpG	-	-	-	0	11	0	2	1
gafD	3	0	0	-	-	1	0	1
Toxins:								
sat	37	57 **	36	40	33	42	32	39
hlyD	30	21	23	0	33 **	23	15	21
hlyF	17	9	17	5	11	15	11	14
vat	0	0	6	15	22	3	11*	5
EAST1	3	6	2	0	0	4	2	3
tsh	0	0	4	20	11	2	13***	3
pic	-	-	-	5	11	0	4*	1
Siderophores:								
fyuA	80	80	68	30	67 *	75*	60	70
iutA	47	63	60	65	56	57	60	58
iroN	37	33	36	0	44** *	36	32	34
ireA	10	0 **	15	-	-	10**	0	7
Capsules:								
*kpsMT***II**	53	53	62	40	33	57	57	57
*kpsMT***III**	0	0	2	-	-	1	0	1
K1	10	3 *	19	15	11	12	19	14
K5	3 *	0	0	-	-	1	0	1
K15	0	3	0	5	0	1	2	1
Protectins:								
traT	53	63	62	56	75	60	66	62
iss	23	13 **	23	30	56	21	53***	31
cvaC	17	13	11	5	11	13	11	12
rfc	0	10 **	4	-	-	5	0	3

Asterisk indicates if the proportion of isolates possessing that VF is significantly different between infection types, as determined by the Fishers exact test: ***P<0.001; **P<0.01; *P<0.05

Table 19 continued

Characteristic	ABU	UC	COMP	GU	GIT	UTI	BLC	Overall
Miscellaneous:								
usp	53	40 *	62	45	67	53	66	57
malX	50	40	43	15	78 ***	44	51	46
pks	30	33	40	30	44	36	40	37
ompT	33	37	28	45 **	0	32	21	29
cnf1	27	6 **	28	24	33	21	21	21
ibeA	17	0 *	6	0	11	7	19*	11
cdtB	3	6	13	5	22	8	9	8
cif	0	0	4	0	10	2	4	3

Asterisk indicates if the proportion of isolates possessing that VF is significantly different between infection types, as determined by the Fishers exact test: ***P<0.001; **P<0.01; *P<0.05

Of the fimbriae, *fimH* was the most prevalent (81%), followed by *pap* (64%). The *iha* gene was by far the most common non-fimbrial adhesin, detected in 49% of isolates. Yersiniabactin (*fyuA*) was the second most prevalent VF (70%) and also the most common siderophore detected, followed by *iutA* (58%). Of the toxins, *sat* was the most frequently detected (39%), followed by *hlyD* (21%) and the avian haemolysin, *hlyF* (14%). The *kpsMTII* (57%) capsules were more common than *kpsMTIII* (1%) capsules, with K1 (14%) the principal K-antigen detected. Sixty-two percent of isolates encoded *traT*, with half of those encoding *iss* (31%), and approximately a quarter encoding *cvaC* (12%). Of the miscellaneous proteins, *usp* was most prevalent (57%), followed by the PAI virulence marker, *malX* (46%), and the *pks* island (37%). *Cnf1* was the most common cyclomodulin (21%), with <10% isolates encoding *cdtB* and *cif*.

Urinary tract infections: *fimH* was significantly more prevalent in urinary isolates (88%, P<0.01) than bloodstream isolates (66%), particularly ABU (97%) and UC (93%) infections. In addition, *hra, bmaE* and *gafD* were only detected in urinary isolates. Of the toxins *sat, hlyD, hlyF* and *East1* were more frequently detected in urinary isolates. In particular, *sat* was more common in UC isolates (57%) versus ABU (37%) and COMP (36%) isolates, but not significantly so. *FyuA* was more frequently detected in ABU and UC (both 80%) isolates than COMP isolates (68%), but also not significantly so.

While *iutA* was less common in ABU (47%) than the other urinary isolates (60-63%) and *iroN* was similarly distributed across all UTI types (33-37%). Of note was *ireA*, which was only detected in urinary isolates (10%, *P*<0.05), but not UC infections. *KpsMTII* genes were detected more frequently in COMP isolates (62%) than other urinary isolates (53%). As for the protectins, *traT* was more common in UC (63%) and COMP (62%) isolates, while *iss* was more frequently detected in COMP and ABU isolates (both 23% vs. 13%). Of the miscellaneous proteins *usp, pks, cnf1, cdtB* and *cif* were more common in COMP isolates, while *malX* and *ibeA* were more common in ABU isolates and *ompT* was more prevalent in UC isolates.

Analysis of UC isolates alone, as an indicator of the VF required to cause infection in non-compromised patients, revealed *iha, sat, iutA, traT* and *ompT* to be more frequently detected. Of these, the association of *iha* and *sat* with UC approached significance (*P*<0.08).

Bacteraemia: As the urinary tract is the most common source of *E. coli* bacteraemia, GU and GIT isolates were compared to identify VFs associated with severe infection, versus those required for colonisation or less severe infections. In contrast to *fimH, pap* appeared more prevalent in GU isolates, but the difference was not significant (80% vs. 44%, *P*=0.07). The adhesins *iha* (60%) and *afa/dra* (35%) were also more common in GU isolates. CS31A adhesins were only detected in bacteraemia isolates, albeit at a low rate (2%), but were detected in GI isolates only and were absent in GU isolates. *Sat* was notably more prevalent in GU isolates (40% vs. 33%), as was *tsh* (20% vs. 11%). However, in GIT isolates, *hlyD* was more common (33% vs. 0%), along with *vat* (22% vs. 15%) and *pic* (11% vs. 5%). Of the siderophores *fyuA* (67% vs. 30%) was significantly more prevalent in GIT isolates, along with *iroN* (44% vs. 0%, *P*<0.01). As for the capsular types, all were more prevalent in GU isolates, while the protectins and miscellaneous proteins were all more prevalent in GIT isolates, except for *ompT* (0% vs. 45%, *P*<0.05). The association of *pap, afa/dra* and *ompT* with GU isolates, approached significance (*P*<0.06).

Analysis of the alternative source bacteraemia isolates (Line, Chest, CSF, SSTI) revealed other associations. The CSF-source isolate belonged to ST95 and encoded *fimH, pap, sfa/foc, hlyF, fyuA,*

iutA, *iroN*, K1, *traT, iss, cvaC, usp, ibeA, pks* and *malX*. Of the Chest-source isolates only *fyuA* was encoded in 100% of isolates, with *fimH* in 80%. In Line-source isolates 100% encoded *usp* only, with 83% also encoding *fimH, fyuA, iss* and *malX*. In SSTI-source isolates 100% of isolates encoded *fyuA, iroN, kpsMTII*, *iss* and *usp*, with 75% also possessing *pap, fimH, iha, ibeA, pks* and *malX*.

Urosepsis: Analysis of VFs more prevalent in COMP and GU isolates may indicate determinants important in urosepsis. Notably, *pap* (P<0.01)*, afa/dra* (P<0.01) and *kpsMTII* were more frequently detected in these isolates, while *fimH* (P<0.01)*, fyuA* (P<0.01) and *cvaC* were lacking.
VF and Abx scores were not significantly different (P>0.05) between the urinary isolates and bloodstream isolates of the 17 pairs (Table 20), except for pair 16 which comprised two separate strains (see Table 13). However, there were some minor differences in VF possession and antibiogram between paired isolates. For example, in one pair the bloodstream isolate had two additional VFs (*afa/dra* and *iha*); while in another pair the urinary isolate was resistant to additional antibiotics (cefoxitin and ertapenem). In addition, *fimH* and *fyuA* were only detected in 13/34 and 8/34 of the paired isolates respectively, despite being detected in 112/120 (93%) and 100/120 (83%) of the remaining 120/154 isolates analysed by PCR.

Table 20 Virulence and resistance scores for the 34 paired urosepsis isolates

Isolate pair	Sample	VF score	Abx score
1	Blood	8	5
	Urine	6	5
2	Blood	12	3
	Urine	11	3
3	Blood	10	5
	Urine	10	5
4	Blood	12	6
	Urine	13	6
5	Blood	10	3
	Urine	9	3
6	Blood	9	11
	Urine	12	10
7	Blood	2	16
	Urine	1	18
8	Blood	12	4
	Urine	14	2
9	Blood	8	2
	Urine	10	3
10	Blood	10	15
	Urine	10	13
11	Blood	9	6
	Urine	7	5
12	Blood	10	6
	Urine	12	5
13	Blood	16	3
	Urine	13	3
14	Blood	12	4
	Urine	10	4
15	Blood	11	4
	Urine	10	4
16	Blood	10	5
	Urine	4	8
17	Blood	15	6
	Urine	15	5

Pathogenicity islands: 134/154 (87%) of the VF isolates possessed ≥1 of the PAIs. The isolates lacking the PAIs were mostly from UTIs (n=17/20) and belonged to phylogroup A (n=11/20), with a VF score ranging from 1-9.

In order of prevalence, 536 IV was the most frequently detected (n=129/154), followed by CFT073 I (n=71/154), CFT073 II (n=68/154), 536 I (n=25/154), J96 II (n=18/154), 536 II (n=13/154), J96 I (n=7/154) and 536 III (n=5/154).

The most common PAI profile, which was detected in 49/154 (32%) isolates, was CFT073 I, CFT073 II and 536 IV. Isolates possessed between 1-7 PAIs.

PAIs were often significantly associated ($P \leq 0.01$) with the presence of other PAIs, including CFT073 I with CFT073 II and 536 I with 536 III. The CFT073 and J96 PAIs were also significantly more prevalent in phylogroup B2 isolates, than any other phylogroup, while 536 III was significantly associated with serogroup ST127 and serogroup O6 isolates (all $P<0.01$).

Scores: Across all isolates, VF scores ranged from 0-18 (median=9) and Abx scores ranged from 0-30 (median=4). Linear regression revealed VF scores were inversely associated with the Abx scores ($P<0.05$), for all isolates, including the paired isolates. VF scores and Abx scores according to patient and isolate variables are listed in table 21.

Of note, Abx scores increased with age and were higher in male patients and bacteraemia isolates, although these observations were not significant. COMP isolates had higher scores than the other urinary isolates, while bacteraemia scores were difficult to interpret according to source due to the variation in sample number.

Table 21 Virulence and resistance scores according to patient demographics and strain characteristics

Variable	Number of isolates	VF score	Abx score
Infection:			
Urinary tract	107	9	4
Bacteraemia	47	9	6 **
UTI type:			
ABU	30	9	3
UC	30	9	4
COMP	47	9	5
BLC type:			
Chest	5	6	9
CSF	1	18	4
GI	9	9	7
GU	20	8	6
Line	6	10	5
SSTI	4	13	5
NK	2	12	2
Phylogenetic group:			
A	26	5	6*
B1	2	7	6
B2	84	11***	4
D	42	7	5
Sequence type:			
ST131	13	10	8**
ST127	9	10	4
ST95	14	12 ***	4 *
ST73	12	12***	3**
ST69	20	7**	4
NK	86	8*	5
Age (years):			
0-10	8	9	3
11-20	6	9	4
21-30	29	9	5
31-40	30	9	4
41-50	14	9	4
51-60	15	10	6
61-70	13	10	5
71-80	15	8	7
81-90	19	9	7
>90	5	9	3

Table 21 continued

Variable	Number of isolates	VF score	Abx score
Sex:			
Female	113	9	4
Male	41	8	7 **
Healthcare setting:			
CAI	122	9	5
HAI	32	10	5
Overall:		9	4

Asterisk indicates if the scores are significantly different according to various patients and strain variables, as determined by the Mann Whitney U test: ***P<0.001; **P<0.01; *P<0.05

Phylogroup: Phylogroup B2 had the highest mean VF score of 11 (P<0.01), while phylogroups D and B1 both had a score of 7. Phylogroup A had the lowest VF score of 5, but also the highest Abx score at 6, along with phylogroup B1 isolates. VFs only detected in phylogroup B2 included *sfa/foc, gafD, clpG, pic, tsh, ireA, cif* and *cdtB*, while *vat* and *ibeA* were detected in phylogroups B2 and D only. The isolate of the novel phylotype (*gadA*$^{+}$, *chuA*$^{-}$, *yjaA*$^{+}$, TspE4.C2^{+}) possessed *pap*, *fimH*, *iha*, *sat*, *ireA*, *iutA*, *usp*, *traT*, *pks* and *malX*. No other isolates in this collection shared this virulence profile.

Sequence type (ST): ST73 and ST95 had the highest VF score (VF=12, P<0.01). ST69 had the lowest score of the 5 lineages (VF=7). All STs had a low Abx score compared with the VF score, with the exception of ST131 isolates, which had a mean VF and Abx score of 10 and 8 respectively. Multiple VFs were detected in at least three of the lineages, including *pap*, *usp*, *malX* and *pks*, but some VFs were also significantly associated with one lineage compared to all others, according to a correlation matrix; ST131 was significantly associated with *iha* (92%, P<0.01) and *iutA* (100%, P<0.01); ST127 was significantly associated with *cnf1* (78%, P<0.01); ST95, the avian lineage, was significantly associated with *cvaC* (42%, P<0.01), *hlyF* (50%, P<0.01) and K1 (57%, P<0.01); ST73 was significantly associated with *sfa/foc* fimbriae (83%, P<0.01), *pks* (100%, P<0.01) and cytolethal distending toxin, *cdtB* (42%, P<0.01); and ST69 was significantly associated with *sat* (80%, P<0.01) plus *ompT* (75%, P<0.01). Despite these differences in VF detection, it was not possible to use VFs to identify

particular lineages or to distinguish them. Analysis of the ST131 virulence repertoire (Table 22) according to the three sub-clones (section 6.3) revealed *afa/dra* and *pap* to be absent from the non-clonal strain, while in the clonal isolates *pap* was present in all ESBL$^+$ isolates, but only in one of the ESBL$^-$ isolates and *cnf1* was detected in three of the ESBL$^+$ isolates only. Median VF scores also varied between the three sub-clones: 8 (clonal, ESBL$^+$), 10 (clonal, ESBL$^-$) and 11 (non-clonal).

Table 22 Virulence factors detected in the ST131 isolates according to sub-clone. VFs that are underlined were identified in >70% of isolates, according to each sub-group, except for non-clonal isolates, of which only one was investigated using the VF PCR assays.

ST131 sub-group	No. of isolates	VF score	Virulence factors (% isolates positive for VF)
Clonal, ESBL+	7	8	*fimH* (57) *pap* (86) *afa/dra* (43) *clpG* (14) *iha* (100) *hlyD* (14) *sat* (43) *pic* (14) *fyuA* (57) *kpsII* (57) *ompT* (29) *usp* (86) *traT* (71) *iss* (57) *cnf1* (43) *malX* (43)
Clonal, ESBL-	6	10	*fimH* (100) *pap* (17) *afa/dra* (17) *iha* (67) *hlyF* (33) *sat* (83) *fyuA* (83) *iutA* (100) *iroN* (17) *kpsII* (83) *usp* (100) *traT* (100) *iss* (50) *ibeA* (50) *cdtB* (17) *cvaC* (17) *malX* (100)
Non-clonal	1	11	*fimH iha sat fyuA iutA kpsII usp traT iss pks malX* (100)

Virulence profiles: Particular clones and pathotypes have been investigated for virulence profiles, with VFs detected in ≥70% of isolates included in the profile (Clark *et al.* 2012). For example, Clonal group A (ST69/D) isolates have been characterised by the presence of *pap*, *iutA*, *traT* and *kps*II and the absence of *sfa/foc*, *afa/dra*, *hlyD*, *cnf*1, *iroN*, *iss* and *malX* (Manges *et al.* 2001).

Using this method ≥70% UK ST131 isolates possessed ≥3 of the following: *fimH, iutA, fyuA*, *iha*, *kpsII*, *usp*, *traT* and *malX*, with some variations according to sub-clone (Table 22). The ST69 profile included *pap, fimH, iutA, iha, sat, traT* and *ompT* but lacked *sfa/foc*, *afa/dra*, *hlyD, malX, cnf1*, *hra, pic, vat, tsh, ireA, usp, ibeA, pks, cif* and *cdtB*.

In addition, 10% of UK isolates possessed APEC traits including *iroN*, *fimH*, *hlyF*, *iss* and *iutA* and 94% possessed ≥2 of the following, defining these isolates as ExPEC according to Johnson and Stell (2000): *pap*, *fimH*, *hlyD*, *fyuA*, *iutA*, *kps*II, *traT* and *malX*.

7.4 Discussion points

It was not possible to evaluate the virulence and resistance profile of 95 selected ExPEC isolates using the *E. coli* combined genotyping microarray.

The ExPEC VF PCR assays designed by Johnson and Stell (2000) revealed the virulence repertoire of 154 UK ExPEC isolates to be highly varied.

Specific VFs were identified within the five major ExPEC lineages that were highly prevalent, but unable to distinguish between these sequence types.

Paired isolates causing urosepsis typically possess the same virulence factors, but during transmission from the urinary tract to the bloodstream, additional factors may be gained or lost.

Evaluation of VFs prevalent in UC cystitis and genitourinary-source bacteraemia, compared to all other isolates, could identify those required for colonisation and those required for virulence.

8. ExPEC virulence in *Galleria mellonella*

8.1 Introduction

Multiple virulence factors have been reported in ExPEC (Johnson and Stell, 2000). Recent studies suggest that *pap, fyuA, iroN, kpsMTII* and *ompT* are important in pyleonephritis strains, with *kpsMTII* associated with particularly severe infection (Kudinha *et al.* 2013b; Marschall *et al*. 2012). Despite this, only the presence of *fyuA* and *pap* have been linked to increased patient mortality (Mora-Rillo *et al.* 2013), while the *pks* island encoding colibactin is suggested to decrease sepsis survival in a mouse model (Marcq *et al*. 2014). Another study investigated the role of O-antigens in ExPEC survival and confirmed that O-antigens increase serum survival, but O6, in particular, facilitates colonisation of the urinary tract (Sarkar *et al*. 2014). Antibiotic resistance and delay in receiving appropriate antibiotic therapy have been strongly linked to patient mortality, specifically the presence of ESBL enzymes (Ku *et al*. 2013; Melzer and Petersen, 2007). These two observations are interesting given that Piatti *et al* (2008) and Cooke *et al* (2010) have demonstrated the inverse relationship between VF expression and antibiotic resistance, suggesting that mortality and severity of infection is due to one or the other, but not both.

UK ExPEC isolates revealed great diversity in their virulence factor (VF) repertoire (see 7.3). Data suggested that *iutA* and *traT* were important VFs in UTIs, while *pap, afa/dra* and *tsh* were important VFs in GU-source bacteraemia and *iha*, *sat* and *ompT* were important in both infection types. Analysis of urosepsis isolates suggested that the presence of *pap, afa/dra* and *kpsMTII* coupled with the absence of *fimH, fyuA* and *cvaC* were significantly associated with these infections. Overall phylogroup B2 strains possessed the highest number of VFs, along with ST73 and ST95 strains and specific VFs were associated with particular sequence types, such as ST131 and *iha*. While statistical analysis has revealed many of these findings to be significant, evaluation of these strains in an infection model would further support the validity of these results.

Various cell lines, mice and *Caenorhabditis elegans* have been used as models of infection for investigating virulence of ExPEC (Bhargava *et al.* 2009; Blango and Mulvey, 2010; Johnson *et al*. 2012a; Lemaitre *et al*. 2013; Totsika *et al*. 2013). Disadvantages include their time-consuming nature, the expense to maintain these cell lines and animal models, but also the ethical requirements that need to be addressed before some of the models can be used. Final instar larvae of the greater wax moth, *Galleria mellonella,* provide a more affordable, high-throughput model that is simple to maintain, can be incubated over a large temperature range (15-37°C) and can be inoculated with exact quantities of test organism and antibiotic (Glavis-Bloom *et al*. 2012). As such, *G. mellonella* have been used in numerous studies to analyse the pathogenesis, virulence and antibiotic treatment of multiple bacterial and fungal pathogens, including *Francisella tularensis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and *Cryptococcus neoformans* (Andrejko and Mizerska-Dudka, 2012; Aperis *et al*. 2007; Hornsey *et al*. 2013; Joyce and Gahan, 2010; Mylonakis *et al*. 2005; Nicoletti *et al*. 2011). Recently *G. mellonella* was also established as a good model for assessing the comparative virulence of clinical *Klebsiella pneumoniae* strains, by analysing larval mortality (Insua *et al*. 2013; Wand *et al*. 2013). In addition, non-pathogenic bacteria demonstrated larval mortality comparable to that seen in PBS-inoculated larvae, suggesting that avirulent strains could be identified using this criteria (Aperis *et al.* 2007; Mylonakis *et al.* 2005).

The innate immune system of *G. mellonella* is comprised of the hemolymph which carries the hemocytes (equivalent to white blood cells in mammals). The hemocytes recognise invading pathogens and perform oxidative killing or encapsulation/nodulisation of these organisms and necrotic tissue (Lionakis, 2011; Ribeiro and Brehelin, 2006). Recognition and encapsulation of these pathogens result in the hemocytes producing phenoloxidase, which is released on cell destruction (Ribeiro and Brehelin, 2006). Phenoloxidase metabolises phenol precursors into quinine molecules, melanin pigments and toxic reactive oxygen and nitrogen species (Nappi and Christensen, 2005). The dark melanin pigments act as a structural immune defence by hardening and preventing pathogens

from spreading further into the larval cuticle, as well as perturbing pathogen enzymes, which would degrade the larval cuticle. This process can be monitored readily by eye because it results in a darkening of the larvae from cream to brown/black (Dubovskiy *et al*. 2013). Lactate dehydrogenase (LDH) is produced by skeletal muscle and erythrocytes during glycolysis. As a result LDH is produced during exercise (Berg *et al*. 2002), but also in tumour cells where glucose metabolism is highly upregulated (Lu *et al*. 2013). During infection apoptosis leads to release of cellular LDH in *G. mellonella*, enabling the level of LDH to act as a marker of cell damage (Wand *et al.* 2013). Therefore, measurement of melanin production and LDH concentration can be used as additional markers of pathogen virulence and tissue damage.

Until 2014 there were no published studies analysing the virulence of ExPEC in the *G. mellonella* model (Williamson *et al*. 2014). Therefore 40 ExPEC strains, encompassing both urinary and bloodstream isolates and the five major ExPEC lineages, were analysed using the *G. mellonella* model to identify patient and strain characteristics associated with larval mortality and to determine how severity of human infection might relate to larval mortality.

8.2 Methods

8.2.1 Isolates for analysis

Forty of the 154 ExPEC isolates (see 7.2.2, appendix E) analysed by the VF PCR assays were investigated in the *G. mellonella* model, as listed in appendix F. From here on these isolates will be referred to as the '*Galleria* isolates'. Patient and strain characteristics for the *Galleria* isolates are listed in table 23. VF PCR results can be found in appendix F.

Table 23 Patient and strain characteristics for the forty ExPEC isolates analysed using the *G. mellonella* infection model

Characteristic		Number of isolates	Total
Sex	Male	14	40
	Female	26	
Urinary tract infection	ABU	7	24
	UC	6	
	COMP	11	
Source of bacteraemia	GU	2	16
	GIT	4	
	CHEST	3	
	LINE	3	
	SSTI	3	
	CSF	1	
Healthcare setting	CAI	33	40
	HAI	7	
Phylogroup	A	10	40
	B1	2	
	B2	21	
	D	7	
Sequence type	131	4	40
	127	4	
	95	4	
	73	3	
	69	3	
	unknown	22	
Scores	VF	1 - 18	
	Abx	1 - 18	

NB: ABU, asymptomatic bacteruria; UC, uncomplicated cystitis; COMP, complicated cystitis/pyleonephritis; GU, genitourinary source; GIT, gastrointestinal source; CSF, cerebral spinal fluid source; CHEST, respiratory tract source; LINE, intravenous line source; SSTI, skin and soft tissue

infection source; CAI, community-associated infection; HAI, hospital-associated infection; VF, virulence factor; Abx, antibiotic resistance.

8.2.2 *G. mellonella* Infection Model

8.2.2.1 Inoculum Preparation

From an overnight culture on ISO agar (Oxoid), a 1 µl loop of culture was taken and inoculated in 3-5ml LB broth (Sigma). Suspensions were incubated overnight, at 37°C, in an Innova 4000 incubator shaker (New Brunswick Scientific; Eppendorf). Cultures were centrifuged at 4000 rpm for 10 minutes in an IEC CL40 centrifuge (Thermo Fisher Scientific) and the supernatant aspirated. Pellets were re-suspended in 3ml PBS. Centrifugation and re-suspension steps were repeated twice more. A serial 1:10 dilution was performed (100 µl pellet suspension in 900 µl PBS), with a final dilution of 1 x 10^7/ml. A 100 µl aliquot of the 1 x 10^7 suspension was spread evenly on an ISO agar plate using an L-shaped spreader (STARLAB, Hamburg, Germany). Inoculated plates were incubated overnight, at 37°C. Colony counts were performed to determine the number of colony forming units (CFU) in all serial dilutions. The most appropriate dilution was selected and diluted to achieve the required inoculum for the *G. mellonella* (Livefood UK Ltd, Rooks Bridge, Somerset, UK) model.

8.2.2.2 Inoculum Test

Prior to analysing the *E. coli* isolates in the *G. mellonella* model an inoculum test was performed to determine the most appropriate inoculum required for the virulence assay. Previous studies have used an inoculum of 10^1-10^7 CFU (Hornsey *et al.* 2013; Nicoletti *et al.* 2011); however, other studies conducted here at the Blizard Institute indicated that an inoculum of 10^4-10^6 was most appropriate for important Gram-negative bacteria (Betts *et al*. 2014; Hornsey *et al.* 2013). Therefore, for this test an inoculum of 1 x 10^4, 1 x 10^5 or 1 x 10^6 of three ExPEC strains was injected into ten larvae each to identify the inoculum that would kill 50% of *G. mellonella* after 24 hours incubation at 37°C. These three strains included the ATCC 25922 type strain (VF score = 4), one clinical isolate with a VF score of two and another clinical isolate with a VF score of seven.

8.2.2.3 Virulence Assay

Ten *G. mellonella* larvae were injected in the first left proleg (Figure 33) with 10^5 CFU/larvae, per test isolate using a 22-gauge gastight syringe (Hamilton, Bonaduz, Switzerland). The UPEC type strain CFT073 was also inoculated into *G. mellonella* larvae as a comparator for the test isolates. Ten larvae were inoculated with PBS as a stress control. Larvae were incubated aerobically at 37°C (for optimal *E. coli* growth conditions) on Grade 1 Whatman™ filter paper (Whatman plc, Maidstone, UK) in extra-deep petri dishes (Thermo Fisher Scientific) for 96 hours. Larvae were considered dead if they did not respond to touch. The virulence assay was performed on three separate occasions with three different batches of larvae. Virulence of the *Galleria* isolates (as determined by the proportion of larvae killed) was analysed according to various patient and strain characteristics to identify variables linked to larval mortality.

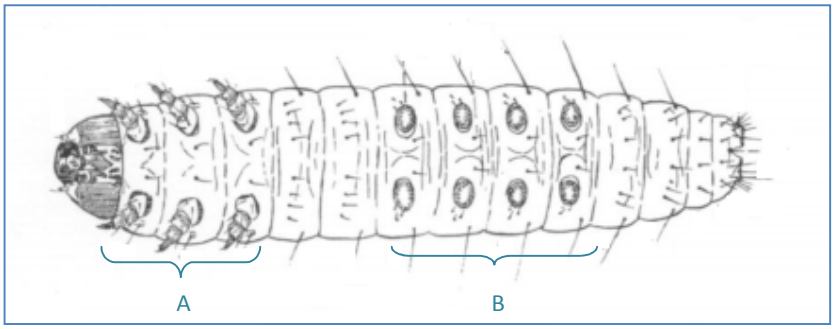


Figure 33 External morphology of the underside of *G. mellonella* **larvae.** A indicates the mesothoracic legs and B indicates the prothoracic legs, or pro-legs. This image was amended from the image by Smith (1965).

8.2.2.4 Melanisation Assay

Galleria larvae were investigated for melanin production, as a marker of the immune response, four hours post-inoculation, as performed by Wand *et al* (2013). Ten *G. mellonella* were injected per strain and incubated, as described above. After four hours the larvae were chilled on ice for 10-15

minutes and surface sterilised with 70% ethanol (Thermo Fisher Scientific). Larvae were sacrificed using a sterile disposable surgical scalpel (Swann-Morton, Sheffield, UK) and hemolymph collected into a pre-chilled eppendorf tube (STARLAB, Germany) containing 10-20 crystals of N-phenylthiourea (Alfa Aesar, Ward Hill, MA, USA) to prevent further melanisation of the hemolymph (Andrejko and Mizerska-Dudka, 2012). A 100 µl aliquot of pooled hemolymph was transferred into a 96-well polystyrene microplate (STARLAB, Germany) and the optical density (OD) measured at a wavelength of 450 nm using an ELx800 absorbance microplate reader (Biotec, Winooski, VT, USA). Uninoculated larvae were used as a background control and their OD subtracted from the OD readings of each of the *Galleria* isolates. The assay was performed in triplicate on three separate days. The highly virulent CFT073 strain was included as a comparator for the test strains.

OD was measured at 450 nm, instead of 490 nm, as described by Wand *et al* (2013). Yellow solutions (hemolymph is yellow) absorb blue light with a wavelength of 450-490 nm (Royal Society of Chemistry, 2013). Therefore, readings at 450 nm, instead of 490 nm, were considered valid and comparable.

8.2.2.5 Lactate Dehydrogenase Assay

Galleria larvae were investigated for lactate dehydrogenase (LDH) production, as a marker of cell damage, four hours post-inoculation, as performed by Wand *et al* (2013). Ten larvae were inoculated, incubated and sacrificed, as described above (see 8.2.4.4). A 50 µl aliquot of pooled hemolymph was transferred to a 96-well polystyrene microplate (STARLAB) and analysed using the CytoTox 96 non-radioactive cytotoxicity assay (Promega), according to the manufacturer's instructions. Briefly, 50 µl assay buffer was added to each well containing hemolymph. The microplate was incubated at room temperature, in the dark, for 30 minutes. After incubation 50 µl of stop solution was added to each well and the OD read using an ELx800 absorbance microplate reader (Biotec), at a wavelength of 450 nm. Uninoculated larvae were used as a background control, as described previously. The assay was performed in triplicate on three separate days. The highly virulent CFT073 strain was included as a comparator for the test strains.

8.2.3 Statistics

All statistical analyses were performed using GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, California, USA).

Association between PAIs and patient/strain characteristics were analysed by Spearman correlation, assuming data were non-parametric. Association between two variables was further analysed using Fishers exact test. A *P*-value ≤0.01 was considered significant for both tests.

Association between larvae survival and melanin/LDH production was measured by linear regression, with a *P*-value ≤0.01 considered as significant. Difference in melanin/LDH production according to patient and strain variables was measured using the unpaired t-test, with a *P*-value ≤0.05 considered significant.

Larvae survival curves have traditionally been analysed by the log rank test (Betts *et al.* 2014; Hornsey *et al.* 2013; Mylonakis *et al.* 2005). However, this test analyses the trend of survival curves, irrespective of time. After discussions with a statistician, relative risk was used to analyse larvae survival 96 hours after inoculation. This test is more appropriate as it analyses the proportion of dead larvae at specific time points and can be used to analyse different variables irrespective of sample size. The relative risk (RR) of larvae death is reported according to various strain and patient variables, with a *P*-value <0.01 considered significant. Variables that generated a RR >5 were analysed further by Spearman correlation to identify those significantly associated with mortality. A *P*-value <0.05 was considered significant.

8.3 Results

G. mellonella Infection Model

A dose of 1 x 10^5 was identified as the most appropriate inoculum for virulence discrimination in this assay (Table 24). The CFT073 control strain affected 100% larvae mortality and the PBS control affected 87% larvae survival after 96 hours. For the 40 *Galleria* isolates, larvae survival ranged from 0% - 100% (Figure 34).

Table 24 Larvae survival 24 hours post-inoculation with 1 x 10^4, 10^5 and 10^6 colony forming units

Isolate	Inoculum	No. surviving larvae after 24hrs
3	1 x 10^4	10
9		10
25922		8
3	1 x 10^5	3
9		3
25922		2
3	1 x 10^6	0
9		0
25922		0

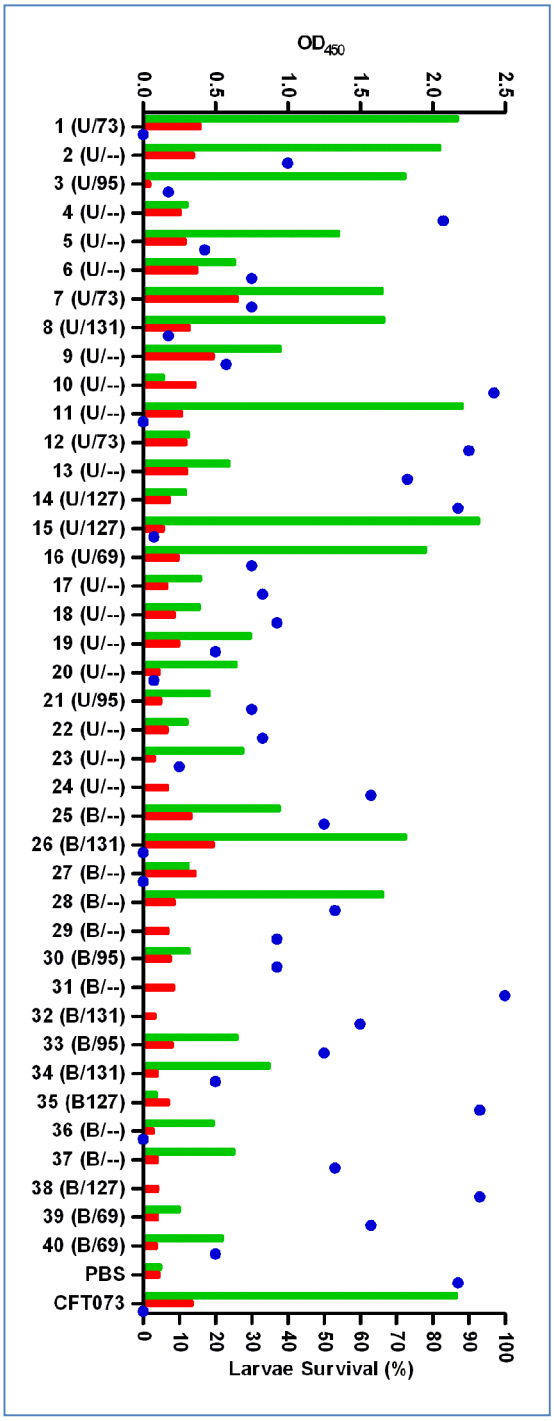


Figure 34 Percentage larvae survival, melanin production and LDH production for each of the 40 ExPEC isolates. Percentage larvae survival, 96-hours post-inoculation is plotted on the lower x-axis (blue dots). Melanin (green bars) and lactate dehydrogenase (red bars) OD readings are plotted on the upper x-axis. For each isolate the specimen type (U, urine or B, bloodstream) and sequence type (131, 127, 95, 73, 69 or unknown; -) are included with the isolate number on the y-axis.

Table 25 *G. mellonella* **mortality and relative risk ratios associated with individual patient and strain variables.** Percentage mortality is calculated from pooled data generated by the three assay replicates. RR is calculated by comparing each variable to the PBS control.

	Variable	Number of isolates	Mortality (%)	RR	*P-value*†
Infection type	U	24	65	4.84	<0.01 (RR 1.17, *P*<0.01)
	B	16	55	4.14	
Setting	CAI	33	64	4.77	<0.01 (RR 1.38, *P*<0.01)
	HAI	7	46	3.46	
Sex	Female	26	62	4.63	<0.01 (RR 1.1, *P*<0.01)
	Male	14	56	4.20	
UTI type	ABU	7	54	1.04	<0.01 (UC vs. COMP RR 1.21 & ABU vs. COMP RR 1.35, *P*<0.01)
	UC	6	61	4.54	
	COMP	11	72	5.41	
Bacteraemia source	GU	2	100	7.50	<0.01 (GU vs. GIT RR 2.4, *P*<0.01)
	GIT	4	40	3.00	
	Line	3	41	3.08	
	Chest	3	32	2.42	
	SSTI	3	76	5.67	
	CSF	1	63	4.75	
Phylogroup	A	10	72	5.40	<0.01 (A vs. B2 RR 1.25, *P*<0.01)
	B1	2	53	4.00	
	B2	21	58	4.32	
	D	7	55	4.11	
ExPEC lineage	131	4	78	5.88	<0.01 (131 vs. 95 RR 1.13, *P*>0.01) (131 vs. 73 RR 1.33, *P*<0.01) (131 vs. 69 RR 1.26, *P*>0.01)
	127	4	31	2.31	
	95	4	69	5.19	
	73	3	59	4.42	
	69	3	62	4.67	

†Direct comparison of RRs of binary variables is included in parenthesis.

Larvae mortality was significantly higher with urinary isolates, particularly COMP isolates, CAIs, isolates from female patients, isolates of phylogroup A and isolates belonging to ST131, closely followed by those of ST95. As for bloodstream isolates, a GU-source affected significantly higher mortality rates than all other foci of infections (all *P* <0.01).

Despite the high VF score of phylogroup B2 isolates, the average mortality rate was similar to isolates of phylogroup B1 and D, with phylogroup A isolates affecting the highest rate of the four phylogroups. However, analysis of the major ExPEC lineages, all of which belong to phylogroup B2 or

D, revealed ST131 isolates to affect higher rates of mortality than phylogroup A isolates (78% vs. 72%). While ST127 isolates were the least virulent, affecting just 31% mortality.

Although the CFT073 (ST73) control affected 100% mortality, the three clinical ST73 isolates affected a mortality of 100%, 70% and <7%.

Larvae mortality was also analysed according to individual VFs, PAIs and scores, as detailed in table 26.

Table 26 *G. mellonella* **mortality and relative risk ratios associated with individual virulence factors, pathogenicity islands and scores.**

	Variable	Number of isolates	Mortality (%)	RR	*P-value*† (if significant)
Fimbriae	*fimH*	30	57	4.30	<0.01
	pap	20	72	5.36	<0.01
	sfa/foc	9	63	4.69	<0.01
	bmaE	3	50	3.75	<0.01
	gafD	1	3	0.25	
Non-fimbrial adhesins	*afa/dra*	2	97	7.25	<0.01
	iha	13	57	4.29	<0.01
	hra	4	61	4.56	<0.01
	clpG	1	40	3.00	
Toxins	*cnf1*	11	65	4.84	<0.01
	cdtB	2	67	5.00	<0.01
	sat	8	58	4.31	<0.01
	vat	1	63	4.75	<0.01
	tsh	2	53	4.00	<0.01
	EAST1	2	12	0.88	
	hlyD	11	62	4.68	<0.01
	hlyF	5	73	5.45	<0.01
Siderophores	*fyuA*	30	58	4.38	<0.01
	iutA	19	65	4.91	<0.01
	iroN	15	68	5.07	<0.01
	ireA	1	70	5.25	<0.01
Capsules	*kpsII*	20	62	4.65	<0.01
	kpsIII	1	63	4.75	<0.01
	K1	6	68	5.13	<0.01
	K5	1	3	0.25	
O-antigen	O6	3	93	7.00	<0.01
	O25	4	78	5.88	<0.01

Table 26 continued

	Variable	Number of isolates	Mortality (%)	RR	*P-value*† (if significant)
Miscellaneous	*usp*	23	57	4.28	<0.01
	traT	21	64	4.82	<0.01
	ompT	4	87	6.50	<0.01
	iss	13	63	4.69	<0.01
	ibeA	4	75	5.63	<0.01
	cvaC	4	71	5.31	<0.01
	pks	16	63	4.72	<0.01
	malX	20	54	4.04	<0.01
Pathogenicity islands	CFT073 I	24	60	4.52	<0.01
	CFT073 II	18	64	4.79	<0.01
	J96 I	7	59	4.39	<0.01
	J96 II	8	73	5.50	<0.01
	536 I	5	41	3.05	<0.01
	536 II	7	81	6.11	<0.01
	536 III	1	97	7.25	<0.01
	536 IV	33	63	4.74	<0.01
High Scores	VF (>9)	18	61	4.58	<0.01
	Abx (>4)	22	62	4.66	<0.01

Note: RR is calculated by comparing each variable to the PBS control.

Of all the virulence factors, isolates possessing *afa/dra*, serogroup O6 or the PAI 536 III (encodes *sfa/foc*, *tsh* and *sat*) were >7x as likely to affect larvae death, than isolates lacking these factors. Isolates possessing *ompT* or 536 II (encodes *hlyA* and *hra*) were >6X more likely to affect larvae death and isolates with *pap*, *hlyF*, *iroN*, *ireA*, K1, serogroup O25, *ibeA*, *cvaC* or J96 II (encodes *pap*, *hlyA*, *cnf1* and *hra*) were >5x more likely to affect larvae death than isolates lacking these determinants (Bidet *et al.* 2005; Dobrindt *et al.* 2002). The remaining virulence factors were associated with a RR ≤5 and gafD, clpG, bmaE, EAST1 and K5, in particular, were associated with low RRs (<4) and low rates of mortality (≤50%).

Isolates with high VF scores or high Abx scores affected similar mortality and RRs. Notably, *malX*, which was considered a marker of virulent ExPEC isolates affected 54% mortality, with a RR <5.

Variables generating a RR >5 were considered to be associated with larvae mortality and were analysed further by Spearman correlation (Figure 35). Of these, genitourinary-source bloodstream isolates, *pap*, PAI 536 II and serogroup O6 correlated with larvae mortality (P<0.05).

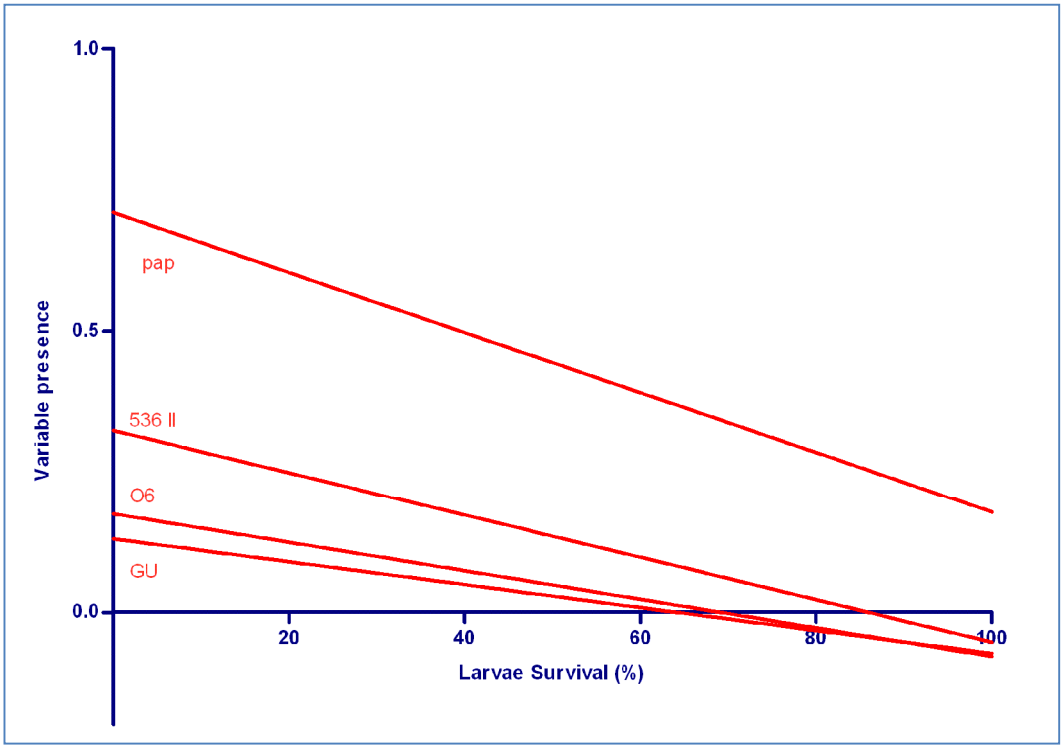


Figure 35 the ExPEC variables significantly associated with larvae mortality. Spearman correlation revealed strains from genitourinary-source bacteraemia or those with pap, 536 II or serogroup O6 to be associated with low larvae survival (P<0.05).

G. mellonella immune response

Melanin production was used as a marker of the *G. mellonella* immune response. The CFT073 control strain induced melanin production with a mean OD reading of 1.69. The *Galleria* isolates induced a mean melanin OD reading ranging from 0-2.32 (Figure 34). Linear regression revealed an inverse association between larvae survival and melanin production (P<0.0001). In addition, urinary isolates induced significantly higher melanin production than bloodstream isolates (P=0.04), with

isolates causing UC inducing the highest mean melanin production (OD 1.48), compared with COMP (OD 0.92) and ABU (OD 0.72) isolates. Of all six known sources of bacteraemia, GU isolates induced the highest mean melanin production (OD 1.06) and GIT isolates the lowest (OD 0.16).
While not significant, phylogroup B2 isolates induced the highest mean melanin production (OD 0.86), followed by phylogroup A isolates (OD 0.80), compared with phylogroup B1 (OD 0.67) and D isolates (OD 0.75). Within the five major lineages ST73 isolates induced the highest mean melanin production (OD 1.38), followed by ST131 (OD 1.09) and ST127 induced the lowest mean melanin production (OD 0.68). Isolates with a VF score >9 also induced higher mean melanin production (OD 1.14) than isolates with a low VF score (OD 0.92). Virulence factors found to significantly induce higher melanin production included P-fimbriae (OD 1.13) and OmpT (OD 1.7)(P<0.01).

LDH production was used as a marker of cell damage as a result of ExPEC infection. The CFT073 control strain induced a mean LDH OD reading of 0.34. Mean LDH OD readings ranged between 0.05-0.98 (Figure 34), but LDH production was found to be unrelated to larvae survival.
LDH production was higher for urinary isolates (OD 0.26 vs. 0.19), particularly COMP isolates (OD 0.28 vs. 0.22 – 0.27); for GU bacteraemia isolates (OD 0.42 vs. 0.09 – 0.25); and for CAIs (OD 0.23 vs. 0.21), but these findings were not significant. However, isolates from men produced significantly more LDH than isolates from women (OD 0.32 vs. 0.21, P=0.05), despite there being no difference in melanin production.
Isolates from phylogroups A and B1 induced higher LDH production than isolates from phylogroups B2 and D. As with melanin, ST73 isolates (OD 0.45) induced the highest LDH production of all the major lineages, followed by ST131 (OD 0.25), with the remaining generating an LDH OD reading of 0.14-0.15. However, these readings were not significant.

Interestingly, isolates lacking *fimH, fyuA* (P=0.03), *hlyF, hra, ibeA* (P=0.04), *iss, cvaC, usp* and *malX* induced higher LDH production than isolates possessing these virulence factors. While possession of *iutA, sat* and *ompT* resulted in higher LDH production compared with isolates that lacked these

virulence factors. Again, however, these findings were not significant. In addition, LDH production was highly similar between isolates with a high VF score (>9) and those with a low VF score (≤9).

8.4 Discussion points

The three most prevalent PAIs (CFT073 I, CFT073 II and 536 IV) encode four frequently detected ExPEC virulence factors; *pap*, *hlyA*, *fyuA* and the marker *malX*. Of these, *pap* was significantly associated with low larvae survival, but the others were not.

Urinary isolates, particularly from complicated infections, were associated with higher mortality rates than bloodstream isolates, in which a GU-source affected the highest mortality compared with all other sources. Community-associated infections were also linked to high mortality, as was phylogroup A and ST131 isolates. Notably, urinary isolates, GU-source and ST73 isolates induced a strong immune response and urinary isolates causing complicated infection, community-associated isolates and GU-source isolates were associated with high levels of cell damage.

Regarding virulence factors, *afa/dra*, *ompT*, serogroups O6 and O25 generated the highest relative risk ratios and mortality, all of which are associated with ST131, except for O6, which is frequently identified in ST73 isolates. ST73 isolates and those possessing *ompT* were also associated with high levels of cell damage. However, only *pap* and O6 were significantly correlated with mortality.

In contrast isolates possessing *fimH, fyuA*, *hlyF, hra, ibeA*, *iss, cvaC, usp* or *malX* were associated with low levels of cell damage, supporting the idea of a colonisation factor, rather than a virulence factor.

Many of the findings from these assays approached significance, but often the number of isolates analysed was too small. Subsequent studies analysing a larger sample population would confirm the significance and validity of these observations. In addition, current and future research involving whole genome sequencing of ExPEC strains could identify novel virulence factors or genetic elements associated with urosepsis, severe infection and high mortality.

The *G. mellonella* infection model provides a simple and effective method for analysing the virulence of ExPEC strains.

9. General discussion and concluding remarks

The UK ExPEC population

There have been no large-scale investigations of UK ExPEC strains for several years (Lau *et al.* 2008b) and recent studies were restricted geographically (Gibreel *et al.* 2012) and by infection type (Horner *et al.* 2014). This study improved on earlier investigations by comparing >1000 urinary and bloodstream isolates, collected from around the UK and Republic of Ireland, between January 2011 and March 2012. As expected the patient populations were similar for these two infection types and the distribution of phylogenetic groups was consistent with previous studies (Banerjee *et al.* 2013a; Bukh *et al.* 2009). However, there were some variations in age, sex, clonal composition and serogroup depending on whether the isolate caused a UTI or bacteraemia.

There was no change, from previous studies, in the patient demographics for urinary strains (Bean *et al.* 2008; Foxman, 2002; Kahlmeter, 2003; Linhares *et al.* 2013), with the majority (89%) causing CAIs, isolated from female patients (84%) aged 21-50 years (55%), with an average age of 40 years. This is unsurprising given the common, tedious nature of UTIs and empirical management, which lends to this infection being diagnosed more frequently in community clinics, GP practices or out-patient departments (Bean *et al.* 2008; Denes *et al.* 2012; Leydon *et al.* 2009).

In contrast to female patients, the average age of men presenting with a UTI was higher (62 years) and the frequency of UTIs in men increased with age, likely a result of the increased risk of prostatitis and instrumentation (e.g. catheter) in this age group (Cornia *et al.* 2006; Etienne *et al*. 2008; Lipsky, 1999). Otherwise the demographics of men presenting with UTI was similar to recent UK and European studies (Bean *et al.* 2008; den Heijer *et al.* 2013; Koeijers *et al.* 2010; Marschall *et al*. 2013). The most recent UK study of UTI in children was conducted in Staffordshire (Chakupurakal *et al.* 2010), reporting approximately 72 paediatric UTIs each year, predominantly in girls. A study in London found 10% (n=1227) of urinary isolates were from patients <16 years (Bean *et al.* 2008),

although the distribution according to patient sex was unknown. Similar to these studies, 8% of urinary strains here were isolated from patients aged <18 years, with the majority from girls. The lack of sufficient clinical information often made it difficult to determine the UTI type caused by the isolates. Together with the criteria for collecting urinary strains, some bias was introduced during isolate collection. Lack of good clinical information supplied with study isolates led to all asymptomatic bacteruria isolates, except one, being collected from asymptomatic pregnant women screened for UTI at their initial antenatal visit. Uncomplicated infections affect both men and women (Bean *et al.* 2008; Kahlmeter, 2003), but the controversy surrounding the classification of UTI in men (it is assumed that men have an anatomical or structural abnormality resulting in infection) led to isolates from male patients being classified as complicated (SIGN, 2006; Lipsky, 1989). Therefore, all uncomplicated isolates, by default, were from female patients only. Further analysis of the clinical information from male patients identified 32/82 (39%) with symptoms of cystitis only and the majority aged 50–78 years (56%), suggesting that approximately 40% of men diagnosed with a UTI have an uncomplicated infection. However, without a complete patient history it cannot be confirmed that these male patients lacked an abnormality, co-morbidity or other complication that would alter their UTI diagnosis. In patients <18 years old complicating factors included urosepsis or recurrent infection, but in adults additional factors included immunocompromising conditions, surgery and instrumentation, suggesting that environmental and host factors are more influential in adult UTIs, but also more common in this age group. Despite the overall isolate collection bias, especially towards uncomplicated cystitis isolates, these strains were typical of the reported patient population (Den Heijer *et al.* 2010; Foxman *et al*. 2000; Kahlmeter, 2003).

ExPEC causing bacteraemia also reflected recent studies, being isolated almost equally from male and female patients, predominantly aged >60 years and prevalence of bacteraemia increased with patient age (Alhashash *et al*. 2013; Health Protection Agency, 2011; Schlackow *et al.* 2012; Wester *et al*. 2013; Williamson *et al*. 2013b). Similar to urinary isolates the majority were CAIs (67%). Despite the collection process for the local bacteraemia strains being skewed toward urinary-source strains,

the non-biased BSAC (Reynolds *et al.* 2008) strains still revealed the genitourinary tract to be the most common source of infection (39%), as identified in multiple UK and European studies (Bukh *et al* .2009; Horner *et al.* 2014; Laupland *et al.* 2008; Wester *et al.* 2013).

Interestingly, respiratory-source strains were more common in male patients than female patients. It is possible that this is due to aspiration pneumonia (Wei *et al*. 2013) and community-acquired pneumonia (Millett *et al*. 2013; van Gageldonk-Lafeber *et al*. 2013) occurring more frequently in men or that the respiratory tract infection was reported as the most common HAI in the UK (Health Protection Agency, 2012c). However, this is unclear here without further investigation of patient histories. Genitourinary, gastrointestinal and respiratory-source isolates were predominantly isolated from patients aged 61–90 years, demonstrating the high number of patients presenting with bacteraemia between these ages and the increased likelihood of co-morbidity at this age (Wester *et al.* 2013).

There has been no major change in the phylogroups dominating ExPEC strains in the UK (Cooke *et al.* 2010). Phylogroups B2 and D were most prevalent in all infection types, with a higher proportion of these two groups in bloodstream isolates than urinary isolates (89% versus 82%). The success of these groups was affirmed by the prevalence of the five major lineages ST131, ST95, ST127, ST73 (all B2) and ST69 (D), which have been reported as frequent causes of extra-intestinal infections in the UK and around the world over the last few years (Abraham *et al*. 2012; Banerjee *et al.* 2013a; Horner *et al.* 2014; Johnson *et al.* 2011; Skjot-Rasmussen *et al.* 2012b). These lineages were identified in a similar proportion of urinary (40%) and bloodstream (44%) isolates, which supports the notion of urinary strains spreading to the bloodstream and causing infection. Occasionally (2%) strains of these sequence types were detected in other phylogroups (e.g. ST131/D, ST127/A, ST95/D, ST69/B1), as identified previously (Johnson *et al.* 2008b; Molina-Lopez *et al.* 2011). These rare strains

likely display traits of these sequence types, but differ enough to be distinguished, such as EAEC strains (ST69/A) with clonal group A (CgA) characteristics that occasionally cause UTIs, but were absent in this study (Olesen *et al.* 2012; Wallace-Gadsden *et al.* 2007).

Regarding infection type, ST95 and ST127 were uniformly distributed among bloodstream and urinary isolates, suggesting universal adaption to multiple anatomical sites or lack of site-specific virulence factors. ST95 strains are known to cause avian and human extra-intestinal infections, with virulence factors (*ibeA*) enabling invasion of the brain endothelium (Homeier *et al.* 2010). This confirms the former conclusion, that these sequence types possess universal virulence factors, as ST95 strains appear specifically adapted to brain tissue, but also possess some of the adhesins and toxins that allow colonisation of the urinary tract or bloodstream (Mora *et al.* 2009). In addition, ST95 was responsible for the only CSF-source bloodstream isolate in this study, also supporting these conclusions. ST127 isolates are reported to possess a high number of virulence factors (Gibreel *et al.* 2012), but are more closely linked to infections in dogs than humans (Johnson *et a.l* 2008b), suggesting this even distribution among extra-intestinal infections is due to low adaptation to human anatomical sites, which is overcome by the virulence factors present .

In contrast ST131 and ST73 were more frequently detected in bloodstream isolates. This mirrors a recent study from Nottingham, UK (Alhashash *et al.* 2013). However, another study from the same region reported ST73 as the predominant clone causing bacteraemia (Horner *et al.* 2014). The former study investigated isolates from HAIs only, while the latter study included both CAIs and HAIs, demonstrating that ST131 is more successful in the hospital setting, while ST73 is better adapted for success in the community. The success of ST73 in the community is confirmed by another study of UPEC in Manchester, UK, which identified this sequence type as the most prevalent in urinary isolates also (Gibreel *et al.* 2012).

Unsurprisingly ST69 was more frequently detected in urinary isolates than bloodstream isolates. As a clone originally identified in college women with uncomplicated cystitis (Manges *et al.* 2001), studies

from around the world indicate that ST69 has remained a significant cause of UTIs (Banerjee *et al.* 2013a; Dias *et al.* 2009; Johnson *et al.* 2011). In conjunction with ST95 these two clones were responsible for the majority of ABU, UC and COMP isolates, reaffirming the adaption of these lineages to the urinary tract.

The geographical spread and distribution of the major lineages around the UK was surprising. As a well characterised multi-drug resistant clone, it was assumed that ST131 would be the most common lineage in bloodstream isolates in each region and country of the UK. However, this was only observed for London, the South-West of England and Wales. The office of National Statistics (http://www.neighbourhood.statistics.gov.uk/) reports that Wales has a higher proportion of people aged >65 years (19.1%) compared with England (16.9%). In addition, of all nine regions of England, the South-West also has the highest proportion of people aged >65 years (20.3% vs. 11.3-18.2%). As this aged population is the most frequently affected by *E. coli* bacteraemia, it is possible that ST131 is prevalent in these regions due to the larger susceptible population. ECDC data also reports that more quinolones are prescribed in Wales than in England, which may be driving the success of ST131 in this country also (European Centre for Disease Prevention and Control, 2013a).
Also of note was the lack of ST69 in Welsh and Scottish isolates. However, as a predominantly uropathogenic clone it is possible that this lineage is not as well adapted for the bloodstream and limited use of trimethoprim in treating UTIs in these countries may prevent this clone from becoming established in these communities and spreading to the bloodstream (Johnson *et al.* 2011).

Defying the general phylogenetic consensus, an unusual phylogroup PCR profile (*gadA*$^{+}$, *chuA*$^{-}$, *yjaA*$^{+}$, TspE4.C2^{+}) was identified in this study. Though uncommon, two other studies have reported this unique profile (Mendonca *et al.* 2011; Skjot-Rasmussen *et al.* 2013). Contrary to these previous reports and the hypothesis that this phylotype was related to ST127, the UK isolate belonged to a novel sequence type, ST3679, and serogroup O18ab. In addition, PFGE analysis (Figure 15) revealed just 40% DNA relatedness between this strain, the four major phylogroups and five major lineages of

this study, indicating a non-ST127 origin. It is possible that this phylotype represents an atypical ExPEC strain or is not ExPEC in origin, as all *E. coli* strains and pathotypes share 40% of their core genome (Welch *et al.* 2002).

Serotyping can facilitate identification of new ExPEC clones, but this method is usually limited to reference facilities and characterisation of small research collections (Blanco *et al.* 1997; Loos *et al*. 2012), with serotyping of ExPEC strains typically focused on internationally recognised clones (Blanco *et al.* 2011; Johnson *et al.* 2006). This potentially reduces detection of atypical clonal isolates and full understanding of the ExPEC population. A benefit of such a large isolate collection included the detection of twenty-one novel ExPEC serogroups in this study, as well as 14 serogroups previously unlinked to the five major clones. Conversely, 14 well-known ExPEC serogroups were frequently (61%) identified in urinary and bloodstream isolates and dominated the five major clones, such as ST95-O2, ST73-O6 and ST131-O25 (Ananias and Yano, 2008; Blanco *et al.* 2011; Emanghoraishi *et al*. 2011; Johnson *et al.* 2008b; Kudinha *et al.* 2013b; Lau *et al.* 2008b; Manges *et al.* 2008; Melzer *et al.* 2008; Skjot-Rasmussen *et al.* 2012a). Serogroup O6 in particular has been identified as an important colonisation factor of the urinary tract, with an additional role in serum resistance (Sarkar *et al.* 2014). Therefore, it is possible that the other common O-antigens have similar functions. Together these results demonstrate the serogroup diversity within ExPEC and confirm the clonal success of many ExPEC strains. Other important serogroups of note included O7 and O75 (Johnson *et al.* 2006; Platell *et al.* 2011), which were not identified in the five major clones, but were identified in urosepsis isolates of ST62 and ST404, both of which are recognised ExPEC clones (Alhashash *et al.* 2013; Berman *et al*. 2014). This observation reaffirms the role of serotyping in identifying clinically significant clones when molecular methods, such as MLST, may be unavailable.

Analysis of the paired urosepsis isolates demonstrated the success of the five major ExPEC lineages and identified other clones circulating within the UK; ST354, ST404, ST405, ST62, ST14 and ST617 (Alhashash *et al.* 2014; Horner *et al.* 2014; Matsumura *et al.* 2012; Mora *et al*. 2011; Novais *et al*.

2013; Smet *et al.* 2010). ST1405 was also detected, but little was discovered about this sequence type except its association with asymptomatic bacteruria (http://mlst.ucc.ie/mlst accessed 26th March 2014), highlighting the importance of understanding ExPEC as a whole, as it cannot be guaranteed that new sequence types will not become prevalent in the future. Generally urosepsis was caused by one strain, however, one patient in this study was simultaneously infected with two different strains (ST14/B2 and ST354/D). It is possible that this phenomenon occurs more frequently in *E. coli* infections than reports suggest (Clermont *et al*. 2013b; Leflon-Guibout *et al*. 2002), especially with the tendency in diagnostic microbiology to select one colony forming unit for identification and analysis if all colonies appear the same. In these cases one strain may benefit from the virulence repertoire expressed by the other strain, despite being avirulent or antibiotic susceptible. This type of symbiotic relationship is particularly evident in biofilms and intracellular communities (Blango and Mulvey, 2010). In the future it would be interesting to analyse multiple *E. coli* colonies from urosepsis patients to determine whether there are any strains or clones that function together.

In summary, this study has confirmed the findings of previous studies by observing no major change in the UK patient population susceptible to *E. coli* bacteraemia and UTIs (Bean *et al.* 2008; Health Protection Agency, 2011). Although, this study does provide the supporting information necessary for classification of UTIs in men to be more defined. In contrast the ExPEC population in the UK is highly diverse, as demonstrated by phylogroup, serogroup, sequence type and PFGE, despite the dominance of the five major ExPEC clones. It is also apparent that the clonal composition of UK isolates differs from other international studies, demonstrating the importance of local routine monitoring of *E. coli*, not just in bacteraemia, but also UTIs, the most common bacterial infection in the community. With the variation in prescribing practices around the world, it is important to understand how this diversity affects antibiotic resistance and to determine whether standard empirical therapy remains valid.

Antibiotic resistance in *Escherichia coli* causing urinary tract infections and bacteraemia, in East London and across the UK

Comparison of antibiotic resistance data from this study with published studies reveals resistance rates to have increased in *E. coli* (Bean *et al.* 2008; Health Protection Agency, 2011; Kahlmeter and Poulsen, 2012; Schito *et al.* 2009), but without any recent or large-scale studies in the UK, it is difficult to understand the changes in urinary isolates on a national level.

The Infectious Diseases Society of America (IDSA) has suggested that antibiotics with resistance rates ≥20% should no longer be prescribed, so as to prevent selection of resistant strains and to minimise the risk of treatment failure (Gupta *et al.* 2011). If this recommendation was adopted, ampicillin, amoxicillin-clavulanate (augmentin), trimethoprim and trimethoprim-sulfamethoxazole should no longer be prescribed for the eradication of UTIs and bacteraemia caused by ExPEC in the UK. Within urinary isolates this high prevalence of resistance was multi-factorial, attributable in part to two ExPEC clones, clonal group A/ST69 (58% trimethoprim-resistant) and ST131 (66% ciprofloxacin-resistant, 62% trimethoprim-resistant), plus the recommended use of trimethoprim-sulfamethoxazole (uncomplicated cystitis) and ciprofloxacin (complicated cystitis/pyelonephritis) which now appear to be inappropriate given these high resistance rates and associated clonal resistance (Gupta *et al.* 2011; Johnson *et al.* 2011; Johnson *et al.* 2010; SIGN, 2006; Barts and the London Trust Antimicrobial Review Group, 2011).

Fortunately nitrofurantoin resistance has remained low (1%), possibly due to the negative impact on bacterial fitness and lack of plasmid-mediated resistance genes targeting this antibiotic (Sandegren *et al*. 2008; Whiteway *et al.* 1998). In addition, nitrofurantoin is highly effective against ESBL-producing isolates (Fournier *et al*. 2013; Livermore *et al*. 2011; Sahm *et al.* 2001), making this antibiotic a viable option for first-line treatment of lower UTIs. Despite 29% of urinary isolates demonstrating amoxicillin-clavulanate resistance, this antibiotic combination could still be used for lower UTIs, including those caused by ESBL-producing isolates and infections in pregnant women. Clavulanate achieves a high concentration in the bladder, enabling eradication of *E. coli* with an

amoxicillin/clavulanate MIC of 64/32 mg/L (Alou *et al.* 2006). EUCAST has now adjusted its clinical breakpoints accordingly (The European Committee on Antimicrobial Susceptibility Testing, 2014) and repeat analysis of the UC study isolates resulted in a shift in resistance rates from 29% to 0%, while ABU resistance rates changed from 28% to 0% also. In children, Chakupurakal *et al* (2010) recommended cefalexin as first-line UTI treatment due to the ampicillin and trimethoprim resistance in this population. Results from this study support this suggestion. For multi-drug resistant isolates that express ESBLs, such as ST131 strains, a carbapenem would be effective (Fournier *et al.* 2013). Especially as carbapenem resistance was rarely reported in UK *E. coli* during this study, despite the circulation of carbapenemase enzymes within this species (Hopkins *et al.* 2014; Woodford *et al.* 2013). However, recent data from Public Health England (Personal communication with Prof. Neil Woodford, September 2014) suggests this low level carbapenem resistance won't last and this proposed treatment of multi-drug resistant isolates will need to be revisited in the future.

Antibiotic resistance in bloodstream isolates was higher than urinary isolates, increasing 2-8-fold in the presence of ESBLs, but comparable to recent UK and European bacteraemia reports (European Centre for Disease Prevention and Control, 2012; European Centre for Disease Prevention and Control, 2013b; Horner *et al.* 2014). However, resistance to the cephalosporins (10%), ciprofloxacin (21%) and aminoglycosides (11 – 26%) was often higher in these UK strains, likely as a result of the higher proportion of ST131 strains causing bacteraemia. A recent UK study linked rising antibiotic resistance to the increasing number of *E. coli* bacteraemias (Schlackow *et al.* 2012). Therefore, it is important that regular monitoring of prescribing practices and infection control policies is performed alongside antibiotic resistance surveillance, to prevent further increases in antibiotic resistance and empirical therapy becoming redundant. Until this happens temocillin and carbapenems are currently recommended as alternative antibiotic options for eradicating multi-drug resistant ExPEC strains causing bacteraemia (Fournier *et al.* 2013; Livermore *et al.* 2011). Another factor in treating bacteraemia is targeting the source of infection, to prevent repeat infection of the bloodstream and to ensure that the prescribed antibiotics will reach effective concentrations at both

sites. The genitourinary tract was the most common source of bacteraemia in this study and urosepsis can be prevented by improving UTI management. One Danish study indicated that higher resistance in their community-associated urosepsis isolates was due to high prescribing in the community (Skjot-Rasmussen *et al.* 2012a). However, in this study hospital-associated bloodstream isolates were more resistant than the community-associated isolates, suggesting this principle is not applicable here, but revision of prescribing practices may reduce the number of *E. coli* isolates that go on to cause urosepsis and reduce antibiotic resistance (Livermore *et al*. 2013).

The BSAC bacteraemia isolates revealed regional variations in antibiotic resistance that could not be completely explained by antibiotic prescription data (Health Protection Agency, 2008). The recent English surveillance programme for antimicrobial utilisation and resistance report from Public Health England reveals a north/south divide in antibiotic consumption in England, with a higher number of prescriptions in the North (Public Health England, 2014a). However, the lack of national guidelines for empirical treatment of *E. coli* bacteraemia lends to clinicians prescribing antibiotics based on their experience, medical specialty and local hospital guidelines, which can vary from clinician to clinician (McNulty *et al.* 2003). Although in London and the South-West of England, the high rates of cephalosporin and fluoroquinolone resistance are likely to be a direct result of the ST131 prevalence in these regions. On a national level, possible reasons for high rates of resistance to cephalosporins, aminoglycosides (both Wales), cefoxitin (Republic of Ireland) and chloramphenicol (Scotland) may include the high prevalence of ST131 (Wales), circulation of AmpC beta-lactamases (Ireland), co-circulation of plasmid-mediated resistance determinants in animal strains (all), the use of chloramphenicol-medicated feed for farm animals (Scotland) and, for the South-West and Yorkshire and Humber regions, high prescriptions of ciprofloxacin and trimethoprim, respectively. Although further investigation would be required to confirm these hypotheses (Frye and Jackson, 2013; Public Health England, 2014a; Woodford *et al*. 2007).

Interestingly, isolates from male patients demonstrated higher rates of resistance compared with female patients, with particular emphasis on ciprofloxacin resistance. Published studies have reported similar observations (Bean *et al.* 2008; Linhares *et al.* 2013), while others contradict this finding, likely as a result of differing prescription practices (Den Heijer *et al*. 2012; SIGN, 2006; Wagenlehner *et al*. 2013b). The logical explanation for this finding lies in the infection types and treatment options for male patients. Prostatitis and bacteraemia occur more frequently in male patients >50 years (Health Protection Agency, 2011; Lipsky *et al.* 2010), with the former often leading to the latter in 46% cases (Etienne *et al.* 2008). In the UK ciprofloxacin is recommended for treating prostatitis (SIGN, 2006) leading to selection and expansion of ciprofloxacin-resistant clones, such as ST131 (Wagenlehner *et al.* 2013b; Williamson *et al.* 2013a). In addition, ciprofloxacin is not the first-line treatment for UTI in female patients and they are not prescribed fluoroquinolones as frequently as male patients (Den Heijer *et al.* 2012; Den Heijer *et al.* 2010; Goettsch *et al.* 2000; Linhares *et al.* 2013). Furthermore, in hospitalised patients ciprofloxacin is avoided to prevent the development of *Clostridium difficile*-associated diarrhoea and prescriptions in this setting have declined over the last few years (Dancer *et al*. 2013; Public Health England, 2014a).

Much of the resistance observed within UK ExPEC isolates was associated with multi-drug resistant clones, such as ST131 and ST69, which belong to phylogroups B2 and D, respectively. However, strains of phylogroup A and B1 were cumulatively more resistant than these virulent phylogroups. This is likely a result of the low numbers of phylogroup A and B1 strains, plus the number of ESBLs (n=13) expressed by these isolates, generating disproportionately high resistance rates.

ESBL prevalence remained low within the urinary (0.6%) and bloodstream (8.8%) isolates (Health Protection Agency, 2011; Kahlmeter and Poulsen, 2012) and, similar to previous studies, CTX-M-15 was the most frequently detected enzyme (Woodford *et al.* 2004; Horner *et al.* 2014; Schito *et al.* 2009), often in conjunction with OXA-1 and/or TEM-1 (Huang *et al.* 2012; Karisik *et al.* 2006; Wang *et al.* 2013). It should be noted that approximately 35-88.2% of all *E. coli* isolates possess the TEM-1

gene, conferring resistance to ampicillin +/- amoxicillin-clavulanate, but in this study only cephalosporin-resistant isolates were investigated for this gene (Olesen *et al*. 2004; Perez-Moreno *et al.* 2004; Thomson and Amyes, 1993). However, the frequency at which *aac(6')-Ib-cr* was detected in ESBL-producing strains has increased to 59%, compared with the 32-51% reported previously in UK isolates (Amin and Wareham, 2009; Wu *et al.* 2013) and was often identified with the widespread aminoglycoside modifying enzyme *aac(3')-IIa* (Jones *et al*. 2008; Lindemann *et al*. 2012; Xiao and Hu, 2012). Notably, *aac(6')-Ib-cr* was not found in phylogroup D strains, along with ESBLs, indicating that the prevalence of these resistance determinants was directly attributable to ST131 strains and the multi-drug resistance plasmids they possess (Calhau *et al.* 2013; Chmielarczyk *et al.* 2013; Olesen *et al.* 2013). In addition, isolates lacked 16S rRNA methyltransferases, which confer pan-resistance to aminoglycosides, confirming that this pan-resistance was a result of multiple AME possession and/or loss of an aminoglycoside transport system or change in membrane potential. It is these additional mechanisms, along with efflux pumps, change in membrane permeability and mutations in the 16S rRNA that likely confer aminoglycoside non-susceptibility in the AME-negative/aminoglycoside-resistant isolates (Ramirez and Tolmasky, 2010). Few *E. coli* isolates are reported to possess *qnrS*, *qepA* or *oqxAB* (Jones *et al.* 2008; Kirchner *et al.* 2011), as confirmed by the results of this study. While these genes may benefit other Enterobacteriaceae, such as *Klebsiella* species (Deepak *et al.* 2009; Younes *et al.* 2011), they are likely redundant in *E. coli* that possess mutations in the DNA gyrase gene and/or *aac(6')-Ib-cr* , as these are sufficient to raise the MICs for survival in the presence of fluoroquinolones (Johnson *et al.* 2013; Kim *et al.* 2013).

Despite this concerning increase in resistance to empirical therapy, a significant proportion of urinary isolates displayed susceptibility profiles that should have resulted in effective eradication of the infection according to current guidelines. In addition, several antibiotic candidates have been identified (e.g. nitrofurantoin, fosfomycin) that could be routinely employed for empirical treatment of multi-drug resistant UTI and bloodstream isolates. Also concerning was the discrepancies between the antibiotic susceptibility testing methods evaluated as part of this study (disc diffusion,

MicroScan and agar dilution), suggesting that antibiotic resistance may be under-reported. However, MICs here are unlikely to be 100% representative of the community and nosocomial ExPEC population, given the lack of good quality clinical information accompanying patient specimens and the typical reasons for referral of urinary isolates (e.g. treatment failure, recurrent infection, complication). Much of the antibiotic resistance seen in these UK isolates could be attributed to major ExPEC clones, especially ST131, which was likely a major contributor. To ensure that this increasing tide in resistance can be controlled, or even reduced, therapeutic strategies targeting the multi-drug resistant clones need to be developed, such as rapid diagnostic tests, as well as changes in prescribing practices which select for these strains.

The UK ST131-O25b clone

Many studies of antibiotic resistance in ExPEC have focused on the ST131 clone (Kudinha *et al.* 2013a; Liss *et al.* 2013; Matsumura *et al.* 2013). This multi-drug resistant clone emerged in the early 2000s, becoming a significant cause of UTIs and bacteraemia, while providing a challenge to empirical antibiotic therapy (Nicolas-Chanoine *et al.* 2008; Peirano and Pitout, 2010). However, in the last few years studies have concentrated on the H30 sub-clade, identified by sequencing of the *fimH* gene, and its associated fluoroquinolone resistance and ESBL expression (Banerjee *et al.* 2013b; Peirano and Pitout, 2014; Price *et al.* 2013). Therefore, it was important to identify and characterise ST131 strains in the UK and establish how they related to previous studies and this new sub-clade.

Multiple PCRs, SNP analyses and sequencing methods have been employed to identify ST131 strains (Blanc *et al.* 2014; Dahbi *et al.* 2013; Olesen *et al.* 2013). In this study a sequence type PCR (Doumith *et al.* 2014) was evaluated as a simple method to identify strains belonging to this clone, which was in turn validated using the published *pabB* SNP PCR (Dhanji *et al.* 2010). Serogrouping, *fimH* sequencing and antibiotic resistance further differentiated UK ST131 strains into three sub-clones based on the presence (clonal) or absence (non-clonal) of both *pabB* SNPs, then by the presence or absence of ESBLs (Table 27).

Sub-clone	ST131	SNPs	ESBL	FQ-R
1	X	X	X	X
2	X	X	X	
3	X			

Table 27 Algorithm for identification of UK ExPEC strains belonging to the three ST131 sub-clones

ST131 as determined by sequence type PCR, presence of SNPs determined by real-time PCR, ESBLs identified by PCR and DNA sequencing and fluoroquinolone resistance (FQ-R).

ST131 was the most frequently detected ExPEC clone in this study and dominated bloodstream isolates (19%), which emulates recent UK and international studies (Horner *et al.* 2014; Johnson *et al.* 2010; Kang *et al.* 2013; Kudinha *et al.* 2013a). However, ST131 (8%) was not the dominant clone in urinary isolates, being surpassed by ST95 (11%) and ST69 (10%), although urinary ST131 strains were mostly isolated from complicated cystitis/pyelonephritis infections. This trend in infection type can be explained by the antibiotic resistance of ST131. UK clonal ST131 strains demonstrated 62% and 77% resistance to trimethoprim and ciprofloxacin, respectively, rendering empirical UTI therapy with these antibiotics mostly ineffective (Gupta *et al.* 2011; SIGN, 2006). This ability to overcome first-line therapy can result in ascension to the kidneys and initiation of a more severe infection (e.g. bacteraemia), which is facilitated by the possession of virulence factors (Rogers *et al.* 2011). The proportion of ciprofloxacin resistance observed here was much higher than recent studies from Europe and North America reporting 26%-40% (Blanco *et al.* 2011; Chmielarczyk *et al.* 2013; Colpan *et al.* 2013), although some studies have reported 93%-100% resistance (Dahbi *et al.* 2013; Olesen *et al.* 2013), but these focused on the ESBL-producing variants of the clone.

The prevalence of CTX-M genes in UK ST131 isolates was also higher (31% vs. 10%-12%) than similar studies describing ST131 strains (Blanco *et al.* 2011; Platell *et al.* 2011) and 92% of CTX-M-positive isolates belonged to the newly designated H30-R sub-clone (Price *et al.* 2013), with the R representing ciprofloxacin resistance. The dominance of CTX-M-15 in these UK strains, reflects the successful circulation of this ESBL in the UK (Woodford *et al.* 2004; Gibreel *et al.* 2012), but CTX-M-

14 and CTX-M-27 genes were also detected; a feature reported in Danish ST131 isolates also (Olesen *et al.* 2013). CTX-M-27 is derived from CTX-M-14 (alanine->glycine substitution at position 725), sharing >98% amino acid identity, and is one of few CTX-M genes with a D240G (aspartic acid->glycine) substitution along with CTX-M-15; together demonstrating the conserved nature of ESBL possession in *E. coli* strains (Bonnet *et al.* 2003; Bonnet, 2004). These CTX-M genes, except two, were encoded on IncF plasmids of FIA or FIA-FIB allelic combinations, many of which have not been described in this clone (http://pubmlst.org/plasmid/). The two exceptional strains carried IncU plasmids and were thought to encode CTX-M-15, the first description of this plasmid type in ST131 strains from the UK, however these plasmids were recently described in ST131 strains from Japan (Matsumura *et al.* 2013). Associated with *qnrS* genes and identified in multi-drug resistant *Aeromonas* species (Cattoir *et al.* 2008; Picao *et al.* 2008), the IncU plasmids identified here also encoded TEM-1, OXA-1 and *aac(6')-Ib-cr*, typical of resistance plasmids in ST131 strains (Dahbi *et al.* 2013; Matsumura *et al.* 2012).

Long established as an identifying feature of the ST131 clone, the O25b antigen was detected in 100% of UK H30-R isolates, as well as in ciprofloxacin-susceptible and non-H30 strains. In contrast to previous studies, (Dahbi *et al.* 2013; Kudinha *et al.* 2013a; Matsumura *et al.* 2013; Olesen *et al.* 2013) O16 was identified in non-clonal isolates only, all of which were ciprofloxacin-susceptible and CTX-M-negative. Importantly, three serogroups novel to ST131 strains within the UK and around the world, were identified (O19a, O136 and O153). These serogroups have been reported in *E. coli* (Ananias and Yano, 2008; Croxen *et al.* 2013; Staaf *et al.* 1999) and, like the IncU plasmids, were likely acquired as a result of the plastic nature of the *E. coli* genome, by horizontal gene transfer of plasmids or high-frequency recombination events of chromosomal DNA fragments (Wiesner *et al*. 2013; Brouwer *et al*. 2013; Chewapreecha *et al.* 2014).

Further evidence of the expansion of this sub-clone lies in analysis of the *fimH* alleles. Historically the H22 allele dominated ciprofloxacin-susceptible ST131 strains (Johnson *et al.* 2013). However, H30 was just as prevalent in ciprofloxacin-susceptible strains here, suggesting that the H30-R sub-clone

may soon encompass all clinically isolated ST131 strains, especially if the use of fluoroquinolones is not restricted further.

Despite the expansion and spread of H30-R ST131 isolates PFGE revealed great heterogeneity, with just 40% DNA relatedness. This differs to previous reports of ST131 strains being highly related (>60%), although these reports were limited geographically (Colpan *et al.* 2013; Gibreel *et al.* 2012; Johnson *et al.* 2009a; Lau *et al.* 2008a; Liss *et al.* 2013), whereas these UK isolates represent the national diversity of ST131. In addition, there was no clustering of traits such as CTX-M genes, serogroups and *fimH* alleles, as seen in previous studies (Lau *et al.* 2008a; Price *et al.* 2013). Together these results revealed how ST131 strains have evolved significantly since the first report of this emerging clone (Nicolas-Chanoine *et al.* 2008) and vary from country to country, despite retaining the molecular traits specifically attributed to this clone. These results also suggest that PFGE should no longer be used to compare such geographically-widespread ST131 collections, given this heterogeneity and the over-complicated characterisation of this clone. Peirano and Pitout (2010) suggested that only the sequence type, ST131, characterized this clone, with sub-clades comprising ciprofloxacin-resistant and/or CTX-M-15 strains. Although, this was before the discovery of ST131-O16 strains and identification of the H30 *fimH* allele (Johnson *et al.* 2013; Matsumura *et al.* 2012). Despite these novel attributes of ST131 strains, results from this study support Peirano's statement (see Table 26 above). However, it is important that the algorithm is re-visited regularly as new traits are identified within this clone.

CTX-M production was always accompanied by ciprofloxacin resistance in this clone (Banerjee and Johnson, 2014), but CTX-M-negative ST131 isolates may also be ciprofloxacin-resistant. Therefore, antibiotic susceptibility needs to be determined before antibiotics can be prescribed rationally to eradicate clonal isolates and prevent treatment failure, except for bacteraemic patients where rapid treatment is required. Development of a point-of-care test that identifies this clone, in a similar manner to urine dipsticks or pregnancy tests, would enable rapid identification of ciprofloxacin-resistant ST131 strains in patients presenting to their GP with a UTI and, potentially, patients with

bacteraemia presenting to accident and emergency departments. It would also facilitate appropriate antibiotic therapy, which in turn would reduce treatment failure and the need to re-visit the GP, as well as prevent the infection ascending to the kidneys and causing bacteraemia. A test that targeted ST131, cefotaxime resistance (as a marker of ESBL) and ciprofloxacin resistance would enable identification of all clonal isolates and distinction of the sub-clades. These patients could then be treated with appropriate antibiotics such as nitrofurantoin (lower UTI) or a carbapenem (bacteraemia or upper UTI) (Gupta *et al.* 2007; Takahashi *et al.* 2009).

Further analysis of this clone, such as examination of the virulence determinants possessed by ST131 strains and the three sub-clones, may help explain the diversity of this clone, whether the 'limited profile' in CTX-M-positive strains (i.e. O25b, ciprofloxacin resistant, H30) includes their virulence repertoire and if these determinants explain the invasiveness of the ST131 clone.

ExPEC virulence determinants

In this study an *E. coli* genotyping microarray was used to simultaneously investigate the antibiotic resistance determinants, serotypes and virulence repertoire of 95 ExPEC isolates. Unfortunately this array did not perform as expected, producing multiple false-positive and false-negative signals, as determined by comparison with data for the same isolates from published PCRs and their MICs. This is not the first time that discrepant results have been reported with these arrays (Dierikx *et al.* 2012; Geue *et al.* 2010; Geue *et al.* 2014; Schilling *et al.* 2012). Possible reasons include microarray printing errors, inefficient primers, excess DNA and low temperatures resulting in non-specific hybridisations, inefficient washing during the hybridisation process, stability of stored reagents and inefficient labelling (Eads *et al.* 2006). The microarray assay was performed according to the manufacturer's instructions, using the reagents and protocols recommended at the time. However, subsequent to this work being performed, Alere Technologies updated the array (http://alere-technologies.com/en/products/lab-solutions/e-coli) and removed several targets. These included *cif, vat, tsh, sat* and *iha.* In this evaluation *sat, tsh* and *iha* were not detected in 34 isolates by the

microarray, while *cif* and *vat* were frequently detected by the microarray (n=62), demonstrating poor specificity, despite the VF PCRs demonstrating contradictory results. Although removal of non-specific targets may improve the performance of the microarray, this does not explain all the ambiguous results. Raising the signal value cut-off above that recommended by the manufacturer (e.g. ≥0.5) has been used by some researchers to reduce discrepancies (Wagner, 2012). However, raising the signal value cut-off did not improve the microarray results in this study. Problems with DNA-based microarray platforms have been reported, suggesting that at present this technology can be problematic for analysing the genetic content of bacterial strains (Bogaerts *et al.* 2011; Cohen *et al.* 2010; Nijhuis *et al.* 2011). Fortunately, whole genome sequencing has advanced in recent years and may provide an improved technique for evaluating the gene content of ExPEC isolates (Subashchandrabose *et al.* 2013).

The VF PCR assays provided an improved method for investigating the VF content of these strains, by including a higher number of ExPEC, DEC and APEC VFs that have been reported in ExPEC strains (Johnson and Stell, 2000). In addition, these assays have been used by multiple international research groups, confirming their reliability (Banerjee *et al.* 2013b; Blanco *et al.* 2013; Lopez-Banda *et al.* 2014; Santos *et al.* 2013).

The majority (94%) of isolates analysed were classified as ExPEC, according to the characteristic VFs *fimH, pap, fyuA, iutA, kpsMTII, traT* and *usp,* and this proportion was in agreement with similar studies (Cooke *et al.* 2010; Micenkova *et al.* 2014; Oteo *et al.* 2014; Piatti *et al.* 2008). However, in contrast to the minimum ExPEC profile defined by Johnson and Stell, *hlyD* and *malX* were less frequently detected and *usp* has become more prevalent (Johnson and Stell, 2000). Notably, the 6% of isolates that lacked the characteristic VFs were generally avirulent (maximum 3 VFs), collected from patients with a complication (diabetic, catheter) and mostly belonged to phylogroup A, suggesting their success was a result of patient compromise rather than virulence. Overall, the UK ExPEC strains demonstrated great variety in their VF repertoire, with the major ExPEC lineages also

differing in their VF content compared to internationally characterised strains (Manges *et al.* 2001; Platell *et al.* 2011).

As well as possessing the highest VF score of the four phylogroups, phylogroup B2 isolates also demonstrated the highest prevalence of pathogenicity islands (PAI). As reported by Calhau *et al* (2013), CFT073 I, CFT073 II and 536 IV were frequently identified together within these isolates. These islands encode *pap*, *hlyA, sat*, *fyuA* and *malX*, supporting the notion that high numbers of PAIs and prevalent virulence factors go hand in hand (Dobrindt *et al.* 2002; Guyer *et al.* 2000; Parham *et al.* 2005a; Guyer *et al.* 1998). Another interesting observation from this study was the association of 536 III with ST127-O6 isolates. This PAI encodes *sfa* and *tsh,* of which *sfa* has been linked to ST127-O6 isolates and was identified in these UK strains (Dobrindt *et al.* 2002; Johnson *et al.* 2008b).

Based on infection-specific differences, virulence factors could be sub-divided into colonisation factors and virulence factors. Identified in 94% of isolates, *fimH, fyuA, iutA, kpsMTII, traT* and *usp* could be considered ExPEC colonisation factors. The role of *fyuA* as a colonisation factor was supported by Johnson and Stell (2000), who identified the importance of *fyuA* in compromised and non-compromised patients. In this study *fyuA* was equally as prevalent as *traT* (66%), which was common to both urinary and bloodstream isolates. Furthermore, *fyuA* was lacking in the paired urosepsis isolates, advocating a colonisation role, along with *fimH* which was also less prevalent (81% vs. 95-100%) than previous studies (Cooke *et al.* 2010; Johnson *et al.* 2005b; Norinder *et al.* 2011).

Whereas *iha* and *sat* were prevalent in both UC and GU isolates, indicating a role in ExPEC virulence in UTIs and bacteraemia, which has been confirmed by the presence of these determinants in EHEC O157:H7, EAEC causing UTIs and the ST131 H30-Rx sub-clone (Banerjee *et al.* 2013b; Bielaszewska *et al.* 2011; Olesen *et al.* 2012).

In urosepsis isolates (COMP and GU), *pap*, *afa/dra* and *ompT* were more frequently detected compared to isolates from other infection types and sources of infection, indicating a urosepsis-

specific virulence role. As discussed, *fyuA* and *fimH* were surprisingly lacking, suggesting a colonisation role. One study suggested that *pap* was required for urosepsis development (Venier *et al.* 2007). However, a more recent study suggested that both *pap* and *afa/dra* are required for the translocation of *E. coli* from the urinary tract to the bloodstream (Szemiako *et al.* 2013), as observed here. Interestingly, another study reported *pap* to be more prevalent in community-associated urosepsis isolates, compared with hospital-associated urosepsis isolates (Skjot-Rasmussen *et al.* 2012a), but this may be a result of UTIs presenting predominantly in the community (Bean *et al.* 2008) and the increased role of catheters in hospital-acquired infections (Melzer and Welch, 2013) ruling out the need for conventional adhesins. Johnson *et al* (2008a) also demonstrated the importance of *ompT* in bacteraemia, with 22% of faecal isolates and 82% of bloodstream isolates encoding this protein, as well as the majority of American ST131 clonal isolates (Colpan *et al.* 2013; Johnson *et al.* 2010). Paired urosepsis isolates were mostly of the same strain, but there were differences between the virulence factors detected in urinary and bloodstream isolates. It is possible that these discrepancies actually represent multiple variants of an ExPEC strain simultaneously causing infection (Clermont *et al.* 2013b; Leflon-Guibout *et al.* 2002) or, that during translocation to the bloodstream, there has been a change in or acquisition of genetic material that improves invasiveness (Mahjoub-Messai *et al.* 2011; Szemiako *et al.* 2013).

As with previous reports, there was an inverse relationship between VF score and Abx score (Cooke *et al.* 2010; Piatti *et al.* 2008), confirming that the success of ExPEC relies on either virulence or antibiotic resistance, but generally not both. The high VF score of phylogroup B2 isolates was likely partly due to the major ExPEC clones within this group (Gibreel *et al.* 2012b), especially as the seven VFs that characterised 94% of UK ExPEC strains were highly prevalent within ST131, ST95, ST73 and ST127 strains. However, virulence profiles specific to these clones (ST131 and ST69) differed to previous reports (Clark *et al.* 2012; Johnson *et al.* 2012a; Manges *et al.* 2001; Platell *et al.* 2011), demonstrating the temporal and geographical variation in ExPEC strains (Table 28). In particular,

ST73 and ST95 isolates possessed the highest number of VFs (n=12) compared with the infamously virulent ST131 clone, which possessed fewer (n=10). Sub-clonal differences in ST131 isolates provided insight into this virulent clone. The non-clonal isolate lacked adhesins seemingly important in virulence (*afa/dra* and *pap*), while the clonal-ESBL^{+} isolates demonstrated lower VF scores, as expected, but possessed *pap* and *iha*, suggesting these adhesins compensated for the lack of virulence factors in highly resistant strains. However, all three sub-clones included a high proportion of isolates with *usp* and *traT*, indicating that these two virulence factors are important in ST131 success. Unfortunately recent ST131 studies have focused primarily on the H30-Rx clone, so it would be interesting to investigate these three sub-clones further, such as in an *in vivo* model, to provide insight into the importance of these virulence factors within the sub-clones.

Table 28 Virulence profiles identified in UK isolates of five major ExPEC clones. Virulence factors included in the profile are detected in ≥70% isolates of each clone.

Clone	Virulence factor profile
ST131	*fimH iha iutA kpsII traT usp*
ST127	*fimH pap cnf1 usp pks malX*
ST95	*fimH pap fyuA usp pks malX*
ST73	*fimH pap sfa/foc iha iutA usp pks*
ST69	*fimH pap fyuA iutA sat traT ompT*

The unusual phylotype isolate was investigated for virulence factors as another means to determine relatedness to ExPEC and ST127. This isolate possessed ten virulence factors included in the PCR, including the ExPEC defining *fimH*, *pap*, *iutA*, *traT* and *malX*, confirming that this isolate likely belongs to this pathotype (Johnson and Stell, 2000). Analysis of ST127 strains revealed the majority (7/9 analysed) to possess *fimH*, *pap*, *usp*, *pks* and *malX*, as detected in this novel isolate. However, this strain also possessed *iha*, *sat*, *ireA*, *iutA* and *traT*, which were less common in ST127, but have been detected frequently in other ExPEC clones. Together these findings suggest that this isolate is potentially a rare hybrid of clones, seen in a similar manner with the recent *E. coli* O104 outbreak

strain (Bielaszewska *et al.* 2011) or is a novel ExPEC variant that has acquired virulence factors commonly detected in other sequence types. The intestines act as a melting pot for *E. coli* strains and pathotypes, enabling regular sharing of genetic material, therefore, it is possible that this unusual phylotype will become more prevalent and expand, just as the major ExPEC clones have. For example, *pic* and EAST1 were detected in a few ExPEC isolates in this study, highlighting the increasing trend of EAEC traits in ExPEC isolates (Olesen *et al.* 2012; Toval *et al.* 2014), as a result of the sharing of genetic material between *E. coli* pathotypes.

Bacteraemia isolates with a GIT-source likely originate from commensal or avirulent *E. coli* in the intestines (Bokranz *et al.* 2005; Johnson *et al.* 1998). With this in mind, it could be argued that isolates with a VF score higher than that of GIT-source isolates (>9) be considered virulent. Closer analysis of the UK ExPEC isolates revealed that 69/154 (45%) of isolates had a VF score >9, of which 63/69 (91%) were phylogroup B2 and 62/69 (90%) belonged to one of the major ExPEC lineages. Therefore, it could be assumed that the remaining 55% of isolates, which are seemingly less virulent, caused infection in the presence of host compromise, antibiotic selection or other environmental factors or were caused by a commensal isolate of phylogroup A/B1. But this was unfounded, with many of these less virulent isolates including major ExPEC clones (ST69, ST95, ST131) or causing urosepsis. Therefore, invasiveness and success seems multi-factorial.

In summary, results from this study demonstrated the link between high VF scores and strain variables, such as phylogroup B2 and ST73, while high resistance scores were linked to male patients, those aged >50 years and bloodstream isolates. Colonisation factors enable ExPEC to infect the urinary tract and, occasionally, the bloodstream, but specific virulence factors are required for urosepsis. As the UK ExPEC population has evolved and diversified, its virulence factor repertoire has also changed, with different pathotypes and clones sharing virulence factors that facilitate success. In order to understand and confirm the importance of these virulence factors, a selection of these strains were analysed in an invertebrate infection model.

ExPEC virulence in *G. mellonella*

Mortality effected by the 40 ExPEC isolates was highly varied; however, particular patient and strain features were significantly associated with larvae mortality.

Isolates from female patients, UTIs and community-associated infections (CAI) all effected slightly higher mortality compared with isolates from male patients, the bloodstream and hospital-associated infections (HAI). More significant than these were isolates of GU-source (100%), ST131 (78%), SSTI-source (76%), complicated UTIs (72%) and phylogroup A (72%), which effected the highest mortality and relative risk ratios of ≥5.40.

The urinary tract has been identified as the most common source of bacteraemia in the UK (Horner *et al.* 2014; Livermore *et al.* 2008). Notably GU isolates also induced the strongest immune response (high melanin) and the most cell damage (high LDH), of all bacteraemia sources, supporting the notion of urosepsis as a severe and often fatal infection, often mediated by an overwhelming immune response (Knowles *et al.* 2014; Ku *et al.* 2013; Melzer and Petersen, 2007; Wagenlehner *et al.* 2013a). Analysis of the urinary isolates identified those causing complicated infections to induce high levels of cell damage, which would be required for transmission to the bloodstream, whereas isolates from uncomplicated cystitis induced a strong immune response that would result in eradication from the bladder. This digression among urinary isolates could possibly be explained by UC isolates expressing more immunogenic virulence factors (Brumbaugh *et al.* 2013), which initiated a strong immune response, compared with the immune evasion factors expressed by COMP and GU isolates, such as capsules and serum resistance proteins (Sarkar *et al.* 2014; Ulett *et al.* 2013).

Interestingly, isolates from male patients produced significantly more cell damage compared with isolates from female patients, whereas isolates from female patients effected higher mortality. The high levels of cell damage associated with isolates from men is probably linked to the high LDH levels induced by COMP isolates, as men tend to present with complicated UTIs, such as prostatitis which is more common in older men (Kizilbash *et al.* 2013; Koeijers *et al.* 2010; Lipsky, 1999). The higher

mortality observed with female patients is likely due to a specific strain characteristic or virulence factor that is more frequently encountered in women than men. It was thought that *pap* may be the responsible virulence factor, but statistical analysis revealed no association between *pap* and female patients. However, *pap* was significantly associated with CAIs and GU isolates ($P<0.01$), both of which were also associated with female patients ($P<0.01$). It is also possible that the higher mortality associated with strains from female patients is due to an unknown genetic trait that has yet to be identified and could be explored in future studies.

The extensive virulence factor repertoire of phylogroup B2 isolates explains the strong immune response observed with this phylogroup, compared with groups A, B1 and D. However, mortality was significantly higher with phylogroup A isolates (72%), compared with phylogroup B2 (58%), even though phylogroup A isolates possessed a lower VF score. This difference can be explained by LDH production, which was higher with isolates of phylogroups A and B1, compared with groups B2 and D, indicating that cell damage plays an important role in larvae mortality, as purported by Wand *et al* (2013) with clinically important *Klebsiella* strains. These findings also suggest that it is not the possession of multiple virulence factors that affect mortality, but other genetic or clonal features.

It was no surprise that ST131 isolates, of all the major ExPEC lineages, effected the highest mortality, as this clone is known to be highly pathogenic and transmissible (Colpan *et al.* 2013; Price *et al.* 2013). However, it was isolates of ST73 that induced the strongest immune response and cell damage, closely followed by ST131 isolates. The virulence of these two lineages may also explain the higher mortality associated with CAIs, rather than HAIs, as both ST131 and ST73 were more prevalent in CAIs in this study. The increased invasiveness of ST73 isolates observed here, compared with ST131, could also explain the high proportion of ST73 isolates causing bacteraemia around the UK (Horner *et al.* 2014). However, an alternative hypothesis is the role of O-antigens in the virulence of these lineages. Serogroups O6 and O25 are frequently detected in ST73 and ST131 isolates respectively (Dahbi *et al.* 2013; Johnson *et al.* 2008b). The O6 antigen has recently been reported as

an important colonisation factor of the urinary tract, with an O6-associated insertion sequence (IS1) specifically linked to this virulence (Alghoribi *et al.* 2014; Sarkar *et al.* 2014). In support, this study identified isolates of serogroup O6 to affect the highest mortality (93%); second only to *afa/dra* (97%) of all the virulence factors, with O25 isolates affecting much lower mortality (78%). Lack of this insertion sequence would also explain the low mortality of ST127 isolates, which also encode the O6-antigen (Johnson *et al.* 2008b), but future work would need to confirm this.

In addition to *afa/dra*, the other virulence factor affecting significantly high mortality, and also cell damage, was *ompT* (87%). Both of these factors were significantly associated with urosepsis isolates (see section 7.3) and have been associated with the ST131-O25b clone, which could explain the high mortality associated with this lineage (Colpan *et al.* 2013; Dahbi *et al.* 2013; Platell *et al.* 2011). Of all the fimbriae detected *pap* was significantly associated with high mortality (72% vs. 3-63%), a strong immune response and was another virulence factor linked to urosepsis in this study. Together these data confirm that *pap*, *afa/dra* and *ompT* are undoubtedly essential in extra-intestinal infection, particularly urosepsis, as demonstrated in previous studies (Johnson *et al.* 2005b; Kariyawasam *et al.* 2006; Mahjoub-Messai *et al.* 2011; Santos *et al.* 2013; Skjot-Rasmussen *et al.* 2012b; Szemiako *et al.* 2013; Tarchouna *et al.* 2013).

In contrast, *fimH* and *fyuA*, which were the two most common virulence factors in urinary isolates, were not associated with mortality, a strong immune response or cell damage; confirming their role as colonisation factors. Yersiniabactin (*fyuA*) has been linked to patient mortality (Mora-Rillo *et al.* 2013), but this study did not support this association. 536 IV, the pathogenicity island which encodes *fyuA*, was frequently detected alongside the islands CFT073 I and CFT073 II, as observed in this study (Calhau *et al.* 2013). However, the combination of these three islands did not affect significantly high mortality, nor did the virulence factors frequently encoded by these islands; *hlyA, iha, sat* and *iutA*, except *pap*; although *iutA* and *sat* were associated with significant cell damage. Interestingly, the pathogenicity islands 536 II and 536 III were associated with high mortality compared to all other

islands. Loss of the 536 III PAI from ST127 strains has been linked to decreased virulence. It was hypothesised that loss of the *iroN* gene, located on this island, caused this drop in virulence, but future work would need to confirm the effect of this island and associated genes on ExPEC virulence (Tourret *et al.* 2010).

Analysis of the virulence factors on 536 II and 536 III found no evidence to support this finding, suggesting that this associated mortality was due to the genomic content or clonal features of isolates encoding these islands, rather than specific virulence factors.
Other determinants associated with low levels of cell damage included *hlyF, hra, usp, ibeA, iss, cvaC* and *malX,* suggesting these factors also function during colonisation rather than as virulence factors. A study comparing ExPEC with non-ExPEC also suggested that *iss* and *cvaC* were not associated with virulence (Santos *et al.* 2013), while the frequent detection of *malX* in GIT-source isolates and those causing asymptomatic bacteruria, conditions generally associated with less virulent ExPEC, also implies that *malX* is no longer a marker of virulence.

Despite the range in mortality, larvae death was not associated with one specific, universal virulence factor. This is particularly apparent with phylogroup B2 isolates, which were frequently reported as expressing a high number of virulence determinants, but did not affect significantly higher mortality than the other major phylogroups. Similarly, a recent Danish study investigating mortality of patients with urosepsis found no association with specific virulence factors or antimicrobial resistance (Skjot-Rasmussen *et al.* 2012a). Notably, ST131 isolates were associated with significantly high mortality, but few possessed *afa/dra* or *ompT*, which were also linked to mortality. Another important feature of ST131, compared to the other major lineages, is that these isolates often have a high VF score and a high Abx score, despite previous studies reporting virulence and resistance to be inversely related (Banerjee *et al.* 2013b; Cooke *et al.* 2010; Ewers *et al.* 2010). This suggests that virulence and mortality, particularly with ST131 isolates, is clonal and determined by particular genomic fragments

or genetic make-up, which together confer ExPEC virulence, rather than antibiotic resistance alone or a specific virulence factor repertoire.

Williamson *et al* (2014) were the first group to publish their findings on ExPEC virulence in the *G. mellonella* model. While they found that this model correlated well with the mouse model and that high aggregate VF scores were associated with mortality, they were limited by their sample population. This study has improved on their initial work by analysing isolates in this model with respect to phylogroup, lineage, patient demographics and various virulence factors. However, more remains to be done to understand the virulence of ExPEC.

Next-generation sequencing has recently entered the forefront of diagnostic and research microbiology (Bialasiewicz *et al.* 2014; Skurnik *et al.* 2013; Underwood *et al.* 2013), providing greater understanding of clinically important ExPEC clones, such as ST131 (McNally *et al.* 2013). The combination of next-generation sequencing and a much larger *G. mellonella* study could confirm the findings reported here, but also determine whether the hypothesis that virulence and mortality of ExPEC is clonal and not defined by one sole variable or feature, is valid.

Concluding remarks

ExPEC remains the most significant cause of urinary tract infections and bacteraemia, in the UK and around the world. However, the diversity of ExPEC strains causing these infections makes antibiotic therapy problematic and vaccine development challenging. This study aimed to improve on current UK and international ExPEC data by: defining the UK ExPEC population and the patient population affected; establishing the antibiograms for urinary and bloodstream isolates; determining the prevalence and characteristics of the ST131 clone in the UK; and defining the virulence factors required for causing the major ExPEC infections; UTI, bacteraemia and urosepsis. It is hoped that results from this study will inform future vaccine development, therapeutic guidelines and facilitate development of diagnostic tests.

This is the first UK study to analyse such a large collection of ExPEC strains, by phylogroup, sequence type, serogroup and virulence factors. The findings of this study agreed with many long established trends in ExPEC, but also identified a number of unique findings.

In agreement with previous studies, it was found that uncomplicated cystitis typically affected women, aged 21-50 years in the community, while UTIs in men occurred later in life and was typically complicated in nature, likely due to prostatitis in this age group. Bacteraemia, however, affected both sexes equally, predominantly in those aged >60 years, and with the genitourinary tract the most frequent source of infection. Phylogroup B2 strains remained predominant in UK ExPEC infections, followed by phylogroup D. The major ExPEC clones ST131, ST127, ST95, ST73 and ST69 accounted for ≥40% of UK isolates, with ST131 and ST95 the most prevalent types in bloodstream and urinary tract infections, respectively. This finding contrasts with recent UK studies which found ST73 to be the most frequent sequence type causing these infections (Gibreel *et al.* 2012; Horner *et al.* 2014), but this might be a result of ST131 becoming more dominant in the interim.

This large-scale study led to identification of 21 serogroups previously unreported in ExPEC, as well as 14 serogroups novel to the major ExPEC clones ST131, ST127, ST95, ST73 and ST69. The internationally recognised ST131 clone is known for possessing a few characteristic traits, including

serogroups O25 and O16 (Johnson *et al.* 2010). However, UK ST131 isolates possessed serogroups previously unreported in this clone, including O19a, O136 and O153, suggesting that UK ST131 isolates are more diverse and have evolved differently from ST131 strains in North America, for example.

Novel to this UK study, an isolate of unknown phylotype was identified. Though reported in Portugal and Denmark (Mendonca *et al.* 2011; Skjot-Rasmussen *et al.* 2013), this variant (of ST3679) had not been recognised in the UK until now. Unlike these previous studies, this strain could not be linked to ST127 isolates, indicating that it might belong to an unknown clone or possibly a novel pathotype that combines intestinal and extra-intestinal traits, similar to Enteroaggregative *E. coli* strains with traits of clonal group A (CgA) (Wallace-Gadsden *et al.* 2007).

Importantly, antibiotic resistance has increased in the ExPEC population, despite the decreased use of specific antibiotic classes (e.g. cephalosporins, ciprofloxacin). For example, comparison of BSAC isolates collected in 2006 with those collected in 2011 revealed that ciprofloxacin resistance had increased from 19% to 21% and gentamicin resistance had increased from 9% to 12%. However, cefotaxime resistance has remained stable, at 11% (Health Protection Agency, 2011). Within urinary *E. coli*, ampicillin resistance has increased from 55% to 74%, trimethoprim resistance has increased from 40% to 47%, ciprofloxacin resistance has remained relatively stable at 12%, but nitrofurantoin resistance has decreased from 6% to 1% (Bean *et al.* 2008). Some of the increased resistance can be attributed to the circulation of multi-drug resistant clones, such as ST131-O25 and ST69-CgA. However, this study identified a high number of ESBLs in phylogroup A isolates, suggesting that phylogroup A isolates contribute significantly to the increased resistance, despite previously reports suggesting these isolates were antibiotic susceptible (Johnson *et al.* 2003; Kawamura-Sato *et al.* 2010). ST617, of phylogroup A, was identified in a patient with urosepsis in this study and possessed an ESBL. This sequence type has been reported as a frequent carrier of ESBLs, indicating that ExPEC studies shouldn't limit themselves to the highly prevalent clones (ST131, ST127, ST95, ST73 and ST69), but also consider those associated with multi-drug

resistance (Kang *et al.* 2013; Novais *et al.* 2012; Peirano *et al.* 2012). The decrease in nitrofurantoin resistance is interesting. However, this study compared multiple antibiotic susceptibility testing methods and found that disc diffusion often identified isolates as nitrofurantoin-resistant, but MICs revealed them to be susceptible; indicating that this decrease in resistance is actually an artifact of two non-comparable testing methods. In addition, the regional variation in antibiotic resistance of bloodstream isolates indicates that a national guideline for *E. coli* bacteraemia is urgently required to decrease antibiotic resistance, decrease the use of last-line antibiotics and introduce formal stewardship of antibiotic prescribing (Public Health England, 2014a). This study also highlighted the importance of conducting regular surveillance of antibiotic resistance in urinary *E. coli*. As an *E. coli* UTI can lead to bacteraemia and urosepsis, it would be logical to target the *E. coli* before it reaches the bloodstream. By reviewing regional and national antibiotic resistance on an annual basis, or semi-regularly, results would inform local resistance rates and prescribing practices, as well as update treatment guidelines accordingly.

Empirical prescribing is one of the possible reasons that the ST131-O25b clone is able to succeed. Another is the ability to evolve and acquire new virulence factors that facilitate infection and success. These UK ST131 isolates possessed many characteristics of this clone, including H30-Rx and CTX-M-15. However, one UK isolate possessed a *fimH* allele typically associated with ST95 (H27) and two other isolates possessed IncU plasmids. These features have not been reported in UK strains and, until recently, IncU plasmids were limited to an ST131-O16 isolate in Japan and multi-drug resistant *Aeromonas* species (Cattoir *et al.* 2008; Matsumura *et al.* 2013; Picao *et al.* 2008; Price *et al.* 2013; Weissman *et al.* 2012). However, this may be because UK studies have not performed such a thorough molecular analysis. The virulence factor repertoire of this clone also varied in the UK, compared with earlier studies in the UK, Europe and Australia, with *fyuA*, *ompT* and *malX* lacking, but *iha*, *iutA* and *traT* highly prevalent and important (Clark *et al.* 2012; Mora *et al.* 2014; Platell *et al.* 2011).

In addition to ST131, the virulence factors that define UK ExPEC differ slightly to those identified by Johnson and Stell (Johnson and Stell, 2000). In the UK, *fimH*, *fyuA, hlyD* and *malX* are less prevalent, while *afa/dra*, *iha*, *sat* and *iroN* seem more important in ExPEC infections, particularly those caused by the major clones. This is partially in agreement with another UK study conducted in Manchester in 2011, which identified fyuA, *hlyD* and *malX* in <50% of isolates (Gibreel *et al.* 2012). This study was one of the first in the UK to analyse a selection of ExPEC strains in the *Galleria mellonella* model. This analysis confirmed that *fyuA* and *fimH* are commensal factors required for establishing an infection, but not the development of severe infections. In addition, this virulence model identified ST131 isolates and serogroup O6 as ExPEC variables associated with increased mortality. The latter finding is in agreement with a study conducted in Manchester, which identified an insertion sequence in the O6-antigen operon of these virulent isolates. The authors linked ST127 isolates to high mortality, compared with isolates of the other major clones, including ST131, as a result of the O6-specific insertion sequence (Alghoribi *et al.* 2014). While these two studies are conflicting, together they highlight the heterogeneity and virulence of ExPEC, but also the diversity of these strains within the same country.

This regional variation indicates that ExPEC studies from around the world are not directly comparable to those conducted in the UK. However, UK studies conducted to date have been limited by their sample size, the range of virulence factors that were investigated and the degree of molecular exploration. Therefore, it is important to review the local resistance and virulence of ExPEC, rather than assuming all ExPEC populations are the same.

Key findings from this study

- In the UK*, E. coli* bacteraemia affects both men and women equally.
- Resistance has increased to first-line (trimethoprim) and second-line (ciprofloxacin and gentamicin) antibiotics used to treat urinary tract infections.
- ST131 and ST95 are the most frequent clones causing bacteraemia and urinary tract infections, respectively.
- UK ST131 isolates are largely similar to those described in North America, except that IncU plasmids and serogroups O19a, O136 and O153 have also been identified here.
- Type 1 fimbriae and yersiniabactin are not essential for severe ExPEC infection, while P-fimbriae and serogroup O6 are associated with mortality.

Future work

This study has increased the knowledge of ExPEC within the UK, but also on an international level. However, it is important that the findings from these studies are confirmed and explored further. Therefore, future work to expand on this study is described below:

1. Virulence – use whole genome sequencing to confirm the virulence factor, phylogrouping, serotyping and sequence typing data. This would also generate more accurate phylogeny data and help characterise the unusual phylotype, plus identify novel virulence factors for colonisation, infection and those associated with heightened mortality.
2. Serogrouping – Determine the serogroup of the remaining 40% of isolates to reaffirm the serogroup diversity of ExPEC isolates causing UTIs and bacteraemia.
3. Urinary ExPEC resistance – similar to the BSAC Bacteraemia Resistance Surveillance Programme, urinary *E. coli* isolates could be collected from around the UK to monitor trends in antibiotic resistance. Or centre-focused projects could be set up in a similar manner, with urine specimens collected directly from GP practices to determine more accurate

community-associated antibiotic resistance rates, as well as cystitis, asymptomatic bacteruria and pyelonephritis antibiograms.

4. ST131 plasmids – isolate and sequence the ST131 plasmids encoding CTX-M genes to identify any differences between those encoding different CTX-M groups. Also compare the IncU plasmids with the IncF plasmids frequently identified in ST131 isolates.
5. ST131 virulence – conduct a larger study in the *Galleria mellonella* model to confirm the findings of this study, but also compare sub-clonal ST131 isolates (e.g. CTX-M-negative vs. CTX-M-positive)
6. ST127 virulence - investigate the O6-associated insertion sequence (IS1), PAI 536 III and *iroN* in ST127 isolates, plus the other major clones, to identify any patterns in highly virulent strains compared with avirulent strains.
7. Develop a point-of-care test for ST131 H30-Rx clone that can be used by GPs, out-patient departments and accident and emergency departments to guide empirical therapy.
8. Vaccine development – use data from whole genome sequencing with the *Galleria mellonella* model to identify potential vaccine targets to prevent *E. coli* urinary tract infection.

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Appendices

Appendix A

Bart's Health NHS Trust, R&D Approval

Appendix B

Materials and Reagents

Media

Agar and broths, once prepared according to the manufacturers' instructions, were autoclaved at 121°C for 15 minutes. At PHE agar and broths were prepared by the Media department. At Queen Mary's agar and broths were prepared personally.

The majority of media was supplied by Oxoid (Basingstoke, UK), except where specified below, and included: Brilliance^{TM} UTI Agar, blood agar, nutrient agar, ISO-Sensitest agar, Mueller-Hinton agar, Luria-Bertani (LB) agar and thiotone Craigie broth.

Media supplied from elsewhere included: Hedley Wright broth (Mast, Bootle, UK), SOC medium (Bioline, London, UK) and LB broth (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK).

Antibiotics (see section 5.2.1.2)

All antibiotics used in this study were supplied by Sigma-Aldrich Company Ltd. (Gillingham, Dorset, UK), except for those specified below:

Cefpirome sulphate (Sanofi-Aventis, Guildford, Surrey, UK)

Ertapenem and imipenem monohydrate (Merck Sharpe and Dohme (MSD) Ltd, Hoddesdon, Hertfordshire, UK)

Lithium clavulanate (GlaxoSmithKline, Brentford, Middlesex, UK)

Meropenem (AstraZeneca, London, UK)

Temocillin (Eumedica, Basel, Switzerland)

Tigecycline (Pfizer, Walton Oaks, Surrey, UK)

Miscellaneous reagents

Formal saline: 160ml formaldehyde (Sigma), 800ml 17% sodium chloride (VWR International Ltd.) and 15.2L distilled water (PHE)

Sterile phosphate buffered saline (PBS) and sterile distilled water: provided by the in-house media departments (Blizard Institute, Queen Mary's & PHE)

DNA and plasmid extraction (see sections 5.2.8 and 6.2.6)

Absolute ethanol (VWR International Ltd.): 70% ethanol prepared by diluting in sterile distilled water

DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany)

Lysis buffer: 0.05M Tris (Sigma), 0.01M EDTA (VWR International Ltd.), 4% sodium-dodecyl-sulphate (SDS) (Sigma), pH 12.45

Neutralisation buffer: 2M Tris (Sigma), pH 7.0

TE buffer: 0.05M Tris (Sigma), 0.01M ethylene-diamine-tetra-acetic acid (EDTA) (VWR International Ltd.), pH 8.0

Solution A: 20mM EDTA (Sigma) and 400mM Tris (Gibco®, Life technologies, Paisley, UK), pH8

Solution B: 0.4M sodium hydroxide (Sigma)

Solution C: 400mM Tris (Life technologies) and 4% SDS (Fluka™, Sigma)

5M sodium chloride (Sigma)

Sterile-filtered water (Sigma)

Sodium acetate, 3M, pH5.5 (Sigma)

Phenol: chloroform: isoamyl alcohol 25:24:1 saturated with 10mM Tris and 1mM EDTA, pH8 (Sigma)

Chloroform (BDH Prolabo®, VWR, UK)

PCR master mix(s) and enzymes (see sections 3.5, 4.2.6, 5.2.7, 6.2.2, 6.2.7 & 7.2.2)

All primers were sourced from Sigma.

FAST BLUE qPCR MasterMix (Eurogentec, Southampton, UK)

MyTaq™ Red Mix (Bioline)

Qiagen® Multiplex PCR kit (Qiagen)

ReddyMix™ PCR Master Mix (Thermo Scientific, MA, USA)

SensiFAST™ SYBR No-ROX kit (Bioline)

PBRT kit (Diatheva, Fano, Italy)

Combined genotyping array reagents (see section 7.2.1)

DNeasy® Blood and Tissue Kit (Qiagen)

RNase A (Qiagen)

PCR reagents: B1 buffer, B2 solution and primer mix (Alere Technologies GmbH, Jena, Germany)

Hybridisation reagents: C1, C2, C3, C4 and D1 buffers (Alere Technologies)

ArrayStrip™ (Alere Technologies)

PFGE buffers and enzymes (see sections 4.2.7 and 6.2.5)

Alkaline lysis buffer: 1% w/v N-lauroyl sarcosine (Sigma), 0.5M EDTA pH 9.5 (VWR International Ltd.)

FastDigest Green Reaction buffer (Thermo Scientific)

First lysis buffer: 6mM tris (hydroxymethyl) aminomethane (Tris) (VWR International Ltd.), 100mM EDTA (VWR International Ltd.), 1M sodium chloride (NaCL) (VWR International Ltd.), 0.5% w/v Brij® 58 (Sigma), 0.2% w/v sodium deoxycholate (Sigma), 0.5% N-lauroyl sarcosine (Sigma), 1mM magnesium chloride ($MgCL_2$) (VWR International Ltd.)

Lambda ladder (50 – 1000 kb), PFG marker (New England BioLabs© Inc., MA, USA)

Lysozyme, from chicken egg white (Sigma)

Proteinase K (Sigma)

SE buffer: 75mM NaCL (VWR International Ltd.), 25mM EDTA (VWR International Ltd.), pH 7.5

TE buffer: 10mM Tris (VWR International Ltd.), 10mM EDTA pH 7.5 (VWR International Ltd.)

TBE buffer: 0.5X TBE (44.5mM Tris, 44.5mM boric acid, 1mM EDTA) (Invitrogen™, Life Technologies)

Xbal FastDigest enzyme (Thermo Scientific)

S1 nuclease and 10X buffer (Promega, Southampton, UK)

Electrophoresis solutions and reagents (see sections 3.6, 4.2.7 and 6.2.5)

Bionic™ 10X buffer (Sigma)

DNA ladder, 100 bp or 123 bp (Invitrogen™, Life Technologies)

Ethidium Bromide, 10 mg/ml (Sigma): dilute 1:10,000

Gelred™ (Biotium, CA, USA): 3X solution, dilute 60 µl in 200 ml 0.1M NaCL (VWR International Ltd.)

MacroSieve Low Melt Agarose (Scientific Laboratory Supplies Ltd, Hessle, UK): 2.04g in 170ml 0.5X TBE (Invitrogen™, Life Technologies)

Sterile-filtered water (Sigma)

Tris-Borate-EDTA (TBE) buffer, 10X (Promega)

TE buffer, 100X (Invitrogen™, Life Technologies): dilute 1:100

UltraPure™ agarose (Invitrogen™, Life Technologies): 1.4g/70ml (2%) or 2.1g/70ml (3%)

UltraPure™ 10X TBE buffer (Invitrogen™, Life Technologies)

DNA cloning and purification kits (see sections 5.2.8 and 6.2.6.3)

Alpha-select electrocompetent cells (Bioline)

Exonuclease I enzyme (New England BioLabs©)

Exonuclease I reaction buffer (New England BioLabs©)

MinElute® PCR purification kit (Qiagen)

QIAprep® Spin miniprep kit (Qiagen)

rAPid alkaline phosphatase reaction buffer (Roche Diagnostics Ltd, Burgess Hill, UK)

rAPid alkaline phosphatase enzyme (Roche Diagnostics Ltd)

TOPO TA cloning® kit (Invitrogen)

Galleria mellonella **model** (see section 8.2.3)

Galleria mellonella (Livefood UK Ltd, Rooks Bridge, Somerset, UK)

30ml universal container (STARLAB GmbH, Hamburg, Germany)

1 µl calibrated loop (STARLAB)

10 µl calibrated loop (STARLAB)

1 ml graduated pipette (STARLAB)

1.5 ml microcentrifuge tube (STARLAB)

Polypropylene L-shaped spreader (STARLAB)

Fisherbrand™ extra-deep disposable petri dishes (Thermo Scientific)

Whatman™, Grade 1 circles, 240mm, filter paper (Whatman plc, Maidstone, UK)

25 µl, 22 gauge, gastight, bevel tip syringe (Hamilton, Bonaduz, Switzerland)

CytoTox 96® non-radioactive cytotoxicity assay (Promega)

Sterile disposable surgical scalpel (Swann-Morton, Sheffield, UK)

96-well polystyrene microplate, round well, flat bottom (STARLAB)

N-phenylthiourea, crystals (Alfa Aesar, Ward Hill, MA, USA)

Absolute ethanol (Thermo Scientific): diluted to 70% as described above

Consumables

Easy pierce heat sealing foil (Thermo Scientific)

GenePulser® 0.1cm cuvettes (Bio-rad, Hemel Hempstead, Hertfordshire, UK)

Microtitre plate, 96-well U bottom, non-sterile (Thermo Scientific)

PCR plate, 96-well low profile, non-skirted (Thermo Scientific)

PCR tube, 8 strip, 0.2 ml flat cap (Thermo Scientific)

PCR microtube with attached cap, 0.2 ml (Sigma)

Polystyrene tube, 15 ml, with conical bottom, blue screw cap (Greiner Bio-one GmbH, Frickenhausen, Germany)

Pro-Lab Diagnostics™ Microbank™ Bacterial and Fungal Preservation System, cryobeads (Thermo Scientific)

Equipment

ArrayMate™ reader (Alere Technologies GmbH)

Boiling bath, SBB14 (Grant Instruments Ltd, Cambridge, UK)

Centrifuge, 5415 R (Eppendorf, Stevenage, UK)

Centrifuge, IEC® MicroMax (Thermo scientific)

Centrifuge, IEC® CL40 (Thermo scientific)

CHEF-DR II chiller system (Bio-Rad)

ELx800 absorbance microplate reader (Biotec, Winooski, VT, USA)

G:Box imaging camera (IMGEN technologies, Alexandria, VA, USA)

GenePulser Xcell™ (Bio-rad)

Grant OLS200 waterbath (Grant Instruments Ltd)

Grant W14 waterbath (Grant Instruments Ltd)

Heraeus™ Pico™ microcentrifuge (Thermo Scientific)

Innova® 2100 and Innova® 4000 (New Brunswick Scientific, Stevenage, UK)

Lightcycler® 2.0 (Roche Diagnostics Ltd)

MALDI-Tof Microflex LT system (Bruker Daltonics, Germany)

Mastercycler (Eppendorf)

MicroScan® WalkAway 96 plus (Siemens, USA)

ND-1000 NanoDrop (Thermo Scientific)

OLS200 shaking waterbath (Grant instruments Ltd)

Rotor-Gene Q (Qiagen)

Touchgene Gradient Thermal Cycler (Techne)

T100™ Thermal cycler (Bio-Rad)

Techne Dri-block® (Techne, Cambridge, UK)

Vortex genie® (Mo Bio Laboratories Inc., CA, USA)

Wide mini-sub cell GT cell electrophoresis tank (Bio-Rad)

Appendix C

PCR Primers

C1 *E. coli* Phylogenetic group

Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
gadA-F	GATGAAATGGCGTTGGCGCAAG	373	Glutamate decarboxylase-α	(Doumith *et al.* 2012)
gadA-R	GGCGGAAGTCCCAGACGATATCC			
chuA-F	ATGATCATCGCGGCGTGCTG	281	Heme transport protein	(Doumith *et al.* 2012)
chuA-R	AAACGCGCTCGCGCCTAAT			
yjaA-F	TGTTCGCGATCTTGAAAGCAAACGT	216	Unknown function, protein	(Doumith *et al.* 2012)
yjaA-R	ACCTGTGACAAACCGCCCTCA			
TSPE4.C2-F	GCGGGTGAGACAGAAACGCG	152	Unknown function, DNA fragment	(Doumith *et al.* 2012)
TSPE4.C2-R	TTGTCGTGAGTTGCGAACCCG			

C2 Five most common ExPEC sequence types

Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
ST131-F	GACTGCATTTCGTCGCCATA	310		(Doumith *et al.* 2014)
ST131-R	CCGGCGGCATCATAATGAAA			
ST127-F	CGCATAACAGGATTGTCTGG	404		This study
ST127-R	GCTATTCTACGGGCATTGTG			
ST95-F	ACTAATCAGGATGGCGAGAC	200		(Doumith *et al.* 2014)
ST95-R	ATCACGCCCATTAATCCAGT			
ST73-F	TGGTTTTACCATTTTGTCGGA	490		(Doumith *et al.* 2014)
ST73-R	GGAAATGGTTGATGTTGGCT			
ST69-F	AGAGAAAGGGCGTTCAGAAT	100		(Doumith *et al.* 2014)
ST69-R	ATCTGGAGGCAACAAGCATA			

C3 *E. coli* Multi-locus sequence typing (Achtman Scheme)

Primer	Sequence (5'-3')	Product size (bp)	Target	Annealing Tm (°C)	Reference
adk-F	ATTCTGCTTGGCGCTCCGGG	583	Adenylate kinase	54	(Wirth *et al.* 2006)
adk-R	CCGTCAACTTTCGCGTATTT				
fumC-F	TCACAGGTCGCCAGCGCTTC	806	Fumarate hydratase C	54	(Wirth *et al.* 2006)
fumC-R	GTACGCAGCGAAAAAGATTC				
gyrB-F	TCGGCGACACGGATGACGGC	911	DNA gyrase, subunit B	54	(Wirth *et al.* 2006)
gyrB-R	ATCAGGCCTTCACGCGCATC				
icd-F	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	878	Isocitrate dehydrogenase	54	(Wirth *et al.* 2006)
icd-R	GGACGCAGCAGGATCTGTT				
mdh-F	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	932	Malate dehydrogenase	60	(Wirth *et al.* 2006)
mdh-R	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT				
purA-F	CGCGCTGATGAAAGAGATGA	816	Adenylosuccinate synthetase	54	(Wirth *et al.* 2006)
purA-R	CATACGGTAAGCCACGCAGA				
recA-F	ATCTACAGAGAAATCCGGCG	780	Recombinase A	60	(Wirth *et al.* 2006)
recA-R	TTTATCGATGCTGAACACGC				

C4 *fimH* allele determination

Primer	Sequence	Product size (bp)	Target	Reference
fimH-F	CACTCAGGGAACCATTCAGGCA	976	Type 1 fimbriae, minor subunit	(Weissman *et al.* 2012)
fimH-M	CGTTGTTTATAATTCGAG			
fimH-R	CTTATTGATAAACAAAAGTCAC			

C5 EAEC PCR (real-time PCR)

Primer	Sequence (5'-3')	Target	Reference
aggR-F	CCATTTATCGCAATCAGATTAA	*aggR* EAEC regulatory gene	(Chattaway *et al.* 2014)
aggR-R	CAATGTATAGAAATCCGCTGTT		
aggR-probe	**Cy5-**CAGCGATACATTAAGACGCCTAAAGGA **-BHQ**		

C6 Sequencing primers: M13

PCR Assay	Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
M13	M13-F	GTAAAACGACGGCCAG	variable	*Lac* promoter and *lac*Zα gene	Invitrogen Cat. No. K4575-40
	M13-R	CAGGAAACAGCTATGAC			

C7 Genes encoding plasmid-mediated quinolone resistance determinants

Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
qnrA-F	CAGCAAGAGGATTTCTCACG	630	Quinolone resistance determinant A	(Ciesielczuk *et al.* 2013)
qnrA-R	AATCCGGCAGCACTATTACTC			
qnrB-F	GGCTGTCAGTTCTATGATCG	488	Quinolone resistance determinant B	(Ciesielczuk *et al.* 2013)
qnrB-R	GAGCAACGATGCCTGGTAG			
qnrC-F	GCAGAATTCAGGGGTGTGAT	118	Quinolone resistance determinant C	(Ciesielczuk *et al.* 2013)
qnrC-R	AACTGCTCCAAAAGCTGCTC			
qnrD-F	CGAGATCAATTTACGGGGAATA	581	Quinolone resistance determinant D	(Cavaco et al. 2009)
qnrD-R	AACAAGCTGAAGCGCCTG			
qnrS-F	GCAAGTTCATTGAACAGGGT	428	Quinolone resistance determinant S	(Cattoir et al. 2007)
qnrS-R	TCTAAACCGTCGAGTTCGGCG			
qepA-F	GCAGGTCCAGCAGCGGGTAG	218	Quinolone efflux pump A	(Yamane et al. 2008)
qepA-R	CTTCCTGCCCGAGTATCGTG			
oqxA-F	CCGCACCGATAAATTAGTCC	313	RND efflux pump protein A	(Ciesielczuk *et al.* 2013)
oqxA-R	GGCGAGGTTTTGATAGTGGA			
aac(6')-Ib-cr-F	ACACGGCTGGACCATA	260	6'-N-acetyltransferase, type Ib-cr allele	(Ciesielczuk *et al.* 2013)
aac(6')-Ib-cr-R	TTGGAAGCGGGGACGGAM			

C8 Genes encoding Beta-lactamases

Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
CMY-F	ACGGAACTGATTTCATGATG	1200*	Full CMY gene and flanking region	(Ahmed and Shimamoto, 2008)
CMY-R	GAAAGGAGGCCCAATATCCT			
CTX-M group-1 F	AAAAATCACTGCGCCAGTTC	415	CTX-M-1 gene	(Woodford, 2010)
CTX-M group-1 R	AGCTTATTCATCGCCACGTT			
CTX-M group-9 F	CAAAGAGAGTGCAACGGATG	205	CTX-M-9 gene	(Woodford, 2010)
CTX-M group-9 R	ATTGGAAAGCGTTCATCACC			
CTX-M group-9 F	CTGATGTAACACGGATTGAC	932	CTX-M-9 entire open reading frame	(Girlich *et al.* 2009)
CTX-M group-9 R	AGCGCCCCATTATTGAGAG			
TEM-F	ATGAGTATTCAACATTTCCG	858	TEM β-lactamase	(Livermore *et al.* 2001)
TEM-R	CCAATGCTTAATCAGTGACG			
SHV-F	TCAGCGAAAAACACCTTG	475	SHV β-lactamase	(M'Zali *et al.* 1996)
SHV-R	TCCCGCAGATAAATCACCA			
OXA-1-like F	GGATAAAACCCCCAAAGGAA	370	OXA-1-like	(Karisik *et al.* 2006)
OXA-1-like R	TGCACCAGTTTTCCCATACA		β-lactamase	
ACC-F	CACCTCCAGCGACTTGTTAC	346	ACC-1 and 2 genes	(Dallenne *et al.* 2010)
ACC-R	GTTAGCCAGCATCACGATCC			
FOX-F	CTACAGTGCGGGTGGTTT	162	FOX-1 and 2-5 genes	(Dallenne *et al.* 2010)
FOX-R	CTATTTGCGGCCAGGTGA			
MOX-F	GCAACAACGACAATCCATCCT	895	MOX-1 and 2, CMY-1 and 8-11 genes	(Dallenne *et al.* 2010)
MOX-R	GGGATAGGCGTAACTCTCCCAA			
DHA-F	TGATGGCACAGCAGGATATTC	997	DHA-1 and 2 genes	(Dallenne *et al.* 2010)
DHA-R	GCTTTGACTCTTTCGGTATTCG			
CIT-F	CGAAGAGGCAATGACCAGAC	538	CMY 2-7, LAT 1-4, BIL-1 genes	(Dallenne *et al.* 2010)
CIT-R	ACGGACAGGGTTAGGATAGY			
EBC-F	CGGTAAAGCCGATGTTGCG	683	ACT-1 and MIR-1 genes	(Dallenne *et al.* 2010)
EBC-R	AGCCTAACCCCTGATACA			

*size varies depending on CMY allele

C9 Genes encoding aminoglycoside modifying enzymes

Primer	Sequence (5'-3')	Product Size (bp)	Target (annealing temperature, °C)	Reference
aph(3')-VI - F	CGGAAACAGCGTTTTAGA	716	3'-phosphotransferase, type VI (49)	(Noppe-Leclercq *et al.* 1999)
aph(3')-VI - R	TTCCTTTTGTCAGGTC			
ant(2'')-Ia - F	T	404	2'-nucleotidyltransferase, type Ia (49)	(Noppe-Leclercq *et al.* 1999)
ant(2'')-Ia - R	CGAGCCTGTAGGACT			
aac(6')-Ih - F	TGCCGATATCTGAATC	407	6'-acetyltransferase, type Ih (58)	(Noppe-Leclercq *et al.* 1999)
aac(6')-Ih - R	ACACCACACGTTCAG			
aac(3)-Ia - F	GACATAAGCCTGTTCGGTT	372	3-acetyltransferase, type Ia (58)	(Noppe-Leclercq *et al.* 1999)
aac(3)-Ia - R	CTCCGAACTCACGACCGA			
aac(3)-IIa - F	ATGCATACGCGGAAGGC	822	3-acetyltransferase, type IIa (58)	(Noppe-Leclercq *et al.* 1999)
aac(3)-IIa - R	TGCTGGCACGATCGGAG			
aac(6')-Ib - F	TATGAGTGGCTAAATCGAT	395	6'-acetyltransferase, type Ib (55)	(Noppe-Leclercq *et al.* 1999)
aac(6')-Ib - R	CCCGCTTTCTCGTAGCA			
aph(3')-Ia - F	CGAGCATCAAATGAAACTGC	623	3'-phosphotransferase, type Ia (55)	(Noppe-Leclercq *et al.* 1999)
aph(3')-Ia - R	GCGTTGCCAATGATGTTACAG			
aacA-aphD - F	GAGCAATAAGGGCATACCAAA	829	Acetyltransferase-phosphotransferase (56)	(Leelaporn *et al.* 2008)
aacA-aphD - R	GTTCCTATTTCTTCTTCACTATCTTCA			
aph(2'')-Ib - F	TCAAATCCCTGCGGTAGTGTA	428	2''-phosphotransferase, type Ib (56)	(Leelaporn *et al.* 2008)
aph(2'')-Ib - R	CGCCAAAATCAATAACTCCAA			
aph(2'')-Ic - F	GAGGGCTTTAGGAATTACGC	125	2''-phosphotransferase, type Ic (56)	(Leelaporn *et al.* 2008)
aph(2'')-Ic - R	ACACAACCGACCAACAGAGG			
aph(2'')-Id - F	TAATCTGCCGAAGCAATCTCA	550	2''-phosphotransferase, type Id (56)	(Leelaporn *et al.* 2008)
aph(2'')-Id - R	TAATCCCTCTTCATACCAATCC			
aadA - F	ACCGTAAGGCTTGATGAAACA	624	3''-adenyltransferase (56)	(Leelaporn *et al.* 2008)
aadA - R	GCCGACTACCTTGGTGATCTC			
aadE - F	GCCCTTGGAAGAGTTAGATAATT	198	6'-adenyltransferase (56)	(Leelaporn *et al.* 2008)
aadE - R	CGGCACAATCCTTTAATAACA			

Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
armA - F	TATGGGGGTCTTACTATTCTGCCTAT	545	16S methyltransferases ArmA	(Fritsche *et al.* 2008)
armA - R	TCTTCCATTCCCTTCTCCTTT			
rmtA - F	CTAGCGTCCATCCTTTCCTC	635	16S methyltransferases RmtA	(Yokoyama *et al.* 2003)
rmtA - R	TTTGCTTCCATGCCCTTGCC			
rmtB - F	TCAACGATGCCCTCACCTC	466	16S methyltransferases RmtB	(Fritsche *et al.* 2008)
rmtB - R	GCAGGGCAAAGGTAAAATCC			
rmtC - F	GCCAAAGTACTCACAAGTGG	752	16S methyltransferases RmtC	(Fritsche *et al.* 2008)
rmtC - R	CTCAGATCTGACCCAACAAG			
rmtD - F	CTGTTTGAAGCCAGCGGAACGC	376	16S methyltransferases RmtD	(Fritsche *et al.* 2008)
rmtD - R	GCGCCTCCATCCATTCGGAATAG			
npmA - F	CTCAAAGGAACAAAGACGG	641	16S methyltransferases NpmA	(Fritsche *et al.* 2008)
npmA - R	GAAACATGGCCAGAAACTC			

C10 Genes encoding rifamycin resistance determinants

Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
arr2/3-F	CTATCATGGAACCAAAGCCA	296	*arr-2* and *arr-3*	(Hopkins *et al.* 2014)
arr2/3-R	CAACGCCAACAATTCTCAAG			
arr4-F	ACATCTACATCGTTGAACCG	190	*arr-4*	(Hopkins *et al.* 2014)
arr4-R	TGAAGATCCTCCAGAGACG			

C11 Cyclomodulins

Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
CDT-s1	GAAAGTAAATGGAATATAAATGTCCG	467	Cytolethal distending toxin B (cdtB), variant II, III & V	(Toth *et al.* 2003)
CDT-as1	AAATCACCAAGAATCATCCAGTTA			
CDT-IIas*	TTTGTGTTGCCGCCGCTGGTGAAA	556	cdtB-II	(Toth *et al.* 2003)
CDT-IIIas*	TTTGTGTCGGTGCAGCAGGGAAAA	555	cdtB-III, cdtB-V	(Toth *et al.* 2003)
CDT-s2	GAAAATAAATGGAACACACATGTCCG	467	cdtB-I, cdtB-IV	(Toth *et al.* 2003)
CDT-as2	AAATCTCCTGCAATCATCCAGTTA			
CDT-Is	CAATAGTCGCCCACAGGA	411	cdtB-I	(Toth *et al.* 2003)
CDT-Ias	ATAATCAAGAACACCACCAC			
CDT-IVs	CCTGATGGTTCAGGAGGCTGGTTC	350	cdtB-IV	(Toth *et al.* 2003)
CDT-IVas	TTGCTCCAGAATCTATACCT			
Cif-F	AACAGATGGCAACAGACTGG	383	Cycle inhibiting factor (cif)	(Marches *et al.* 2003)
Cif-R	AGTCAATGCTTTATGCGTCAT			
CNF1-F	GGGGGAAGTACAGAAGAATTA	1112	Cytotoxic necrotising Factor 1 (cnf1)	(Toth *et al.* 2003)
CNF1-R	TTGCCGTCCACTCTCACCAGT			

*use CDT-s1 as antisense primer

C12 Genes encoding ExPEC Virulence Factors

Primer Mix 1	Sequence (5'-3')	Product Size (bp)	Target	Reference
PAI-F	GGACATCCTGTTACAGCGCGCA	930	*malX* virulence marker	(Johnson and Stell, 2000) updated protocol (2010)
PAI-R	TCGCCACCAATCACAGCCGAAC			
papA-F	ATGGCAGTGGTGTCTTTTGGTG	717	P-fimbriae, minor subunit A	
papA-R	CGTCCCACCATACGTGCTCTTC			
K15-F	ACGGATTCACGACAAAGCTC	581	K15 capsule	(Schneider *et al.* 2004)
K15-R	GGCAAATATCGCTTGGGTTA			
fimH-F	TCGAGAACGGATAAGCCGTGG	508	Type 1 fimbriae, adhesin	(Johnson and Stell, 2000) updated protocol (2010)
fimH-R	GCAGTCACCTGCCCTCCGGTA			
kpsIII-F	TCCTCTTGCTACTATTCCCCCT	392	Type III capsule	
kpsIII-R	AGGCGTATCCATCCCTCCTAAC			
papEF-F	GCAACAGCAACGCTGGTTGCATCAT	326	P-fimbriae, subunits E and F	
papEF-R	AGAGAGAGCCACTCTTATACGGACA			
Vat-F	AGAGACGAGACTGTATTTGC	289	Vacuolating toxin	(Srinivasan *et al.* 2003)
Vat-R	GTCAGGTCAGTAACGAGCAC			
ireA-F	GATGACTCAGCCACGGGTAA	254	*ireA* siderophore	(Johnson and Stell, 2000) updated protocol (2010)
ireA-R	CCAGGACTCACCTCACGAAT			
Ibe10-F	AGGCAGGTGTGCGCCGCGTAC	171	Invasion of brain endothelium protein	
Ibe10-R	TGGTGCTCCGGCAAACCATGC			

Primer Mix 2	Sequence (5'-3')	Product Size (bp)	Target	Reference
Cnf1-F	GGGGGAAGTACAGAAGAATTA	1112	Cytotoxic necrotising factor 1	(Toth *et al.* 2003)
Cnf1-R	TTGCCGTCCACTCTCACCAGT			
fyuA-F	TGATTAACCCCGCGACGGGAA	787	Yersiniabactin	(Johnson and Stell, 2000) updated protocol (2010)
fyuA-R	CGCAGTAGGCACGATGTTGTA			
iroN-F	AAGTCAAAGCAGGGGTTGCCCG	667	*iroN E. coli* siderophore	
iroN-R	GACGCCGACATTAAGACGCAG			
clbB-F	GATTTGGATACTGGCGATAACCG	579	*Pks* PAI marker *clbB*	(Nougayrede *et al.* 2006)
clbB-R	CCATTTCCCGTTTGAGCACAC			
bmaE-F	ATGGCGCTAACTTGCCATGCTG	507	Blood group M adhesin	(Johnson and Stell, 2000) updated protocol (2010)
bmaE-R	AGGGGGACATATAGCCCCCTTC			
Sfa-F	CTCCGGAGAACTGGGTGCATCTTAC	410	S-fimbriae	
Sfa-R	CGGAGGAGTAATTACAAACCTGGCA			
iutA-F	GGCTGGACATCATGGGAACTGG	302	Aerobactin	
iutA-R	CGTCGGGAACGGGTAGAATCG			
Allele III-F	GGCCTGCAATGGATTTACCTGG	258	P-fimbriae, *papG* adhesin allele III	
Allele III-R	CCACCAAATGACCATGCCAGAC			
Hra-F	CGAATCGTTGTCACGTTCAG	162	Heat-resistant agglutinin	(Srinivasan *et al.* 2003)
Hra-R	TATTTATCGCCCCACTCGTC			

Primer Mix 3	Sequence (5'-3')	Product Size (bp)	Target	Reference
Pic-F	GGGTATTGTCCGTTCCGAT	1200	Protein involved in intestinal colonisation	(Wallace-Gadsden *et al.* 2007)
Pic-R	ACAACGATACCGTCTCCCG			
hlyD-F	CTCCGGTACGTGAAAAGGAC	904	Alpha-haemolysin	(Johnson and Stell, 2000) updated protocol (2010)
hlyD-R	GCCCTGATTACTGAAGCCTG			
rfc-F	ATCCATCAGGAGGGGACTGGA	788	Serogroup O4 regulatory gene	
rfc-R	AACCATACCAACCAATGCGAG			
ompT-F	ATCTAGCCGAAGAAGGAGGC	559	Outer membrane protein T	
ompT-R	CCCGGGTCATAGTGTTCATC			
Allele I'-F*	CTACTATAGTTCATGCTCAGGTC	479	P-fimbriae, *papG* adhesin allele I'	
Allele I'-R	CTGCATCCTCCACCATTATCGA			
Allele I-F*	TCGTGCTCAGGTCCGGAATTT	461	P-fimbriae, *papG* adhesin allele I	
Allele I-R	TGGCATCCCCCAACATTATCG			
Iss-F	CAGCAACCCGAACCACTTGATG	323	Increased serum survival protein	
Iss-R	AGCATTGCCAGAGCGGCAGAA			
Kii-F	GCGCATTTGCTGATACTGTTG	272	Group 2/II capsule, except K2	
Kii-R	CATCCAGACGATAAGCATGAGCA			
papC-F	GTGGCAGTATGAGTAATGACCGTTA	205	P-fimbriae, subunit C	
PapC-R	ATATCCTTTCTGCAGGGATGCAATA			

*if positive for allele I' and/or allele I, repeated in separate simplex PCRs to identify allele

Primer Mix 4	Sequence (5'-3')	Product Size (bp)	Target	Reference
gafD-F	TGTTGGACCGTCTCAGGGCTC	952	G-fimbriae	(Johnson and Stell, 2000) updated protocol (2010)
gafD-R	CTCCCGGAACTCGCTGTTACT			
K5-F	GCCACCAACTGTCGCAAAA	809	K5 capsule	(Srinivasan *et al.* 2003)
K5-R	TGTCGCCCAAACAAAAAGATT			
cvaC-F	CACACACAAACGGGAGCTGTT	679	Colicin V plasmid	(Johnson and Stell, 2000) updated protocol (2010)
cvaC-R	CTTCCCGCAGCATAGTTCCAT			
cdtB-F	GAAAATAAATGGAACACACATGTCCG	466	Cytolethal distending toxin	(Toth *et al.* 2003)
cdtB-F'	GAAAGTAAATGGAATATAAATGTCCG			
cdtB-R	AAATCTCCTGCAATCATCCAGTTA			
cdtB-R'	AAATCACCAAGAATCATCCAGTTA			
focG-F	CAGCACAGGCAGTGGATACGA	364	F1C-fimbriae, adhesin	(Johnson and Stell, 2000) updated protocol (2010)
focG-R	GAATGTCGCCTGCCCATTGCT			
traT-F	GGTGTGGTGCGATGAGCACAG	290	*traT* serum resistance protein	
traT-R	CACGGTTCAGCCATCCCTGAG			
Allele II-F	GGGATGAGCGGGCCTTTGAT	190	P-fimbriae, *papG* adhesin allele II	
Allele II-R	CGGGCCCCCAAGTAACTCG			

Primer Mix 5	Sequence (5'-3')	Product Size (bp)	Target	Reference
G allele 123-F	CTGTAATTACGGAAGTGATTTCTG		P-fimbriae, *papG* adhesin alleles 1, 2 & 3	(Johnson and Stell, 2000) updated protocol (2010)
G allele 1-R	TCCAGAAATAGCTCATGTAACCCG	1140		
G allele 2&3-R	ACTATCCGGCTCCGGATAAACCAT	1070		
iha-F	CTGGCGGAGGCTCTGAGATCA	829	*Iha* adhesin	
iha-R	TCCTTAAGCTCCCGCGGCTGA			
afa-F	GGCAGAGGGCCGGCAACAGGC	594	Afimbrial adhesin	
afa-R	CCCGTAACGCGCCAGCATCTC			
hlyF-F	TCGTTTAGGGTGCTTACCTTCAAC	444	Avian haemolysin	(Morales *et al.* 2004)
hlyF-R	TTTGGCGGTTTAGGCATTCC			
tsh-F	CCGTACACAAATACGACGG	300	Temperature-sensitive haemagglutinin	Johnston, B. & Menard, M., 2010. Unpublished.
tsh-R	GGATGCCCCTGCAGCGT			
sfaS-F	GTGGATACGACGATTACTGTG	244	S-fimbriae	(Johnson and Stell, 2000) updated protocol (2010)
sfaS-R	CCGCCAGCATTCCCTGTATTC			
K1-F	TAGCAAACGTTCTATTGGTGC	153	K1 capsule	
K1-R (Kii-R)	CATCCAGACGATAAGCATGAGCA			

Primer Mix 6	Sequence (5'-3')	Product Size (bp)	Target	Reference
sat-F	GCAGCTACCGCAATAGGAGGT	937	Secreted autotransporter toxin	(Johnson and Stell, 2000) updated protocol (2010)
sat-R	CATTCAGAGTACCGGGGCCTA			
clbN-F	GTTTTGCTCGCCAGATAGTCATTC	733	*Pks* PAI marker *clbN*	(Nougayrede *et al.* 2006)
clbN-R	CAGTTCGGGTATGTGTGGAAGG			
F17-F	CGGAGCTAATACTGCATCAACC	615	F17 fimbriae	(Johnson and Stell, 2000) updated protocol (2010)
F17-R	TGTTGATATTCCGTTAACCGTAC			
kpsII-F (Kii-F)	GCGCATTTGCTGATACTGTTG	570	Type II capsule	
kpsII-R	AGGTAGTTCAGACTCACACCT			
uidA-F	GCGTCTGTTGACTGGCAGGTGGTGG	508	Beta-D-glucuronidase	
uidA-R	GTTGCCCGCTTCGAAACCAATGCCT			
usp-F	ACATTCACGGCAAGCCTCAG	440	Uropathogenic-specific protein	
usp-R	AGCGAGTTCCTGGTGAAAGC			
clpG-F	GGGCGCTCTCTCCTTCAAC	384	CS31A adhesin	
clpG-R	CGCCCTAATTGCTGGCGAC			
afaE8-F	CTAACTTGCCATGCTGTGACAGTA	302	Afimbrial adhesin VIII	
afaE8-R	TTATCCCCTGCGTAGTTGTGAATC			
astA-F	CCATCAACACAGTATATCCG	100	EAST1 toxin	
astA-R	GGTCGCGAGTGACGGCTTTG			

C13 ExPEC Pathogenicity Islands

Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
CFT073 I-F	GGACATCCTGTTACAGCGCGCA	930	*malX*	(Johnson and Stell, 2000) updated protocol (2010)
CFT073 I-R	TCGCCACCAATCACAGCGAAC			
CFT073 II-F	ATGGATGTTGTATCGCGC	400	ORF38 – ORF39 of CFT073 II, GenBank accession no. AF447814	(Sabate *et al.* 2006)
CFT073 II-R	ACGAGCATGTGGATCTGC			
J96 I-F	TCGTGCTCAGGTCCGGAATTT	461	*papGI* allele I	(Johnson and Stell, 2000) updated protocol (2010)
J96 I-R	TGGCATCCCACATTATCG			
J96 II-F	ATGAAAACATGGTTAATGGG	2300-2412	*hlyD* and *cnf1* fragment	(Sabate *et al.* 2006)
J96 II-R	GATATTTTTGTTGCCATTGGTTAC			
536 I-F	TAATGCCGGAGATTCATTGTC	1800	1.9 and 1.10 (Dobrindt, 2002)	(Dobrindt *et al.* 2002)
536 I-R	AGGATTTGTCTCAGGGCTTT			
536 II-F	CATGTCCAAAGCTCGAGCC	1000	Orf1 up and orf1 down (Dobrindt, 2002)	(Dobrindt *et al.* 2002)
536 II-R	CTACGTCAGGCTGGCTTTG			
536 III-F	CGGGCATGCATCAATTATCTTTG	162-200	*sfaA*	(Sabate *et al.* 2006)
536 III-R	TGTGTAGATGCAGTCACTCCG			
536 IV-F	AAGGATTCGCTGTTACCGGAC	287-300	*Irp2*	(Karch *et al.* 1999)
536 IV-R	TCGTCGGGCAGCGTTTCTTCT			

C14 Plasmid replicon typing

Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
HI1-F	GGAGCGATGGATTACTTCAGTAC	471	*parA-parB*	(Carattoli *et al.* 2005)
HI1-R	TGCCGTTTCACCTCGTGAGTA			
HI2-F	TTTCTCCTGAGTCACCTGTTAACAC	644	iterons	(Carattoli *et al.* 2005)
HI2-R	GGCTCACTACCGTTGTCATCCT			
I1-F	CGAAAGCCGGACGGCAGAA	139	RNAI	(Carattoli *et al.* 2005)
I1-R	TCGTCGTTCCGCCAAGTTCGT			
X-F	AACCTTAGAGGCTATTTAAGTTGCTGAT	376	*ori γ*	(Carattoli *et al.* 2005)
X-R	TGAGAGTCAATTTTTATCTCATGTTTTAGC			
L/M-F	GGATGAAAACTATCAGCATCTGAAG	785	*repA, B, C*	(Carattoli *et al.* 2005)
L/M-R	CTGCAGGGGCGATTCTTTAGG			
N-F	GTCTAACGAGCTTACCGAAG	559	*repA*	(Carattoli *et al.* 2005)
N-R	GTTTCAACTCTGCCAAGTTC			
FIA-F	CCATGCTGGTTCTAGAGAAGGTG	462	iterons	(Carattoli *et al.* 2005)
FIA-R	GTATATCCTTACTGGCTTCCGCAG			
FIB-F	GGAGTTCTGACACACGATTTTCTG	702	*repA*	(Carattoli *et al.* 2005)
FIB-R	CTCCCGTCGCTTCAGGGCATT			
W-F	CCTAAGAACAACAAAGCCCCCG	242	*repA*	(Carattoli *et al.* 2005)
W-R	GGTGCGCGGCATAGAACCGT			
Y-F	AATTCAAACAACACTGTGCAGCCTG	765	*repA*	(Carattoli *et al.* 2005)
Y-R	GCGAGAATGGACGATTACAAAACTTT			
P-F	CTATGGCCCTGCAAACGCGCCAGAAA	534	iterons	(Carattoli *et al.* 2005)
P-R	TCACGCGCCAGGGCGCAGCC			
FIC-F	GTGAACTGGCAGATGAGGAAGG	262	*repA2*	(Carattoli *et al.* 2005)
FIC-R	TTCTCCTCGTCGCCAAACTAGAT			

Primer	Sequence (5'-3')	Product Size (bp)	Target	Reference
A/C-F	GAGAACCAAAGACAAAGACCTGGA	465	*repA*	(Carattoli *et al.* 2005)
A/C-R	ACGACAAACCTGAATTGCCTCCTT			
T-F	TTGGCCTGTTTGTGCCTAAACCAT	750	*repA*	(Carattoli *et al.* 2005)
T-R	CGTTGATTACACTTAGCTTTGGAC			
K/B-F	GCGGTCCGGAAAGCCAGAAAAC	160	RNAI	(Carattoli *et al.* 2005)
K-R	TCTTTCACGAGCCCGCCAAA			
K/B-F	GCGGTCCGGAAAGCCAGAAAAC	159	RNAI	(Carattoli *et al.* 2005)
B/O-R	TCTGCGTTCCGCCAAGTTCGA			

Appendix D

Microarray Targets

	Target Group	Array target	Target name, host (if applicable)
Controls	*E. coli* **Controls**	gad	Glutamate decarboxylase
		ihfA	Integration host factor, subunit A (various sp.)
		gapA	Glyceraldehydes-3-phosphate (various sp.)
	Staining control	biotin	Biotin label
Virulence Factors	**Adhesins**	nfaE	Non-fimbrial adhesin
		efa1	EHEC factor for adherence (EHEC)
		Iha	Enterobactin receptor/adhesin (EHEC/UPEC)
		Saa	STEC autoagglutinating adhesin (STEC)
		rpeA	Adhesin (rabbit-specific EPEC)
		CS31A	CS31A adhesin (animal-pathogenic ETEC)
		lngA	CS21 adhesin (ETEC)
	Toxin	astA	EAST1 (EAEC)
		cdtB	Cytolethal distending toxin B
		cnf1/2/3	Cytotoxic necrotising factor 1, 2 & 3
		hlyA	Haemolysin A
		hlyE	Haemolysin E
		ipaD	Invasive plasmid antigen D (EIEC, *Shigella* sp.)
		ipaH	Invasive plasmid antigen H (EIEC, *Shigella* sp.)
		ltcA	Heat-labile enterotoxin (*Vibrio* & *Citrobacter* sp.)
		Pet	Plasmid encoded toxin (EAEC)
		senB	Enterotoxin (*Shigella* sp.)
		Sta	STa, heat-stable enterotoxin (ETEC)
		Stb	STb, heat-stable enterotoxin (ETEC)
		stx1a	Shiga-toxin 1 (EHEC/STEC)
		stx1b	Shiga-toxin 2 (EHEC/STEC)
		virF	Virulence transcription factor (*Shigella* sp., EAEC)
		subA	Subtilase cytotoxin subunit A (EHEC/STEC)
		toxB	Toxin B (EHEC/STEC)
		Tsh	Temperature sensitive haemagglutinin (APEC)
	Bacteriocin	Cba	Colicin B
		Cda	Colicin D
		Ccl	Cloacin
		celB	Colicin E lysis gene
		Cma	Colicin M
		mchB/C/F	Microcin H47
		mcmA	Microcin M
	Miscellaneous	katP	Catalase peroxidise (EHEC/STEC)

	Target Group	Array target	Target name, host (if applicable)
	Fimbriae	K88	K88 fimbriae (ETEC)
		bfpA	Bundle forming pilus (EPEC)
		cfaC	Colonisation factor antigen C
		cofA	CFA major pilin subunit
		F17	F17 fimbriae (animal-pathogenic ETEC)
		fanA	K99 fimbriae (animal-pathogenic ETEC)
		fasA	987P fimbriae (animal-pathogenic ETEC)
		fedA	F107 fimbriae (animal-pathogenic ETEC)
		fedF	F18 fimbriae (animal-pathogenic ETEC/STEC)
		fim41a	F41 fimbriae (animal-pathogenic ETEC)
		lngA	Longus type IV pilus (ETEC)
		perA	Plasmid encoded regulator of BFP (EPEC)
		prfB	P-related fimbriae
		sfaS	S fimbriae
		papB	P-fimbriae
		lpfA	Long polar fimbriae (EHEC/EPEC)
	Siderophores/iron acquisition	ireA	Iron-regulated element
		iroN	Novel siderophore
		hemL	Heme biosynthesis gene
	Serum resistance	Iss	Increased serum survival protein
	Type III Secretion System (T3SS) proteins	Cif	Cycle inhibiting factor
		eaaA	Serine protease (commensal *E. coli*)
		eatA	Serine protease (ETEC)
		epeA	Serine protease (EHEC)
		espA	Serine protease (EHEC/EPEC)
		espB	Serine protease (EHEC/EPEC)
		espC	Serine protease (EHEC/EPEC)
		espF	Serine protease (EHEC/EPEC)
		espI	Serine protease (EHEC/EPEC)
		espJ	Serine protease (EHEC/EPEC)
		espP	Serine protease (EHEC/EPEC)
		nleA	Non-LEE encoded protein (EHEC/EPEC)
		sepA	Secreted protein (*Shigella* & *Salmonella* sp. E. coli)
		Vat	Vacuolating autotransporter toxin
		Pic	Protein involved in intestinal colonisation (EAEC)
		Sat	Secreted autotransporter toxin (DAEC)
		sigA	Serine protease (*Shigella* sp.)
		tccP	Tir cytoskeleton coupling protein (EHEC/EPEC)
		Tir	Translocated Intimin receptor (EHEC/EPEC)
		eae	Intimin (EHEC/EPEC)
	T2SS	etpD	Serine protease (EHEC/STEC)
Genome	**DNA fragment**	IS285	Insertion sequence 285 (*Yersinia* sp.)

	Target Group	Array target	Target name, host (if applicable)
Antibiotic Resistance	**Aminoglycosides**	armA	16S rRNA methyltransferase
		npmA	16S rRNA methyltransferase
		Grm	Gentamicin resistance methylase
		rmtA/B/C/D	16S rRNA methyltransferase
		Rrs	16S rRNA (mutations conferring resistance)
		strA/B	Streptomycin resistance protein
		aac3I	3'-*N*-acetyltransferase
		aac3Iva	3'-*N*-acetyltransferase
		aac3Ia	3'-*N*-acetyltransferase
		aac3Ib	3'-*N*-acetyltransferase
		aac3Ic	3'-*N*-acetyltransferase
		aac3Ie	3'-*N*-acetyltransferase
		aac6II	6'-*N*-acetyltransferase
		aac6IIa	6'-*N*-acetyltransferase
		aac6IIc	6'-*N*-acetyltransferase
		aac6Ib	6'-*N*-acetyltransferase
		aac6	6'-*N*-acetyltransferase
		aac-aph	*N*-Acetyltransferase-*O*-phosphotransferase
		aadA1	3'-*O*-nucleotidyltransferase
		aadA12	3'-*O*-nucleotidyltransferase
		aadA13	3'-*O*-nucleotidyltransferase
		aadA15	3'-*O*-nucleotidyltransferase
		aadA2	3'-*O*-nucleotidyltransferase
		aadA23b	3'-*O*-nucleotidyltransferase
		aadA3	3'-*O*-nucleotidyltransferase
		aadA4	3'-*O*-nucleotidyltransferase
		aadA5	3'-*O*-nucleotidyltransferase
		aadB	2'-*O*-nucleotidyltransferase
		aphA	3'- *O*-phosphotransferase
	Rifampin	arr-1	ADP-ribosylating transferase (*Mycobacterium* sp.)
		arr-4	ADP-ribosylating transferase (*Pseudomonas* sp.)
		arr-5	ADP-ribosylating transferase (*Klebsiella* sp.)
		arr-6	ADP-ribosylating transferase (*Pseudomonas* sp.)
	Erythromycin	Mrx	Macrolide inactivation operon protein
		Mph	Macrolide phosphotransferase
		ereA	Erythromycin esterase A
		ereB	Erythromycin esterase B
		ermB	Erythromycin rRNA methyltransferase
	Streptogramin	vatE	Streptogramin acetyltransferase
		Sph	Streptomycin phosphotransferase
	Chloramphenicol	catB	Chloramphenicol acetyltransferase
		cmlA1	Chloramphenicol efflux pump
		floR	Florphenicol exporter protein

	Target Group	Array target	Target name, host (if applicable)
Antibiotic Resistance	**Trimethoprim & Sulfamethoxazole**	Dfr	Dihydrofolate reductase (trimethoprim)
		Sul	Dihydropteroate synthase (sulfamethoxazole)
	Tetracycline	Tet	Tetracycline resistance protein
	Quinolones	qepA	Quinolone efflux pump
		qnrB	DNA gyrase/topoisomerase IV protection protein
		qnrD	DNA gyrase/topoisomerase IV protection protein
		qnrS	DNA gyrase/topoisomerase IV protection protein
	Glycopeptides	Ble	Bleomycin resistance protein
	Broad-spectrum penicillins (Beta-lactamase)	tem1	Beta-lactamase TEM-1
		oxa	Beta-lactamase OVA variants
		shv1	Beta-lactamase SHV-1
		LAP-1	Beta-lactamase LAP-1
		OKP	Beta-lactamase (*Klebsiella* & *Serratia* sp.)
		CTX-M gp 1	Extended-spectrum beta-lactamase
		CTX-M gp 2	Extended-spectrum beta-lactamase
		CTX-M gp 8	Extended-spectrum beta-lactamase
		CTX-M gp 9	Extended-spectrum beta-lactamase
		CTX-M gp 26	Extended-spectrum beta-lactamase
		CTX-M gp 64	Extended-spectrum beta-lactamase
		veb1	Extended-spectrum beta-lactamase VEB-1
		pse1	Extended-spectrum beta-lactamase PSE-1
		per2	Extended-spectrum beta-lactamase PER
		len1	Extended-spectrum beta-lactamase (*Klebsiella*)
		ges1	(*Pseudomonas* sp.)
		carb	Extended-spectrum beta-lactamase CARB
		Cep	Beta-lactamase (*Aeromonas* sp.)
		kluc	Extended-spectrum beta-lactamase KLUC
	Broad-spectrum penicillins (AmpC beta-lactamase)	cmy	AmpC beta-lactamase CMY
		acc1/2	AmpC beta-lactamase ACC-1 & ACC-2
		act1	AmpC beta-lactamase ACT-1
		mox	AmpC beta-lactamase, MOX family
		Fox	AmpC beta-lactamase, FOX family
		dha1	AmpC beta-lactamase DHA-1
		Mir	AmpC beta-lactamase MIR
	Broad-spectrum penicillins (Metallo beta-lactamase)	KHM-1	Metallo beta-lactamase (*Citrobacter* sp.)
		Gim1	Metallo beta-lactamase, German-origin
		IMP	Metallo beta-lactamase
		VIM	Metallo beta-lactamase
		KPC	Metallo beta-lactamase
		Sfh1	Metallo beta-lactamase (*Serratia* sp.)

	Target Group	Array target	Target name, host (if applicable)
	Broad-spectrum penicillins (Carbapenemase)	sme1	Carbapenemase (*Serratia* sp.)
		spm1	Carbapenemase, Brazil-origin
		imi3	Carbapenemase IMI
Antibiotic Resistance	**Integrons**	intI1	Class I integrase
		intI2	Class II integrase
	dnaE	dnaE	DNA polymerase III alpha-subunit
Serotype	**Flagellin (fliC gene)**	H01	H1-antigen
		H02	H2-antigen
		H04	H4-antigen
		H05	H5-antigen
		H06	H6-antigen
		H07	H7-antigen
		H08	H8-antigen
		H09	H9-antigen
		H10	H10-antigen
		H11	H11-antigen
		H12	H12-antigen
		H14	H14-antigen
		H15	H15-antigen
		H16	H16-antigen
		H18	H18-antigen
		H19	H19-antigen
		H20	H20-antigen
		H21	H21-antigen
		H23	H23-antigen
		H24	H24-antigen
		H25	H25-antigen
		H26	H26-antigen
		H27	H27-antigen
		H28	H28-antigen
		H28/46	H28/46-antigen
		H29	H29-antigen
		H30	H30-antigen
		H31	H31-antigen
		H32	H32-antigen
		H33	H33-antigen
		H34	H34-antigen
		H37	H37-antigen
		H38	H38-antigen
		H39	H39-antigen
		H41	H41-antigen
		H42	H42-antigen
		H43	H43-antigen
		H45	H45-antigen
		H46	H46-antigen
		H48	H48-antigen

	Target Group	Array target	Target name, host (if applicable)
Serotype	**Flagellin (fliC gene)**	H49	H49-antigen
		H51	H51-antigen
		H52	H52-antigen
		H56	H56-antigen
	O-antigen flipase (wzx) & polymerase (wzy)	O103	O103-antigen flipase & polymerase
		O104	O104-antigen flipase & polymerase
		O111	O111-antigen flipase & polymerase
		O113	O113-antigen flipase & polymerase
		O114	O114-antigen flipase & polymerase
		O121	O121-antigen flipase & polymerase
		O128	O128-antigen flipase & polymerase
		O15	O15-antigen flipase & polymerase
		O157	O157-antigen flipase & polymerase
		O172	O172-antigen flipase & polymerase
		O26	O26-antigen flipase & polymerase
		O55	O55-antigen flipase & polymerase
		O6	O6-antigen flipase & polymerase
		O7	O7-antigen flipase & polymerase
		O79	O79-antigen flipase & polymerase
		O8	O8-antigen flipase & polymerase
		O86	O86-antigen flipase & polymerase
		O9	O9-antigen flipase & polymerase
		O91	O91-antigen flipase & polymerase
	Other	fl-H40	H40-antigen
		fl-H-NM	H-antigen non-motile
		fljA	Phase-1 flagellin repressor
		flkA-H03	H3 flagellin, non-fliC locus
		flkA-H53	H53 flagellin, non-fliC locus
		flmA-H54	H54 flagellin, non-fliC locus
		isla29-O145	O145 genomic island
		rfbE-O157	O157 ABC transporter
		rfbU-O157	O157 glycosyltransferase
		sil1-O157	O157 silver resistance gene
		sil-inv-O145	O145 silver resistance gene
		wbdA-O9	O9 mannosyltransferase gene
		wbdA-O9a	O9a mannosyltransferase gene
		wbd-O111	O111 glycosyltransferase gene
		wbd-O79	O79 glycosyltransferase gene
		wzm-O52	O52 ABC transporter
		wz-O101	O101 O-antigen alternative gene

Appendix E

Microarray isolates

No.	Study No.	Specimen	Type/source	Age (years)	Sex	Setting	Phylo.	Serogroup	ST	VF PCR
1	002	Blood	GU	73	M	HAI	A	O162	NK	Y
2	008	Blood	NK	25	M	HAI	A	NT	NK	
3	190	Blood	GU	90	F	CAI	B2	O25	131	Y
4	192	Urine	COMP	90	F	CAI	B2	O25	131	Y
5	218	Urine	COMP	83	M	CAI	A	NT	NK	Y
6	219	Blood	GU	83	M	CAI	A	NT	NK	Y
7	295	Blood	CHEST	80	M	CAI	D	O31	NK	Y
8	348	Blood	GU	88	M	CAI	B2	O25	131	Y
9	349	Urine	COMP	88	M	CAI	B2	O25	131	Y
10	445	Blood	GIT	61	M	HAI	A	NT	NK	Y
11	520	Blood	NK	77	M	HAI	B2	O25	131	
12	561	Blood	GIT	55	M	HAI	A	O8	NK	Y
13	025	Urine	COMP	22	F	CAI	D	O44	69	Y
14	026	Blood	GU	22	F	CAI	D	O44	69	Y
15	090	Blood	GU	88	F	CAI	D	NT	1405	Y
16	091	Urine	COMP	88	F	CAI	D	NT	1405	Y
17	152	Urine	COMP	87	F	CAI	D	O7	62	Y
18	153	Blood	GU	87	F	CAI	D	O7	62	Y
19	187	Urine	COMP	52	F	CAI	B2	NT	405	Y
20	188	Blood	GU	52	F	CAI	B2	NT	405	Y
21	189	Blood	GU	81	M	CAI	B2	O2	95	Y
22	197	Urine	COMP	81	M	CAI	B2	O2	95	Y
23	230	Urine	COMP	69	F	HAI	B2	O2	73	Y
24	231	Blood	GU	69	F	HAI	B2	O2	73	Y
25	293	Blood	GU	44	F	CAI	D	NT	405	Y
26	294	Urine	COMP	44	F	CAI	D	NT	405	Y
27	366	Urine	COMP	32	F	CAI	B2	O75	404	Y
28	367	Blood	GU	32	F	CAI	B2	O75	404	Y
29	494	Blood	GU	63	M	HAI	B2	O6	127	Y
30	495	Urine	COMP	63	M	HAI	B2	O6	127	Y
31	536	Blood	GU	88	F	CAI	B2	O6	73	Y
32	537	Urine	COMP	88	F	CAI	B2	O6	73	Y
33	538	Blood	GU	31	F	CAI	D	O125ab	69	Y
34	539	Urine	COMP	31	F	CAI	D	O125ab	69	Y
35	542	Blood	GU	31	F	HAI	D	O11	69	Y
36	543	Urine	COMP	31	F	HAI	D	O11	69	Y

No.	Study No.	Specimen	Type/source	Age (years)	Sex	Setting	Phylo.	Serogroup	ST	VF PCR
37	548	Blood	GU	27	F	CAI	B2	O18ac	14	Y
38	549	Urine	COMP	27	F	CAI	D	O153	354	Y
39	566	Urine	COMP	26	F	CAI	B2	NK	95	Y
40	567	Blood	GU	26	F	CAI	B2	NK	95	Y
41	3352	Blood	GU	87	F	CAI	D	NK	NK	
42	3381	Blood	NK	33	F	CAI	B2	NK	95	Y
43	3403	Blood	GIT	21	F	CAI	B2	O1	95	
44	3413	Blood	NK	63	M	HAI	B2	O16	NK	
45	3437	Blood	GU	26	F	CAI	B2	NK	95	
46	3456	Blood	GU	88	F	CAI	B2	NK	73	
47	3465	Blood	GU	35	F	CAI	B2	NK	95	
48	3487	Blood	NK	31	F	HAI	D	NK	NK	
49	3497	Blood	GU	57	F	CAI	B2	NK	73	
50	3517	Blood	GU	25	F	CAI	B2	O1	95	
51	3544	Blood	NK	43	F	CAI	B2	NT	95	
52	3566	Blood	GU	88	F	CAI	B2	O25	131	
53	3713	Blood	NK	81	M	CAI	B2	O25	131	
54	3856	Blood	NK	69	F	HAI	B2	O25	131	
55	007	Blood	NK	39	M	CAI	B1	NK	NK	
56	156	Urine	COMP	44	F	CAI	B2	O6	NK	Y
57	302	Urine	COMP	56	M	CAI	D	NT	NK	Y
58	384	Urine	COMP	54	F	CAI	A	O12	NK	Y
59	020	Urine	COMP	75	F	HAI	A	O135	NK	
60	113	Urine	COMP	79	M	HAI	A	O1	NK	Y
61	463	Urine	COMP	23	M	HAI	B2	NK	NK	Y
62	065	Urine	ABU	18	F	CAI	A	O5	NK	Y
63	100	Urine	ABU	24	F	CAI	B1	O8	NK	
64	226	Urine	ABU	64	F	CAI	D	NT	NK	
65	354	Urine	ABU	30	F	CAI	B1	NT	NK	
66	370	Urine	ABU	24	F	CAI	B2	NK	NK	Y
67	049	Urine	UC	77	F	HAI	B2	NT	NK	
68	058	Urine	UC	26	F	CAI	B1	NT	NK	
69	108	Urine	UC	32	F	CAI	B1	O8	NK	
70	122	Urine	COMP	61	M	CAI	B1	O8	NK	
71	017	Urine	COMP	6	F	CAI	D	O17	69	
72	024	Urine	UC	3	F	CAI	D	O86	NK	
73	085	Urine	UC	6	F	CAI	D	O135	NK	Y
74	109	Urine	UC	7	F	CAI	B2	NT	NK	
75	125	Urine	COMP	8	F	HAI	B2	O7	NK	
76	014	Urine	UC	98	F	CAI	D	O102	NK	
77	107	Urine	UC	99	F	CAI	B2	O4	NK	Y
78	285	Urine	COMP	90	M	HAI	B2	O25	131	

No.	Study No.	Specimen	Type/source	Age (years)	Sex	Setting	Phylo.	Serogroup	ST	VF PCR
79	425	Urine	COMP	93	F	HAI	B2	NK	NK	
80	478	Urine	COMP	91	M	CAI	B2	O25	131	
81	087	Urine	ABU	29	F	CAI	B2	O6	NK	Y
82	150	Urine	UC	59	F	CAI	B1	NT	NK	
83	157	Urine	COMP	60	M	CAI	B2	O18ac	NK	
84	193	Urine	UC	23	F	CAI	A	NT	NK	
85	420	Urine	COMP	63	F	CAI	D	NK	NK	
86	182	Urine	ABU	26	F	CAI	A	O62	NK	
87	201	Urine	COMP	37	F	CAI	B2	O39	NK	
88	240	Urine	COMP	56	M	CAI	B2	NT	NK	
89	316	Urine	COMP	46	M	CAI	B2	O25	NK	
90	422	Urine	ABU	35	F	CAI	B2	NK	NK	Y
91	028	Urine	UC	40	F	CAI	A	O93	NK	
92	122	Urine	COMP	61	M	CAI	B1	O8	NK	
93	253	Urine	COMP	57	M	CAI	A	O20	NK	Y
94	356	Urine	UC	34	F	CAI	A	NT	NK	Y
95	374	Urine	UC	74	F	CAI	B2	NK	NK	Y
96	25922	Control	NA	NA	NA	NA	NA	NA	NA	

NA, not applicable; NK, unknown; NT, non-typeable; GU, genitourinary source; GIT, gastrointestinal source; CSF, cerebral spinal fluid source; CHEST, respiratory tract source; LINE, intravenous line source; SSTI, skin and soft tissue infection source; ABU, asymptomatic bacteruria; UC, uncomplicated cystitis; COMP, complicated cystitis/pyelonephritis; CAI, community-associated infection; HAI, hospital-associated infection; VF PCR, isolates also analysed for virulence factors using the six multiplex PCR assays; Y, yes

Virulence factor PCR isolates

No.	Specimen Type/ source	Age	Sex	Setting	Phylo.	Sero.	ST	CFT073 I	CFT073 II	J96 I	J96 II	536 I	536 II	536 III	536 IV	Array
1	U ABU	32	F	CAI	B2	O25	131	•								
2	U ABU	18	F	CAI	A	O5	NK								•	Y
3	U ABU	29	F	CAI	B2	O6	NK	•	•	•	•				•	Y
4	U ABU	23	F	CAI	D	NT	NK								•	
5	U ABU	26	F	CAI	D	NT	69									
6	U ABU	35	F	CAI	D	O77	69								•	
7	U ABU	20	F	CAI	B2	O119	NK	•							•	
8	U ABU	22	F	CAI	B2	O158	73	•	•		•	•			•	
9	U ABU	31	F	CAI	B2	O25	131		•						•	
10	U ABU	29	F	CAI	D	O17	69	•							•	
11	U ABU	38	F	CAI	D	NT	69									
12	U ABU	29	F	CAI	D	NK	69								•	
13	U ABU	34	F	CAI	B2	O6	127	•	•		•	•	•	•	•	
14	U ABU	24	F	CAI	B2	NK	NK		•		•				•	Y
15	U ABU	37	F	CAI	B2	NK	NK									
16	U ABU	32	F	CAI	B2	NK	NK	•	•		•				•	
17	U ABU	35	F	CAI	B2	NK	NK								•	Y
18	U ABU	41	F	CAI	D	NK	NK								•	
19	U ABU	22	F	CAI	D	NK	NK		•						•	
20	U ABU	35	F	CAI	A	O141	NK									
21	U ABU	19	F	CAI	D	NK	NK								•	
22	U ABU	20	F	CAI	B2	NK	NK								•	
23	U ABU	35	F	CAI	A	NK	NK									
24	U ABU	29	F	CAI	B2	NK	NK								•	
25	U ABU	31	F	CAI	B2	O6	127	•				•		•	•	
26	U ABU	26	F	CAI	D	NK	NK								•	
27	U ABU	27	F	CAI	A	NK	NK									
28	U ABU	29	F	CAI	A	NK	NK									
29	U ABU	37	F	CAI	B2	NK	NK	•	•			•	•		•	
30	U ABU	29	F	CAI	B2	NK	NK	•	•	•					•	
31	U COMP	75	M	CAI	A	O150	NK									
32	U COMP	79	M	HAI	A	O1	NK									Y
33	U COMP	41	M	CAI	B2	O2	73	•	•	•					•	
34	U COMP	37	F	CAI	D	O73	69									
35	U COMP	44	F	CAI	B2	O6	NK	•	•						•	Y
36	U COMP	80	M	HAI	B2	O22	73	•	•		•	•			•	
37	U COMP	27	F	CAI	B2	O25	95	•	•						•	
38	U COMP	54	M	HAI	B2	O1	95	•	•						•	

No.	Specimen	Type/ source	Age	Sex	Setting	Phylo.	Sero.	ST	CFT073 I	CFT073 II	J96 I	J96 II	536 I	536 II	536 III	536 IV	Array
39	U	COMP	48	F	HAI	B2	O25	127	•	•						•	
40	U	COMP	57	M	CAI	A	O20	NK								•	Y
41	U	COMP	67	M	CAI	B2	NT	127	•	•	•	•	•			•	
42	U	COMP	56	M	CAI	D	NT	NK								•	Y
43	U	COMP	49	F	CAI	B2	NK	NK	•	•		•	•			•	
44	U	COMP	71	F	CAI	B2	NK	NK		•		•				•	
45	U	COMP	54	F	CAI	A	O12	NK								•	Y
46	U	COMP	50	F	CAI	A	O135	NK								•	
47	U	COMP	62	F	CAI	B2	O25	131		•						•	
48	U	COMP	61	M	CAI	B1	O8	NK									
49	U	COMP	79	F	CAI	B2	NK	NK		•		•				•	
50	U	COMP	65	M	HAI	B2	O25	131		•						•	
51	U	COMP	52	F	CAI	B2	NT	95		•						•	
52	U	COMP	36	M	HAI	D	O153	69	•							•	
53	U	COMP	23	M	HAI	B2	NK	NK								•	Y
54	U	COMP	1	F	CAI	B2	O2	95	•	•						•	
55	U	COMP	27	F	CAI	B2	NK	NK		•			•			•	
56	U	COMP	73	M	CAI	B2	O6	127	•	•			•		•	•	
57	U	COMP	80	F	HAI	B2	NK	NK	•	•			•			•	
58	U	COMP	23	F	CAI	B2	NK	NK								•	
59	U	COMP	82	M	CAI	B2	NK	NK								•	
60	U	COMP	84	F	CAI	D	NK	NK								•	
61	U	COMP	22	F	CAI	D	O44	69								•	Y
62	U	COMP	88	F	CAI	D	NT	1405	•								Y
63	U	COMP	87	F	CAI	D	O7	62								•	Y
64	U	COMP	52	F	CAI	B2	NT	405	•							•	Y
65	U	COMP	90	M	CAI	B2	O25	131	•	•		•		•		•	Y
66	U	COMP	81	F	CAI	B2	O2	95	•							•	Y
67	U	COMP	83	M	CAI	A	NT	617	•							•	Y
68	U	COMP	69	F	HAI	B2	O2	73	•	•			•			•	Y
69	U	COMP	44	F	CAI	D	NT	405	•							•	Y
70	U	COMP	88	M	CAI	B2	NT	131		•		•				•	Y
71	U	COMP	32	F	CAI	B2	O75	404		•						•	Y
72	U	COMP	63	M	HAI	B2	O6	127	•	•			•		•	•	Y
73	U	COMP	88	F	CAI	B2	O6	73				•	•			•	Y
74	U	COMP	31	F	CAI	D	O125ab	69		•						•	Y
75	U	COMP	31	F	HAI	D	O11	69								•	Y
76	U	COMP	27	F	CAI	D	O153	354								•	Y

No.	Specimen	Type/ source	Age	Sex	Setting	Phylo.	Sero.	ST	CFT073 I	CFT073 II	J96 I	J96 II	536 I	536 II	536 III	536 IV	Array
77	U	COMP	26	F	CAI	B2	O4	95		•			•	•		•	Y
78	U	UC	35	F	HAI	B2	O4	73	•	•						•	
79	U	UC	25	F	HAI	D	O77	69									
80	U	UC	65	F	CAI	B2	O6	73	•	•	•	•		•		•	
81	U	UC	33	F	CAI	D	O77	69								•	
82	U	UC	49	F	CAI	A	O87	NK									
83	U	UC	56	F	CAI	B2	O4	95	•	•		•		•		•	
84	U	UC	6	F	CAI	D	O135	NK									Y
85	U	UC	99	F	CAI	B2	O4	NK	•								Y
86	U	UC	29	F	CAI	B2	O18ac	NK	•	•						•	
87	U	UC	40	F	CAI	A	O78	NK	•							•	
88	U	UC	36	F	CAI	D	NT	69								•	
89	U	UC	32	F	CAI	B2	O6	73	•	•						•	
90	U	UC	35	F	CAI	D	O77	69								•	
91	U	UC	7	F	CAI	A	NT	NK								•	
92	U	UC	18	F	CAI	B2	O75	NK		•						•	
93	U	UC	5	F	CAI	B1	NT	NK								•	
94	U	UC	40	F	CAI	B2	O18ac	95	•	•						•	
95	U	UC	9	F	CAI	D	O153	69								•	
96	U	UC	23	F	CAI	A	O8	NK								•	
97	U	UC	34	F	CAI	A	NT	NK								•	Y
98	U	UC	74	F	CAI	B2	NK	NK								•	Y
99	U	UC	78	F	CAI	A	O7	NK									
100	U	UC	10	F	CAI	A	O20	NK									
101	U	UC	50	F	HAI	B2	NK	NK		•						•	
102	U	UC	27	F	CAI	D	NK	NK								•	
103	U	UC	46	F	CAI	D	NK	NK								•	
104	U	UC	21	F	CAI	D	NK	NK									
105	U	UC	70	F	CAI	B2	NK	NK		•		•				•	
106	U	UC	8	F	CAI	A	NK	NK								•	
107	U	UC	28	F	CAI	A	NK	NK								•	
108	B	GU	73	M	HAI	A	O162	NK	•	•						•	Y
109	B	GIT	43	M	HAI	B2	O22	NK	•	•			•			•	
110	B	CHEST	76	M	HAI	B2	NT	NK								•	
111	B	CHEST	80	M	CAI	D	O31	NK	•							•	Y
112	B	GIT	75	M	CAI	D	O31	NK	•							•	
113	B	GIT	61	M	HAI	A	NT	NK	•								Y
114	B	GIT	55	M	HAI	A	O8	NK									Y

No.	Specimen	Type/ source	Age	Sex	Setting	Phylo.	Sero.	ST	CFT073 I	CFT073 II	J96 I	J96 II	536 I	536 II	536 III	536 IV	Array
115	B	CSF	0	M	HAI	B2	NK	95	•	•						•	
116	B	CHEST	99	F	CAI	D	NK	NK								•	
117	B	GU	83	M	CAI	D	NK	NK	•							•	
118	B	GIT	60	F	CAI	B2	O25	131	•	•						•	
119	B	LINE	43	M	HAI	B2	NK	95	•	•			•	•		•	
120	B	NK	33	F	CAI	B2	NK	95	•	•			•	•		•	Y
121	B	GIT	91	F	HAI	B2	NK	95	•	•						•	
122	B	LINE	54	F	CAI	B2	O25	131	•	•			•	•		•	
123	B	GU	52	M	CAI	B2	O25	131	•	•						•	
124	B	LINE	19	M	HAI	B2	NK	127	•								
125	B	CHEST	35	M	HAI	B2	O25	131	•	•						•	
126	B	SSTI	45	F	CAI	B2	NK	NK	•	•	•					•	
127	B	GIT	78	M	CAI	D	NK	NK									
128	B	SSTI	88	F	HAI	B2	NK	NK	•	•						•	
129	B	GIT	64	F	HAI	B2	O2	73	•	•			•			•	
130	B	GIT	64	M	CAI	B2	O27	127	•	•	•		•	•		•	
131	B	LINE	55	M	HAI	A	O36	69									
132	B	SSTI	90	F	CAI	A	O11	69								•	
133	B	CHEST	98	M	CAI	B2	NK	NK	•	•						•	
134	B	LINE	98	F	CAI	D	NK	NK		•						•	
135	B	NK	60	M	CAI	B2	O25	131	•	•						•	
136	B	LINE	59	F	HAI	B2	O6	73	•	•				•		•	
137	B	SSTI	73	M	HAI	B2	O18ab	NK	•	•						•	
138	B	GU	22	F	CAI	D	O44	69								•	Y
139	B	GU	88	F	CAI	D	NT	1405	•							•	Y
140	B	GU	87	F	CAI	D	O7	62								•	Y
141	B	GU	52	F	CAI	B2	NT	405	•							•	Y
142	B	GU	81	F	CAI	B2	O2	95	•							•	Y
143	B	GU	90	M	CAI	B2	O25	131	•	•		•		•		•	Y
144	B	GU	83	M	CAI	A	NT	617	•							•	Y
145	B	GU	69	F	HAI	B2	O2	73	•	•			•			•	Y
146	B	GU	44	F	CAI	D	NT	405	•							•	Y
147	B	GU	88	M	CAI	B2	NT	131	•	•			•			•	Y
148	B	GU	32	F	CAI	B2	O75	404		•						•	Y
149	B	GU	63	M	HAI	B2	O6	127	•	•			•		•	•	Y
150	B	GU	88	F	CAI	B2	O6	73				•	•			•	Y

No.	Specimen	Type/ source	Age	Sex	Setting	Phylo.	Sero.	ST	CFT073 I	CFT073 II	J96 I	J96 II	536 I	536 II	536 III	536 IV	Array
151	B	GU	31	F	CAI	D	O125ab	69								•	Y
152	B	GU	31	F	HAI	D	O11	69								•	Y
153	B	GU	27	F	CAI	B2	O18ac	14	•	•						•	Y
154	B	GU	26	F	CAI	B2	O4	95		•			•	•		•	Y

B, bloodstream isolate; U, urinary isolate; NK, unknown; NT, non-typeable; GU, genitourinary source; GIT, gastrointestinal source; CSF, cerebral spinal fluid source; CHEST, respiratory tract source; LINE, intravenous line source; SSTI, skin and soft tissue infection source; ABU, asymptomatic bacteruria; UC, uncomplicated cystitis; COMP, complicated cystitis/pyelonephritis; CAI, community-associated infection; HAI, hospital-associated infection; Array, isolates also analysed by microarray; Y, yes

Appendix F

Galleria isolates

No.	Specimen	Type/source	Age (years)	Sex	Setting	Phylo.	Sero.	ST	VF PCR
1	B	CHEST	76	M	HAI	B2	NT	NK	*fyuA*
2	U	UC	65	F	CAI	B2	O6	73	*fimH pap sfa/foc iha hlyD sat fyuA iutA iroN kpsII usp traT pks malX*
3	U	UC	49	F	CAI	A	O87	NK	*fimH sat*
4	U	UC	56	F	CAI	B2	O4	95	*fimH pap hra hlyD cnf1 fyuA iroN kpsII usp pks malX*
5	U	UC	6	F	CAI	D	O135	NK	*fimH* EAST1 *kpsII*
6	U	ABU	29	F	CAI	B2	O6	NK	*fimH pap sfa/foc hlyD cnf1 fyuA iroN kpsII usp ompT pks malX*
7	U	COMP	79	M	HAI	A	O1	NK	*fimH*
8	U	COMP	41	M	CAI	B2	O2	73	*fimH pap sfa/foc iha cdtB hlyD sat fyuA iutA iroN usp traT pks malX*
9	B	GU	90	F	CAI	B2	O25	131	*pap iha cnf1 iutA kpsII ompT iss*
10	U	COMP	90	F	CAI	B2	O25	131	*pap afa/dra iha cnf1 iutA kpsII usp ompT iss*
11	U	COMP	83	M	CAI	A	NT	617	*traT*
12	B	GU	83	M	CAI	A	NT	617	*traT*
13	U	ABU	20	F	CAI	B2	O119	NK	*fimH gafD iha sat fyuA iutA kpsII* K5 *usp malX*
14	U	COMP	57	M	CAI	A	O20	NK	*fimH pap bmaE hra cnf1 hlyD fyuA iutA traT pks*
15	U	ABU	22	F	CAI	B2	O158	73	*fimH pap sfa/foc iha cnf1 hlyD fyuA usp traT pks malX*
16	U	UC	5	F	CAI	B1	NT	NK	*fimH pap iha fyuA iutA kpsII*
17	U	COMP	67	M	CAI	B2	NT	127	*fimH bmaE hra cnf1 hlyD fyuA kpsII usp pks malX*
18	B	CHEST	80	M	CAI	D	O31	NK	*fimH fyuA iutA kpsII usp traT malX*
19	U	ABU	34	F	CAI	B2	O6	127	*fimH pap sfa/foc cnf1 hlyD fyuA iroN usp pks malX*
20	U	UC	9	F	CAI	D	O153	69	*fimH pap iha sat fyuA iutA kpsII traT ompT*

No.	Specimen	Type/source	Age (years)	Sex	Setting	Phylo.	Sero.	ST	VF PCR
21	B	GIT	75	M	CAI	D	O31	NK	*fyuA iutA traT malX*
22	U	COMP	54	F	CAI	A	O12	NK	*fimH fyuA kpsII traT*
23	U	COMP	50	F	CAI	A	O135	NK	*fimH fyuA kpsIII*
24	U	COMP	61	M	CAI	B1	O8	NK	*fimH pap hlyF iutA iroN traT iss*
25	U	ABU	41	F	CAI	D	NK	NK	*fimH fyuA traT*
26	U	COMP	1	F	CAI	B2	O2	95	*fimH pap hlyF fyuA iutA iroN ireA kpsII K1 usp traT iss cvaC pks malX*
27	U	ABU	35	F	CAI	A	NK	NK	*fimH*
28	U	COMP	27	F	CAI	D	O153	354	*fyuA usp ibeA*
29	U	ABU	29	F	CAI	B2	NK	NK	*fimH sfa/foc bmaE hra cnf1 hlyD sat fyuA iroN usp traT pks malX*
30	B	CSF	0	M	HAI	B2	NK	95	*fimH pap sfa/foc cdtB hlyF fyuA iutA iroN kpsII* K1 *usp traT ibeA iss cvaC pks malX*
31	B	CHEST	99	F	CAI	D	NK	NK	*fimH fyuA*
32	B	GIT	60	F	CAI	B2	O25	131	*clpG iha sat fyuA iutA usp traT malX*
33	B	GIT	91	F	HAI	B2	NK	95	*fimH pap hlyF vat fyuA iutA iroN kpsII* K1 *usp traT iss cvaC pks malX*
34	B	LINE	54	F	CAI	B2	O25	131	*fimH pap iha cnf1 hlyD sat fyuA iutA kpsII usp traT iss malX*
35	B	LINE	19	M	HAI	B2	NK	127	EAST1 *iutA usp traT iss malX*
36	B	SSTI	45	F	CAI	B2	NK	NK	*fimH pap sfa/foc afa/dra iha hlyF tsh fyuA iutA iroN kpsII* K1 *usp ibeA iss cvaC pks malX*
37	B	SSTI	88	F	HAI	B2	NK	NK	*fimH pap iha fyuA iroN kpsII* K1 *usp traT ibeA iss malX*
38	B	GIT	64	M	CAI	B2	O27	127	*fimH pap sfa/foc iha cnf1 hlyD tsh fyuA iroN usp iss pks malX*
39	B	LINE	55	M	HAI	A	O36	69	*fimH fyuA iroN kpsII usp iss pks*
40	B	SSTI	90	F	CAI	A	O11	69	*pap fyuA iutA iroN kpsII* K1 *usp traT iss pks*

NB. B, bacteraemia; U, urinary tract infection; F, female; M, male; CAI, community-associated infection; HAI, hospital-associated infection; NK, unknown; NT, non-typeable; GU, genitourinary source; GIT, gastrointestinal source; CSF, cerebral spinal fluid source; CHEST, respiratory tract source; LINE, intravenous line source; SSTI, skin and soft tissue infection source; ABU, asymptomatic bacteruria; UC, uncomplicated cystitis; COMP, complicated cystitis/pyelonephritis

Pathogenicity islands in the *Galleria* isolates

No.	CFT073 I	CFT073 II	J96 I	J96 II	536 I	536 II	536 III	536 IV
1								Y
2	Y	Y	Y	Y		Y		Y
3								
4	Y	Y		Y		Y		Y
5								
6	Y	Y	Y	Y				Y
7								
8	Y	Y	Y					Y
9	Y	Y		Y		Y		Y
10	Y	Y		Y		Y		Y
11	Y							Y
12	Y							Y
13	Y							Y
14								Y
15	Y	Y		Y	Y			Y
16								Y
17	Y	Y	Y	Y	Y			Y
18	Y							Y
19	Y	Y		Y	Y	Y	Y	Y
20								Y
21	Y							Y
22								Y
23								Y
24								
25								Y
26	Y	Y						Y
27								
28								Y
29	Y	Y	Y					Y
30	Y	Y						Y
31								Y
32	Y	Y						Y
33	Y	Y						Y
34	Y	Y			Y	Y		Y
35	Y							
36	Y	Y	Y					Y
37	Y	Y						Y
38	Y	Y	Y		Y	Y		Y
39								
40								Y