THE MOLECULAR EPIDEMIOLOGY OF BLA-CTX-M ANTIBIOTIC RESISTANCE GENES IN THE FAECAL MICROBIOME OF HUMANS ACQUIRING EXTENDED-SPECTRUM BETA LACTAMASE-PRODUCING ESCHERICHIA COLI

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ABSTRACT

ESBL prevalence is increasing globally and travellers visiting South Asia have high rates of acquisition of CTX-M-producing-*E. coli* (CTX-M-EC). A prospective observational cohort study of volunteers traveling from the UK to South Asia was undertaken to determine the mechanism of CTX-M-producing *E. coli* (CTX-M-EC) acquisition. CTX-M-EC was acquired by 16/18 (89%) of volunteers, and polyclonal acquisition of CTX-M-EC was seen in 8/15 volunteers, suggesting multiple acquisition events during travel. CTX-M-EC clones were detectable in faecal samples at six months after travel for 6/6 volunteers. Indistinguishable pre-travel non-CTX-M-EC were found in post-travel faecal samples after CTX-M-EC had been lost in 5/15 cases. Therefore, pre-travel non-CTX-M *E. coli* remain as a minority population in the gut until the CTX-M-EC are lost. Ten plasmids were sequenced using WGS with short and long reads. Plasmid transfer after filter-mating occurred in CTX-M-EC from 45% of volunteers, suggesting that conjugation also has a role in the human gut. However, in-vivo horizontal transfer of a $bla_{CTX\text{-}M}$ plasmid was not detected. Plasmids are closely related to those previously sequenced, isolated from humans, animals and the natural environment. Determining the mechanism of spread of CTX-M-EC will underpin infection prevention and control practices, lay a foundation for future research, and avert excess mortality in the future.

DEDICATION

To all my family and friends,

especially Mum and Ogwyn, my sister Joanna, and my grandmother, Enid.

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PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

PUBLICATIONS

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Bevan ER, Jones AM, Hawkey PM. Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. J Antimicrob Chemother. 2017 Aug 1;72(8):2145-2155.

CONFERENCE PRESENTATIONS

Edward R. Bevan, Matthew J. Powell, Christopher M. Thomas, Laura J.V. Piddock, Peter M. Hawkey. Molecular Characterisation of Plasmids Encoding *bla*$_{CTX-M}$ from Fecal *Escherichia coli* in Travelers Returning to the UK from South Asia. American Society for Microbiology Microbe 2019, San Francisco, USA, 20-24th June, 2019. Poster #5480.

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Matthew J. Powell, Edward R. Bevan, Christopher M. Thomas, Laura J.V. Piddock, Peter M. Hawkey. The conjugative ability of E.coli bla-CTX-M plasmids from returning travellers. Proceedings of the 27th European Congress of Clinical Microbiology and Infectious Diseases, Vienna, Austria, 22-25th April 2017. Oral presentation #OS0489.

Edward R. Bevan, Alan McNally, Christopher M. Thomas, Laura J.V. Piddock, Peter M. Hawkey. Investigating the mechanism of acquisition of CTX-M-producing E. coli in the faecal microbiome of travellers. British Society for Antimicrobial Chemotherapy, Spring Meeting, London, 14th March 2017. Poster.

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LIST OF ABBREVIATIONS

3GC	Third generation cephalosporin
AIEC	Adherent invasive *E. coli*
AMR	Antimicrobial resistance
BLAST	Basic local alignment search tool
BRIG	BLAST ring image generator
BWA	Burrows-Wheeler aligner
COMABT	The carriage of multiresistant bacteria after travel
CPE	Carbapenemase-producing Enterobacteriaceae
CTX-M-EC	CTX-M-producing *E. coli*
EAEC	Enteroaggregative *E. coli*
ECOR	E. coli reference collection
EDTA	Ethylenediamine tetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBL	Extended spectrum beta lactamase
ESPAUR	English surveillance programme for antimicrobial utilisation and resistance
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extra-intestinal pathogenic *E. coli*
FMT	Faecal microbiota transplantation
GLASS	Global antimicrobial resistance surveillance system
HGT	Horizontal gene transfer
HUS	Haemolytic uraemic syndrome
IS	Insertion sequence
LBB	Luria-Bertani broth
LMPA	Low melting point agarose
MDR	Multi-drug resistant
MEGA	Molecular evolutionary genetics analysis
MESA	Middle Eastern or South Asian
MIC	Minimum inhibitory concentration
MLEE	Multi-locus enzyme electrophoresis
MLST	Multi-locus sequence typing
NCBI	National Center for Biotechnology Information (USA)
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PG	Phylogenetic group
QUAST	Quality assessment tool for genomic assemblies
RAPD	Randomly amplified polymorphic DNA
RAST	Rapid annotation using subsystems technology
RFLP	Restriction fragment length polymorphism
SDW	Sterile distilled water
SMART	Study for monitoring antimicrobial resistance trends

SNP	Single nucleotide polymorphism
ST	Sequence type
TA	Toxin-antitoxin
TBE	tris(hydroxymethyl)aminomethane-borate-EDTA
UPEC	Uro-pathogenic *E. coli*
UTI	Urinary tract infection
WHO	World Health Organisation

Chapter 1: Introduction

1.1 *Escherichia coli*

1.1.1 Origins and discovery

Escherichia coli (*E. coli*) was first described as part of the intestinal flora of a neonate, and named '*Bacterium coli commune'* by the German physician, Theodore Escherich (Escherich, 1885, English translation 1988), then subsequently renamed in honour of Escherich (Castellani and Chalmers, 1919).

E. coli is a rod-shaped gram negative bacterium which is part of the family *Enterobacteriaceae* within the order Enterobacteriales and sits in the class *Gammaproteobacteria* (Adeolu *et al.*, 2016). *E. coli* are motile, non-spore forming, facultative anaerobes, and are usually lactose fermenting bacteria. They form part of the faecal microbiome in mammals (Edwards and Ewing, 1972), and make up <1% of the normal faecal microbiome of humans (Arumugam *et al.*, 2011).

1.1.2 *E. coli* classification and typing

Early investigations into the taxonomic position of *E. coli* were carried out using DNA hybridisation and polynucleotide sequence divergence (Brenner *et al.*, 1972). The earliest use of O antigen serotyping to differentiate *E. coli* strains was carried out in the early 1940s (Kauffmann, 1943; Orskov *et al.*, 1977). Subsequent serotyping schemes were developed utilising the H and K antigens (Orskov *et al.*, 1977).

The differentiation of *E. coli* strains using gel electrophoresis of enzymes produced by each strain (Milkman, 1973), led to the first system for phylogenetic analysis, named multilocus enzyme electrophoresis (MLEE) (Selander and Levin, 1980). In order to carry out phylogenetic analysis, a large *E. coli* reference collection (ECOR) was compiled, which included both pathogenic and commensal strains from humans and animals, aiming to capture the diversity of the *E. coli* species (Ochman and Selander, 1984). The MLEE approach was then applied to the ECOR collection, resulting in the designation of four major *E. coli* phylogenetic groups: A, B1, B2, and D (Selander *et al.*, 1987). Restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) (Desjardins *et al.*, 1995) superseded MLEE for *E. coli* strain discrimination, but confirmed the 4-group phylogenetic designation (Chaudhuri and Henderson, 2012).

The widespread uptake of PCR in clinical and research laboratories led Clermont and colleagues to develop a PCR-based assay for designation of *E. coli* into one of the four phylogenetic groups (A, B1, B2 and D), based on the amplification of three conserved genes (Clermont, Bonacorsi and Bingen, 2000). The most widely used nucleotide-based scheme for *E. coli* typing is multilocus sequence typing (MLST) which was devised for a number of bacterial species, including *E. coli* (Maiden *et al.*, 1998).

MLST uses PCR to determine the variants of seven or eight housekeeping genes, resulting in the grouping any particular strain into a numbered ‘sequence type (ST),’ allowing greater strain discrimination than phylogenetic grouping in isolation (Maiden *et al.*, 1998; Clermont

et al., 2013). Typing schemes, such as MLST which are based on so-called 'housekeeping' genes in *E. coli*, are limited by the high rate of core genome homologous recombination, which often includes the genes used in the typing scheme, and furthermore using seven or eight genes will never be representative of whole genomic diversity (Chaudhuri and Henderson, 2012). The target genes used in MLST schemes are derived from the original MLEE profiles (Maiden *et al.*, 1998).

Since the publication of the whole genome sequence of *E. coli* K-12 (Blattner *et al.*, 1997), whole genome sequencing of bacterial genomes using high-throughput short-read sequencers has become highly accessible and affordable. The large number of freely available bioinformatic tools for the analysis of draft genomes, means that typing strains using WGS datasets for research purposes or as part of clinical outbreak investigations, has become commonplace.

Typing strains to high resolution is now possible using WGS draft genomes of different *E. coli* strains and comparing the number of single nucleotide polymorphisms (SNPs) that differ between each test strain and the reference genome. SNP-typing has now become the gold standard method in investigation of *E. coli* population structure. Intergenic SNPs are classified as either synonymous, which have no effect on the amino acid sequence, and non-synonymous, which lead to a change in the amino acid sequence.

1.2 Clinical significance of *E. coli*

Pathogenic strains of *E. coli* affecting humans can be broadly divided into: (1) strains which cause gastroenteritis in humans, and (2) strains which cause urinary tract infections, intra-abdominal infections, or biliary infections, any of which may lead to *E. coli* bacteraemia.

1.2.1 *E. coli* causing gastrointestinal disease

The terms used to classify intestinal pathogenic *E. coli* are largely derived from whether or not the strain produces exotoxin, the resulting disease process, or the pathophysiological changes in the gut.

Enteropathogenic *E. coli* (EPEC) and Enteroaggregative (EAEC) are associated with remodelling of enterocytes leading to distinct macroscopic and microscopic changes in the gut epithelium. Adherent invasive *E. coli* (AIEC) is notable for its strong association with Crohn's disease. Enterohaemorrhagic *E. coli* (EHEC) and entero-invasive *E. coli* (EIEC) cause gastrointestinal haemorrhage resulting from production of Shiga toxin. Extreme disease can lead to haemolytic uraemic syndrome (HUS) and death. A very similar disease process may occur in gastroenteritis due to *Shigella* spp. Enterotoxigenic *E. coli* (ETEC) produce heat-stable and heat-labile enterotoxins, and are a significant cause of diarrhoea in the developing world (Chaudhuri and Henderson, 2012).

1.2.2 Extra-intestinal pathogenic *E. coli* (ExPEC)

Extra-intestinal pathogenic *E. coli* (ExPEC) make up the biggest burden of *E. coli* disease in the developed world, including the UK, and are the subject of the remainder of this thesis. The vast majority ExPEC strains include uropathogenic *E. coli* (UPEC) which are the commonest cause of urinary tract infection (UTI). ExPEC are also commonly implicated in intra-abdominal infections such as diverticulitis, appendicitis, as well as biliary tract infections. ExPEC infections may be localised to these anatomical locations. However, severe disease often leads to bacteraemia and severe sepsis, which carries a high mortality.

Particular *E. coli* phylogenetic groups (PG) are associated with extra-intestinal infection. In immunocompetent hosts, phylogenetic groups leading to ExPEC infection are usually B2 and D (Dale and Woodford, 2015). The most predominant clonal group of ExPEC is MLST type ST131 which has become globally disseminated, (Price, Johnson and Aziz, 2013), although other STs have been associated with uropathogenicity (Dale and Woodford, 2015). The role of successful ExPEC clones in the spread of $bla_{CTX\text{-}M}$ genes will be discussed in chapter 2, section 2.5.2.

1.3 Antimicrobial chemotherapy

Since the discovery and mass production of the first antibiotics in the 20th Century, (Ehrlich, 1913; Fleming, 1929; Domagk, 1935; Chain *et al.*, 1940), a plethora of both natural and synthetic agents have been utilised in human medicine. Unfortunately, for each drug

utilised, a corresponding resistance mechanism exists, which usually limits the usefulness of the antibiotic (Figure 1.1) (CDC, 2013).

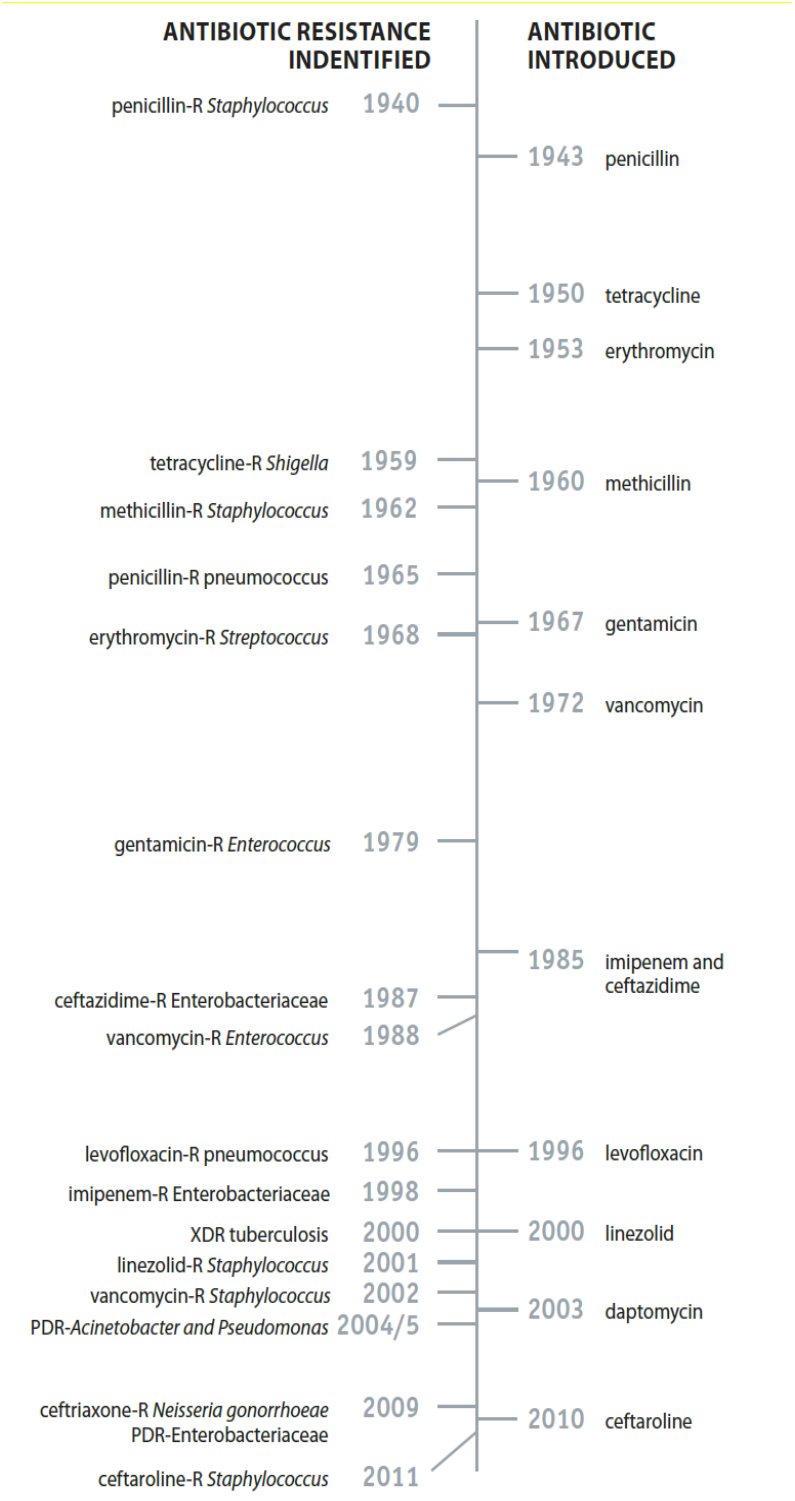


Figure 1.1. Timeline of antibiotic corresponding AMR mechanism discovery.
Adapted from: *Antibiotic resistance threats in the United States,* (CDC, 2013)

Following the discovery and widespread use of antibiotics in the early 20th Century, a plethora of antibiotics were developed, including semi synthetic agents such as amoxicillin (Rolinson and Stevens, 1961), and mould-derived compounds such as cephalosporins (Burton and Abraham, 1951; Bo, 2000).

Beta lactam antibiotics, which include cephalosporins, are a large family of agents with a beta-lactam ring. Beta lactams bind to D-alanyl-D-alanine carboxypeptidase, in the bacterial cell wall, leading to inhibition of cell division, lysis, and cell death (Ogawara, 1981).

The most widely used semi-synthetic penicillin, amoxicillin, is useful in the treatment of infections due to gram positive bacteria such as community acquired pneumonia, as well as infections due to susceptible gram-negative bacteria such as urinary tract infection. (Sutherland, Croydon and Rolinson, 1972). Amoxicillin is often used in combination with the beta-lactamase inhibitor clavulanic acid, (co-amoxiclav) in the modern era.

In the present day, the mainstays of managing *E. coli* bacteraemia depend on local epidemiology of antibiotic-resistance but may involve amoxicillin, co-amoxiclav, piperacillin/tazobactam, gentamicin, ciprofloxacin or carbapenems (Hawkey *et al.*, 2018).

1.4 Antimicrobial resistance in *E. coli*

1.4.1 Antimicrobial susceptibility testing

Antimicrobial susceptibility is determined through measurement of the minimum inhibitory concentration (MIC). This is the minimum antibiotic concentration required to achieve inhibition of bacterial growth. This is traditionally determined using broth microdilution. However, in the modern hospital microbiology laboratory, susceptibilities are determined using e-test strips or automated methods such as the VITEK2.

Antibiotic breakpoints are set after the analysis of a bacterial population and the susceptibility profile for each particular antibiotic. Breakpoints are decided after expert review by international committees such as the European Committee on Antimicrobial Susceptibility Testing, which publish annual interpretive criteria and guidance on susceptibility testing (EUCAST, 2018).

1.4.2 Early reports of antibiotic resistance in Enterobacteriaceae

The heavy use of a new antibiotic has often led to the development of corresponding antimicrobial resistance, and this has historically, been the case in the treatment of gram-negative bacterial infections. The plethora of new antibiotics being developed led to the discovery of antimicrobial resistance mechanisms. New antibiotics with gram negative activity used in human medicine such as streptomycin (Schatz, Bugie and Waksman, 1944), tetracycline (Duggar, 1948), and chloramphenicol (Eherlich *et al.*, 1947), showed early evidence of acquired antibiotic resistance.

Early reports of antibiotic resistance in *E. coli* were described in the context of patients treated with streptomycin for tuberculosis. Faecal *E. coli* were found to be streptomycin-resistant (Hamburger *et al.*, 1951), due to the presence of antibiotic target site mutations in ribosomal RNA (Springer *et al.*, 2001).

Chloramphenicol was discovered by Ehrlich in the late 1940s (Eherlich *et al.*, 1947) and was the first broad spectrum antibiotic, which is still in widespread use today. Resistance to chloramphenicol is usually mediated via acetylation and inactivation by chloramphenicol acetyltransferases (CATs) which are usually found on mobile genetic elements (Maclaren and Shann, 2010).

Tetracycline resistance is usually due to efflux pumps encoded on mobile genetic elements, including plasmids or due to target mutations leading to ribosomal modification (Eisen, 2010). The prevalence of tetracycline resistance was low in the years before tetracycline was used in clinical practice, being around 2% in Enterobacteriaceae isolated between 1917 and 1954 (Datta and Hughes, 1983). However, tetracycline is no longer used in routine clinical practice for treating infections due to Enterobacteriaceae, due to the high rates of resistance in this family of bacteria.

In the early days of antimicrobial chemotherapy, ampicillin and amoxicillin were often used in the treatment of *E. coli* infections, and their use acted as a driver for the emergence of resistant strains. Resistance to ampicillin and amoxicillin in *E. coli*, encoded by the TEM beta-

lactamase, was found to be transferrable, encoded on the newly discovered plasmid, R1 (Datta and Richmond, 1966)

1.4.3 Extended-spectrum beta lactamases

After the introduction of cephalosporins into clinical practice, resistance to these agents became widespread. During the 1980s, cephalosporins including ceftazidime, cefotaxime and ceftriaxone were used heavily as broad-spectrum empirical antibiotics. This overuse led to the dissemination of resistance mediated by extended spectrum beta lactamases (ESBLs) (Livermore and Hawkey, 2005).

Extended spectrum beta lactamases (ESBL) are enzymes which hydrolyse the beta lactam ring of penicillins and third-generation cephalosporins (3GC). ESBLs are defined as any acquired beta-lactam-hydrolysing enzyme which confers increased resistance to oxyimino-cephalosporins in comparison to other members of its genetic family (Livermore, 2008). They are mainly produced by Enterobacteriaceae, including *Escherichia coli* and *Klebsiella pneumoniae.* ESBL-producing strains may be part of the normal bowel flora, but can also be pathogenic, causing ExPEC infections including bacteraemia, which carry an increased mortality (Davies, 2013).

Classification of beta lactamases is based on two schemes: (1) The molecular classification based on amino acid sequence; and (2) functional classification based on substrates and clinical phenotype (Bush and Jacoby, 2010). The first ESBLs originated by mutations from the

TEM and SHV family of beta-lactamases (Kliebe *et al.*, 1985), but by far the most common ESBL is the CTX-M family, which will be described in detail in the following sections.

1.4.4 Surveillance of AMR in *E. coli*

There has long been a paucity of surveillance of AMR globally, particularly in Enterobacteriaceae. The most useful hospital-based surveillance data comes from several publications for The Study for Monitoring Antimicrobial Resistance Trends (SMART), which collates isolates from intra-abdominal infections from different world regions (Hsueh *et al.*, 2010; Chaudhuri *et al.*, 2011; Hawser *et al.*, 2011; Lob *et al.*, 2015). A SMART study from the Asia-Pacific region in 2008 showed that ESBL-producing *E. coli* rates were particularly high for India (61%), China (59%), Thailand (53%), Singapore (12.3%), and Malaysia (2.9%).

In 2015 the World Health Organisation announced the inception of the Global Antimicrobial Resistance Surveillance System (GLASS) which published an initial report. However, notably there is not yet any GLASS-data for the UK, or any South Asian countries, relevant to this thesis (WHO, 2017). However, the European Antimicrobial Resistance Surveillance Network (EARS-Net) has been operational since 1998, providing country-by-country epidemiological data on resistance rates for various bacteria including *E. coli* (ECDC, 2018). Figure 1.2 is a heat map from ECDC showing the latest European epidemiology of 3rd-generation cephalosporin resistant isolates across Europe (ECDC, 2018). There is a particular problem in Southern and Eastern Europe, where 3GC resistance prevalence for E. coli is up to 50% in some countries.

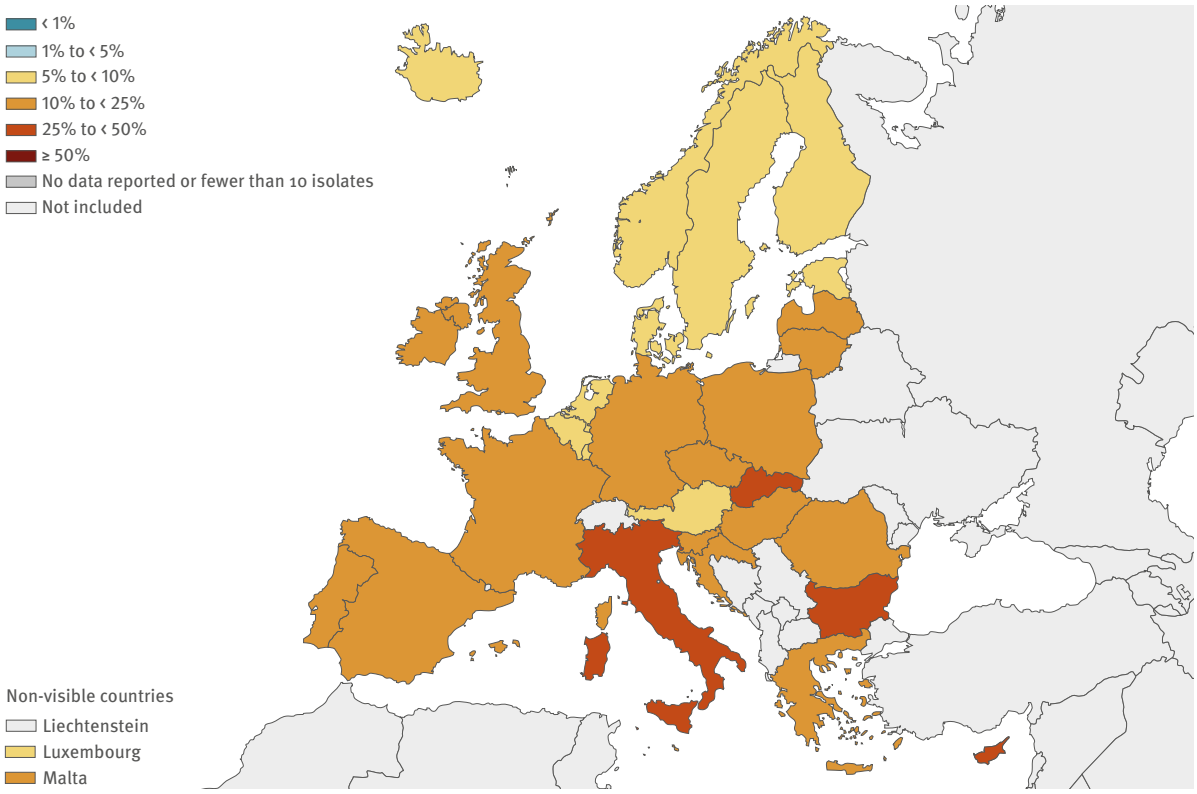


Figure 1.2. Percentage of third-generation cephalosporin-resistant invasive E. coli isolates, 2017 (ECDC, 2018)

1.4.5 UK incidence of *E. coli* bacteraemia

The incidence of *E. coli* bacteraemia in the UK is rising, and the reasons for this are likely to be the combination of an aging population with increasingly complex medical needs. One third of all cases of bacteraemia in the UK are due to *E. coli* and the source of infection in 50% is the urinary tract (Abernethy *et al.*, 2017). Risk factors strongly associated with *E. coli* bacteraemia are recent antibiotic exposure and urinary catheterisation, and the majority of cases are community-acquired rather than hospital-acquired infections (Abernethy *et al.*, 2017). The rate of *E. coli* bacteraemia in England increased by 22% between 2012 and 2017, and is forecast to rise by a further 22% by 2021 if no action is taken (Bhattacharya *et al.*, 2018). Mortality associated with *E. coli* bacteraemia is high, even with appropriate treatment. The all-cause 30-day case fatality rate of *E. coli* bacteraemia was 14.7% in 2016/17 (Bhattacharya *et al.*, 2018). The UK government has set a target to reduce the incidence of gram negative bacteraemia by 50% by 2021 (NHS Improvement, 2017).

1.4.6 Antimicrobial-resistant *E. coli* in the UK

Antimicrobial resistance (AMR) in England is subject to a national surveillance programme, The English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR) (Public Health England, 2018). For *E. coli*, ESPAUR reports rates of resistance to ciprofloxacin, gentamicin, third-generation cephalosporins, carbapenems, piperacillin/tazobactam, and co-amoxiclav (Public Health England, 2018). Although the proportion of resistant E. coli isolates causing bacteraemia has remained stable, there is an overall increase in antibiotic resistant strains due to the increased rate of total *E. coli* bacteraemia (Figure 1.3) (Hawkey *et al.*, 2018; Public Health England, 2018).

Most concerning is the rise in incidence of infections due to Extended spectrum beta lactamses (ESBLs), which encode resistance to third generation cephalosporins and to most beta-lactam antibiotics. There is no mandatory surveillance of clinical ESBL-producing bacteria in the UK, however, the prevalence of community carriage for England as a whole has been estimated to be 7.3% (McNulty *et al.*, 2018). Since gut colonisation is a reservoir of *E. coli* strains and colonisation with ESBL-producers usually precedes clinical infection (Asir *et al.*, 2015), epidemiological surveillance of ESBL community colonisation is important.

numbers of bloodstream infections caused by pathogens resistant to 1 or more key antibiotics increased from 12,250 in 2013 to 16,504 in 2017, a rise of 35% (Figure 2.3). As shown in Figure 2.3, and in more detail in Table 2.2 for infections that occurred in 2017, the burden of antibiotic-resistant bloodstream infections is particularly marked for those caused by Enterobacteriaceae, particularly *E. coli*, as they are the infections with the highest incidence, comprising 84.4% of the total. The burden of resistant infections

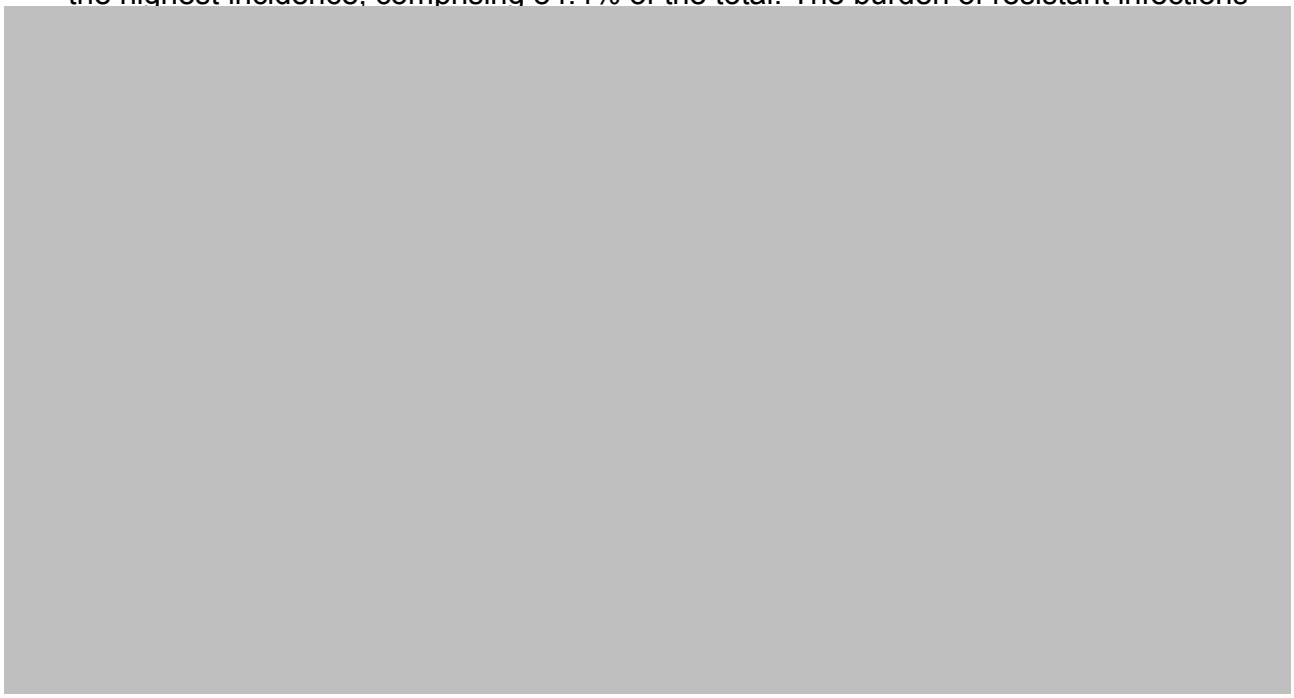


Figure 2.3 Estimated trends in burden of bloodstream infections due to antibiotic-resistant pathogens in England, 2013 to 2017

Figure 2.3 Estimated burden of bloodstream infections due to AMR bacteria in England. Adapted from the resistant pathogens in England, 2013 to 2017.

1.5 Plasmids and horizontal gene transfer

Horizontal gene transfer (HGT) is the process by which genetic material passes between closely or distantly related organisms, and can take place in prokaryotes and eukaryotes (Gogarten, Gogarten and Olendzenski, 2009). For the purpose of this thesis, HGT will be referred to in terms of transfer between bacteria. HGT typically occurs in one of three ways: (1) by transformation of DNA, that is, uptake of DNA from the extracellular environment; (2) by transduction, whereby DNA is transferred by bacteriophage; (3) by movement of mobile genetic elements such as plasmids, transposons, integrons or insertion sequences (Thomas and Nielsen, 2005).

Plasmids are extra-chromosomal, self-replicating DNA molecules which are usually double stranded and circular (Gogarten, Gogarten and Olendzenski, 2009). It has been argued that plasmids constitute a distinct life-form, because they are autonomous and have their own discrete evolutionary lineages (Carattoli, 2013). Plasmids vary in size from 1 kbp – 500kbp, with variation in copy number per bacterium, whereby smaller plasmids often exist in high copy number. Conversely, the copy number of larger plasmids may only be 1-2 per cell.

Plasmids are found naturally in bacteria, and often encode genes which are beneficial to the host such as antibiotic (Datta and Richmond, 1966) or metal resistance genes (Smith, 1967), virulence factors allowing the host cell to be pathogenic (Elwell and Shipley, 1980), or genes allowing the bacterium to adapt to stressful external conditions (Winther and Gerdes, 2011). In addition, plasmids often encode the machinery necessary for horizontal gene transfer by

conjugation, as well as maintenance systems to ensure inheritance in future bacterial progeny, which is especially important for plasmids in Enterobacteriaceae (Carattoli, 2009; Gogarten, Gogarten and Olendzenski, 2009).

Plasmid compatibility grouping was a classification originally devised by grouping plasmids into categories based on their ability to co-exist in the same bacterium (Datta and Hedges, 1971), and subsequently developed into a PCR based typing system (Carattoli *et al.*, 2005). Since the widespread availability of WGS, and in particular long read sequencing, whole plasmid sequencing has become possible (Ashton *et al.*, 2015; Lemon *et al.*, 2017).

Conjugation is a process by which a portion of DNA, usually a circular plasmid, passes from one bacterium to another by direct cell-to-cell contact (Cabezón *et al.*, 2014). The first description of antibiotic resistance gene transfer by conjugation was made in relation to clinical isolates of Shigella spp. in Japan (Watanabe and Fukasawa, 1961). Conjugative plasmids encode the genes required for transfer (the tra operon) which proteins necessary for formation of the bacterial pilus. The pilus, sometimes referred to as the 'sex-pilus' is a protrusion of the bacterial cell membrane and cell wall, allowing contact with a neighboring bacterium, thus allowing horizontal gene transfer of the plasmid (Cabezón *et al.*, 2014).

Several reports have demonstrated the in vivo transfer of antimicrobial resistance genes by plasmid conjugation in the gut of animals (Stecher *et al.*, 2012; Card *et al.*, 2017) and humans (Smith, 1969; Neuwirth *et al.*, 2001; Goren *et al.*, 2010; Mata *et al.*, 2010; Naseer *et al.*, 2012; Knudsen *et al.*, 2018). The first demonstration of plasmid transfer between strains

in the human gut was demonstrated in an elegant experiment by Herbet William Smith who consumed bacterial cultures and then analysed serial faecal cultures, showing that in-vivo conjugation had occurred from a consumed *E. coli* strain, onto a pre-existing host *E. coli* (Smith, 1969). More recently, Knudsen and colleagues used WGS to show that horizontal transfer of a plasmid bearing $bla_{CTX\text{-}M\text{-}1}$ occurs between different *E. coli* clones in the gut of a patient with cystic fibrosis (Knudsen *et al.*, 2018).

1.6 The domination of CTX-M

1.6.1 Origin of CTX-M enzymes

The commonest and most globally predominant of ESBLs are the CTX-M family of enzymes. Humeniuk and colleagues demonstrated that *Kluyvera ascorbata,* associated with the rhizosphere, carries the chromosomal bla_{KLUA} gene which confer resistance to 3GCs.The bla_{KLUA} gene and the flanking regions are highly related to $bla_{CTX\text{-}M}$ enzymes and the flanking sequences carried on plasmids (Humeniuk *et al.*, 2002). Each main cluster of CTX-M genotypes has a corresponding progenitor gene sharing homology with different *Kluyvera* spp. (Zhao and Hu, 2012).

Chromosomal bla_{KLUA} from *K.ascorbata* share 100% homology with plasmid mediated $bla_{CTX\text{-}M\text{-}2}$ (Di Conza et al. 2002). Similarly, CTX-M-14 originates from a chromosomal gene in *K. georgiana* (Olson *et al.*, 2005) which also share close homology with CTX-M-8 (Poirel, Ka and Nordmann, 2002), and CTX-M-25 (Rodriguez *et al.*, 2010). The CTX-M group 1 variant $bla_{CTX\text{-}M\text{-}3}$ originated from *K. ascorbata* (Rodríguez *et al.*, 2004), whereas CTX-M-37 is derived from

K. cryocrescens (Decousser, Poirel and Nordmann, 2001). It is likely that the insertion sequence ISEcp1, frequently found upstream of $bla_{\text{CTX-M-14}}$ (Kim *et al.*, 2011) and $bla_{\text{CTX-M-15}}$ (Dhanji, Patel, *et al.*, 2011) in human isolates of *E.coli* played a key role in the mobilisation of the progenitor ESBL genes from *Kluyvera* spp. (Humeniuk *et al.*, 2002).

CTX-M was first identified in in Germany (Bauernfeind, Grimm and Schweighart, 1990), France (Bernard *et al.*, 1992; Karim *et al.*, 2001), and South America (Bauernfeind *et al.*, 1992) . They have become globally disseminated, with CTX-M-15 and CTX-M-14 being the predominant subtypes. CTX-M-15 is the most common ESBL in the UK and globally. The first description of this enzyme from multiple centres in India in the early 2000s, suggests that it originated from there (Ensor *et al.*, 2006). CTX-M-14 is also globally distributed, especially in China and the surrounding regions, probably originating in China, being first described there in 1998 (Chanawong *et al.*, 2002).

The increasing prevalence of the CTX-M ESBLs since 2000 (Woerther *et al.*, 2013), presents huge challenges to healthcare with restricted options to treat infections caused by CTX-M-producing bacteria. This has led to increased use of carbapenems, (Laxminarayan *et al.*, 2013), leading to the emergence and spread of carbapenemase-producing Enterobacteriaceae (Hawkey, 2015). Developing countries with low levels of sanitation, provide opportunities for the transfer of antimicrobial resistance (AMR) genes, including *bla*-CTX-M, between humans, animals and the natural environment (Wellington *et al.*, 2013; Pehrsson *et al.*, 2016).

1.6.2 Global spread of CTX-M

Since first being described, the global prevalence of CTX-M-producing Enterobacteriaceae has grown significantly (Woerther *et al.*, 2013; Bevan, Jones and Hawkey, 2017). The spread and global dissemination is due to a combination of interlinked factors. These can be divided into two inter-linked categories: (1) Features of $bla_{\text{CTX-M}}$ which include the genetic context, the role of plasmids and *E. coli* clonal lineages; and (2) anthropogenic factors which include human travel, environmental pollution with human and animal waste, and antibiotic misuse in humans and animals. The transfer pathways for AMR genes, especially ESBLs, between, humans, animals, food and the environment are outlined in Figure 1.4. The global epidemiology and drivers for the geographical and temporal shifts in $bla_{CTX\text{-}M}$ genotypes is covered in detail in chapter 2 of this thesis.

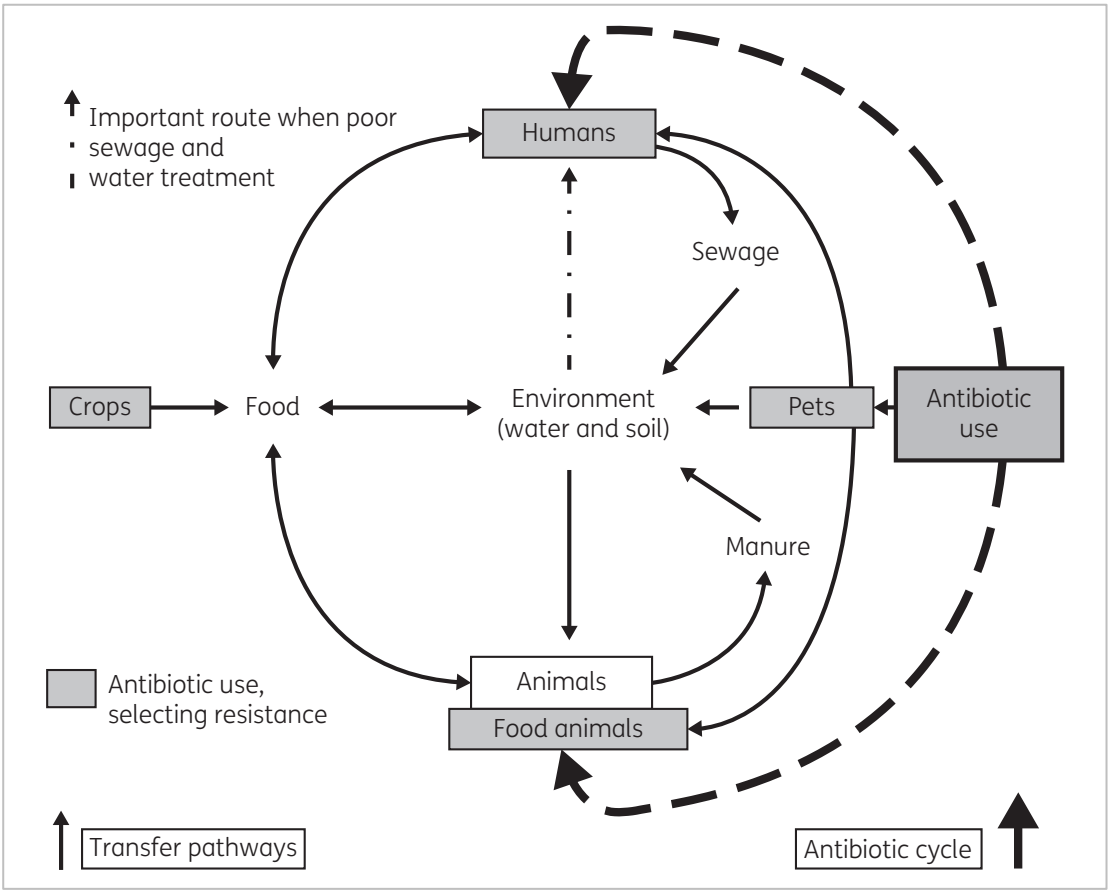


Figure 1.4 The spread of AMR bacteria can be attributed to a network of interactions between humans, animals, and the natural environment. Adapted from The Garrod Lecture, 2017 (Hawkey, 2018).

1.7 Global dissemination of ESBL-producing organisms via human travel

A literature review of studies which assessed the ESBL-acquisition rate in returning travellers was carried out (Table 1.1). The first prospective traveller study of this kind was carried out by Kennedy and Collignon in 2008-2009 (Kennedy and Collignon, 2010). They recruited 102 healthy volunteers in Australia, and found a 21.6% acquisition rate of ESBL- and AmpC producing *E. coli*, and a 57% acquisition rate for those who travelled to the Indian subcontinent (Kennedy and Collignon, 2010).

Since the work of Kennedy and Collignon, there have been 21 travel studies, with the highest acquisition rates reported in studies which included travel to 'South Asia,' 'the Indian subcontinent,' or 'India.' The largest of these studies recruited 2001 healthy volunteers who travelled from The Netherlands to a variety of destinations, including 90 participants who visited India (Arcilla *et al.*, 2017). Arcilla found an overall ESBL-Enterobacteriaceae acquisition rate of 34.3%, but this was 89% in those who travelled to India (70/79), and 75% who visited the South Asia WHO region (Figure 1.5) (Arcilla *et al.*, 2017).

Figure 1.5. Rates of acquisition of ESBL-producing Enterobacteriaceae by Dutch travellers.
The highest rate of acquisition is in individuals who travel to South Asia (75%)

Reference	Study Year	Volunteer No.	Country of Origin	Destinations	Sample Collection Time Points	CTX-M prevalence (%)			*Bla*-$_{CTX-M}$ Genotype, all regions (Group)	*Bla*-$_{CTX-M}$ carriage duration post travel for all regions	Risk Factors Identified	*E. coli* clonal analysis	Plasmid analysis
						Pre-travel	**Post travel acquisition for all countries**	**Post travel acquisition for India**					
(Peirano *et al.*, 2017)	2012-2014	116	CAN	South Asia: India (83%),	Before and after (7 days and 6 months)	6	64	73 (66/90)	CTX-M-15	21% going to South Asia colonised at 6 months	India, meals with locals, antibiotic use during travel	NA	NA
(Arcilla *et al.*, 2017)	2012-2013	2001	NED	South Asia, Southeast Asia East Africa, South America	Before and after travel, 1,3,6,12 months after	6.1	34.3	89 (70/79)	CTX-M-15 (G1) CTX-M-14 (G9)	11.3% positive at 12 months	Diarrhoea, previous colitis, quinolone intake, consuming street food.	NA	NA
(Barreto Miranda *et al.*, 2016)	2013-2014	211	GER	India, Southeast Asia, Africa, Central America	After travel, 6 months, 12 months	N/A	72.1	72^ (69/96)	Group 1 and Group 9 CTX-M	28% (17/61) positive at 6 months	India	NA	NA
(Pires *et al.*, 2016)	Not stated	15	SWI	India	Before and after travel, 3 and 6 months	20	100	100 (15/15)	CTX-M-15 (G1) CTX-M-14 (G9)	40% (6/15) at 6 months	NA	Yes	IncF plasmids in 80% isolates
(Vading *et al.*, 2016)[Ψ]	2013-2015	188	SWE	South Asia, North Africa, Middle East	Before and after travel	6.9	32	49.2^ (30/61)^{MCR}	CTX-M-15 (G1) CTX-M-14 (G9)	NA	Indian subcontinent, North Africa, diarrhoea, antibiotics	Yes	NA
(E A Reuland *et al.*, 2016)	2012-2013	445	NED	Global	Before and 2 weeks after travel	6.1	23.4^{+}	55^ (27/49)	CTX-M-15, CTX-M-14 and CTX-M-27 commonest	NA	Asia, GI disorders, antibiotics	NA	NA
(Mizuno *et al.*, 2016)	2012	57	JAP	Global	Pre and post travel	14	44.9	55^ (6/11)	CTX-M-15, CTX-M-14 and CTX-M-27	NA	NA	NA	NA
(Bengtsson-Palme *et al.*, 2015)	2010-2014	18	SWE	India	After travel only	NA	66.1	66.1 (12/18)	CTX-M-15 (G1)	NA	NA	NA	NA
(Ruppé *et al.*, 2015)	2012-2015	574	FRA	Tropical regions only	Before and after; up to 12 months post	11.6	50.9%	85.4^{+} (53/62)	Group 1 CTX-M Also one each of NDM-1, OXA-48, OXA-181	2.2% after 12 months; 3.7% for Asia	Asia, Sub-Saharan Africa, diarrhoea, antibiotics	NA	NA
(Kantele *et al.*, 2015)	2009-2010	430	FIN	Asia Middle East, Sub-Saharan Africa	Before and after travel	1.2	21	45.9^ 28/61	CTX-M-15 (G1) CTX-M-14 (G9)	zero positive at 12 months	South Asia, diarrhoea, antibiotics	NA	NA
(Angelin *et al.*, 2015)	2010-2014	99	SWE	Global	Before after travel	7	35.4	70^ (21/30)	NA	NA	South Asia, South East Asia	NA	NA

Table 1.1. Traveller studies performed assessing the acquisition of ESBL-producing Enterobacteriaceae.

Reference	Study Year	Volunteer No.	Country of Origin	Destinations	Sample Collection Time Points	CTX-M prevalence (%)			*Bla*-$_{CTX-M}$ Genotype, all regions (Group)	*Bla*-$_{CTX-M}$ carriage duration post travel for all regions	Risk Factors Identified	*E. coli* clonal analysis	Plasmid analysis
						Pre-travel	**Post travel acquisition for all countries**	**Post travel acquisition for India**					
(Lübbert *et al.*, 2015)	2013-2014	205	GER	Global	Before and after travel	6.8	30.4	73.3 (11/15)	CTX-M-15 predominated	8.6% (3/35) positve at 6 months	India and SE Asia, digestive disorders	NA	NA
(Valverde *et al.*, 2015)	2011-2012	86	SPA	Tropical/subtropical regions	After travel only	NA	31.4	NA	CTX-M-15 CTX-M-14	NA	NA	Yes	NA
(von Wintersdorff *et al.*, 2014)	2010-2012	122	NED	Southeast Asia, South Asia	Before and after travel	9	58.1	58.1^ (18/31)	CTX-M-15	NA	India	NA	NA
(Yaita *et al.*, 2014)	2011-2012	68	JAP	South Asia	After travel only	NA	26	71.4 (10/14)	CTX-M-15	NA	India	NA	NA
(Kuenzli *et al.*, 2014)	2012-2013	179	SWI	Indian Subcontinent only	Before and immediately after	5	69.4	86.8 (59/68)	CTX-M-15 only	NA	India/Bhutan/Nepal, stay with relatives, ice cream or pastries	Yes	NA
(Paltansing *et al.*, 2013)	2011	370	NED	South Asia, Southeast Asia, Sub-Saharan Africa	Before and after travel, 6 months after	8.6	30.5*	72^ (18/25)	CTX-M-15 CTX-M-14	16.8% positive at 6 months	South Asia and East Asia	Yes	NA
(Ostholm-Balkhed *et al.*, 2013)	2008-2009	262	SWE	South Asia, East Africa, Central/S. America	Before and after	2.4	31.2*	71.4 (10/14)	CTX-M-15 CTX-M-14 predominated	NA	Asia, Indian subcontinent and N. America, age, fever, GI upset	NA	NA
(Weisenberg *et al.*, 2012)	2009-2010	28	USA	Asia, America, Middle East	Week before and week after travel	4	25	NA	CTX-M-14 CTX-M-15	NA	NA	Yes	NA
(Tängdén *et al.*, 2010)	2007-2009	101	SWE	Global	Before and after travel	1	24	88 (7/8)	CTX-M-15	24% at 6 months (5/21)	India, diarrhoea	NA	NA
(Tham *et al.*, 2010)	2007-2008	242	SWE	Global	After travel, with diarrhoea	NA	28	79% (11/14)	Group 1 and 9 CTX-M	NA	India or Egypt, or rest of world versus Europe	Yes	NA
(Kennedy and Collignon, 2010)	2008-2009	102	AUS	Asia, Middle East, Africa, Central/South America	Before and after up to 6 months	1.9	21.6*	57^ (8/14)	NA	18% positive at 6 months	Asia, S. America, Africa, antibiotic consumption, digestive disorders	NA	NA

Table 1.1 Continued.

AUS, Australia; CAN, Canada; FIN, Finland; FRA, France; GER, Germany; JAP, Japan; NED, the Netherlands; SPA, Spain; SWE, Sweden; SWI, Switzerland. NA: not analysed. EPE: ESBL-producing Enterobacteriaceae.
*Includes ESBL and AmpC enzymes. +includes CPE and AmpC. ^Indian subcontinent. ^{MCR}One CTX-M-EC strain also carried mcr-1, presumed acquisition after travel to Thailand

1.7.1 Risk factors for acquisition of ESBL-producing Enterobacteriaceae

The most widely reported risk factor which is strongly associated with ESBL acquisition in returning travellers is, visiting India, (Tham *et al.*, 2010; Kuenzli *et al.*, 2014; von Wintersdorff *et al.*, 2014; Yaita *et al.*, 2014; Lübbert *et al.*, 2015; Barreto Miranda *et al.*, 2016; Peirano *et al.*, 2017) 'Indian subcontinent,' (Tängdén *et al.*, 2010; Ostholm-Balkhed *et al.*, 2013; Vading *et al.*, 2016) or 'South Asia' (Paltansing *et al.*, 2013; Angelin *et al.*, 2015; Kantele *et al.*, 2015). This particular association with ESBL-acquisition and travel to India and the surrounding region can be explained by high population density, increased human travel, and contamination of drinking water supplies with faecal waste (Bevan, Jones and Hawkey, 2017) (see section 1.5.6 in this thesis).

The mode of acquisition of Enterobacteriaceae is likely to be through drinking contaminated food and water, therefore a number of studies have considered the dietary habits of travellers in risk factor analyses. Specific risk factors which were significantly associated with acquisition were consuming meals with locals (Peirano *et al.*, 2017), consuming street food (Arcilla *et al.*, 2017), or eating ice cream/pastries (Kuenzli *et al.*, 2014).

Antibiotic use during travel has also been found to be a risk factor for acquisition of ESBL-producing bacteria (Kennedy and Collignon, 2010; Kantele *et al.*, 2015; Ruppé *et al.*, 2015; E. A. Reuland *et al.*, 2016; Vading *et al.*, 2016; Arcilla *et al.*, 2017; Peirano *et al.*, 2017). Fluoroquinolone consumption was also found to be an independent risk factor for acquisition (Arcilla *et al.*, 2017). It is not surprising that antibiotic use is a risk factor for acquisition, as this would result in (1) direct selection of ESBL-producing bacteria; and (2)

disruption of the normal faecal microbiome, allowing colonization of new strains. The association of fluoroquinolone use and acquisition of ESBL-producers, points towards a mechanism of co-selection, where resistance genes to fluoroquinolones are present on MDR cassettes which also include ESBL genes.

Diarrhea during travel, which is also related to the disruption of the normal faecal flora, has also been found to be a risk factor for ESBL-acquisition (Ostholm-Balkhed *et al.*, 2013; Ruppé *et al.*, 2015; Vading *et al.*, 2016; Arcilla *et al.*, 2017). Travellers with pre-existing gastrointestinal disorders were also more likely to acquire ESBL-producers (Kennedy and Collignon, 2010; Lübbert *et al.*, 2015; E. A. Reuland *et al.*, 2016). Therefore, host gut inflammation in travellers with an underlying condition, such as inflammatory bowel disease, probably results in higher rates of colonization with travel-acquired bacteria.

Travellers diarrhea is usually caused by bacterial, parasites or viruses, and is usually self-limiting. However, the resulting acute gut inflammation is likely to explain why diarrhea is a risk factor for acquisition. A hypothesis follows that a 'more inflamed' gut, is more susceptible to colonization. In a mouse colitis model, gut inflammation was associated with Enterobacterial blooms and increased tendency for HGT (Stecher *et al.*, 2012), although murine gut flora is not representative of the human faecal microbiome. A human metagenomic study of the post-travel faeces of 35 Swedish travellers found that travel results in a spike in abundance of the phylum *Proteobacteria* (which includes the Enterobacteriales), (Bengtsson-Palme *et al.*, 2015). Increased abundance of clinically

relevant Enterobacteriales in the gut may then provide greater opportunities for resistance gene or strain acquisition.

1.7.2 Duration of carriage of ESBL-producers

The post-travel duration of carriage was determined in a number of studies (Table 1.1) (Kennedy and Collignon, 2010; Tängdén *et al.*, 2010; Paltansing *et al.*, 2013; Kantele *et al.*, 2015; Lübbert *et al.*, 2015; Ruppé *et al.*, 2015; Barreto Miranda *et al.*, 2016; Pires *et al.*, 2016; Arcilla *et al.*, 2017; Peirano *et al.*, 2017). The longest period of follow-up was 12 months. Colonization rates at the 12 month post-travel time point varied considerably between studies, with rates of 0% (Kantele *et al.*, 2015); 2.2% (Ruppé *et al.*, 2015); and 11.3% (Arcilla *et al.*, 2017).

Colonisation rates are variable between studies, which reflects differing host microbiome factors, as well as the differing ability of strains to colonise the faecal microbiome, which is defined as the microbial content of a faecal sample. Travel to Asia was associated with significantly longer carriage duration ($p = <0.001$) at 12 months post-travel compared with travel to other regions (Ruppé *et al.*, 2015). Therefore, it seems likely that regions associated with high ESBL acquisition rates such as India, are also associated with a longer duration of ESBL-strain carriage, compared to other regions. It is logical that strains that are easily acquired by travellers in these 'high risk' regions are also good at long term colonisation of the faecal microbiome.

1.7.3 Onward transmission after travel

Two studies have investigated whether ESBL-producing organisms can be passed to ESBL-negative household members post-travel (Paltansing *et al.*, 2013; Arcilla *et al.*, 2017).

However, only one study showed that onward transmission to close contacts does occur (Arcilla *et al.*, 2017). The lack of research in this area may be due to the more complex study design, high sample size requirement, and more detailed ethical considerations in broadening the numbers of people screened for ESBL-producing organisms. Arcilla et al found that 168 ESBL-negative co-habitants of ESBL-positive travellers were 'at-risk' of ESBL-acquisition. The acquisition rate was found to be 7.7% (13/168), and the risk of onward transmission to a household member was calculated to be 12% (Arcilla *et al.*, 2017).

There are a number of limitations to the aforementioned onward transmission data (Arcilla *et al.*, 2017). Firstly, no typing of ESBL-producers was carried out, e.g. MLST or WGS os isolates. Only ESBL-PCRs including CTX-M grouping PCRs were carried out, which confirmed that acquired strains by householders had the same *bla* genotype, as the corresponding ESBL-positive traveller (Arcilla *et al.*, 2017). Proving onward transmission would require SNP typing of Enterobacterial strains to demonstrate person-to-person transfer. Secondly, there is likely to be a degree of bias in that returning travellers and their household contacts would be 'unblinded' and fully aware of the possibility of ESBL-strain acquisition. Therefore, entry into the study is likely to have had an effect on the hand-washing habits of participants, leading to a potential underestimate of 'real-life' transmission rates in the home after travel.

The importance of onward transmission of ESBL-producing strains depends on the baseline ESBL-prevalence in the country under question. In the Netherlands, the baseline community prevalence of ESBL/AmpC-producing Enterobacteriaceae is 5.2% (Teunis *et al.*, 2018), and in the UK, CTX-M-EC community carriage is 7.3% (McNulty *et al.*, 2018). However, when one considers urban cosmopolitan areas in the UK, such as central Birmingham, the rate of CTX-M-EC colonisation is 16%. The reason behind such a high rate in a UK city, compared to the UK as a whole, is likely due to the high rates of travel between Birmingham and South Asia (McNulty *et al.*, 2018).

1.7.4 Acquisition of carbapenemase-producing Enterobacteriaceae and mcr-1-producers

Carbapenems and colistin, are last resort antibiotics used for the treatment of MDR-gram negative infections, and have been put on the 'reserve' antibiotic list by the WHO (WHO, 2015). Therefore, research into the global spread and epidemiology of carbapenemase-producing Enterobacteriaceae (CPE) and mcr-1 producing bacteria, is of critical importance. CPE and mcr-1 do not fall into the category of ESBLs and are not the focus of this thesis. However, the increase in worldwide prevalence of ESBL-producing Enterobacteriaceae drives the prescription of carbapenems and colistin, which in turn propagates CPE and mcr-1 (Bevan, Jones and Hawkey, 2017).

Acquisition of CPE by travellers is much less frequent than acquisition of ESBL producers (Arcilla *et al.*, 2017). As part of the COMBAT study (Arcilla *et al.*, 2017), Hattem and colleagues described a CPE travel-related acquisition rate of 0.3% (6/2001), which included

bla$_{IMI-2}$, *bla*$_{NDM-1}$, *bla*$_{OXA-244}$ and *bla*$_{OXA-48}$ (Hattem *et al.*, 2016). These CPE acquisitions were in travellers who did not have any healthcare exposure during their excursions (Hattem *et al.*, 2016). The same research group found that 0.9% of CTX-M-EC isolates (6/633 isolates screened) also possessed the plasmid-mediated *mcr-1* gene (Arcilla *et al.*, 2015).

The importance of onward transmission for CPE has been demonstrated by Nordmann et al, who showed that outbreaks of NDM-1-producing isolates in Western Europe and North America can be traced back to the Indian subcontinent (Nordmann *et al.*, 2011; Nordmann, Naas and Poirel, 2011). Episodes of infections with NDM-1-producers in the UK are mostly related to patient-repatriations from overseas hospitals with high rates of CPE (Public Health England, 2013). Due to the low rates of acquisition by travellers, formal screening of patients with a recent travel history, without hospitalization abroad, is not routinely carried out.

1.7.5 Molecular analysis of travel-acquired strains

A limited number of traveller studies carried out bacterial strain typing and plasmid analysis. PCR-based multilocus sequence typing (MLST) of selected isolates was reported in a limited number of studies (Weisenberg *et al.*, 2012; Paltansing *et al.*, 2013; Valverde *et al.*, 2015; Pires *et al.*, 2016), while one study used phylogenetic grouping PCR only (Vading *et al.*, 2016). PCR plasmid replicon typing was also performed in a few traveller studies (Tham *et al.*, 2010; Kuenzli *et al.*, 2014; Pires *et al.*, 2016).

No previous traveller studies could be identified where whole genome sequencing of isolates pre- or post-travel had been carried out. WGS in necessary to answer several important

questions relating to the dynamics of acquisition of CTX-M-EC: (1) Is the CTX-M plasmid transferred onto a pre-travel commensal *E. coli* strain? (2) How many *E. coli* clones do travellers acquire? (3) Do CTX-M-EC completely displace pre-travel commensal *E. coli*? (4) What are the characteristics of the plasmids carrying CTX-M in post travel *E. coli*?

This thesis will address these questions, providing a unique insight into the mechanism and dynamics of acquisition of CTX-M-producing *E. coli* in healthy travellers.

1.8 Hypotheses

Epidemiology of CTX-M beta-lactamases: temporal and geographical shifts in genotype (CHAPTER 2)

1. Plasmids play a key role in the dissemination of *bla*$_{CTX}$ genotypes. Epidemic plasmids are responsible for the horizontal gene transfer of *bla*$_{CTX-M}$ genes.
2. Internationally successful *E. coli* clonal lineages have also led to the global dissemination of particular *bla*$_{CTX-M}$ genotypes
3. A number of anthropogenic factors drive the spread of CTX-M-producing Enterobacteriaceae. These factors can be subdivided:
    a. The role of food animals carrying CTX-M determinants and the overuse of antibiotics in food animals
    b. Poor sanitation in the developing world leads to the contamination of the environment and drinking water supply with human faecal waste
    c. The natural environment which contains human and animal strains of Enterobacteriaceae, acts as a reservoir of *bla*$_{CTX-M}$ genes, and provides the opportunity for the emergence of novel genotypes.

Traveller Study Volunteer recruitment, demographics, and acquisition of CTX-M-EC (CHAPTER 3)

1. The rate of acquisition of CTX-M-EC will be similar to previous studies with volunteers travelling from developed countries to South Asia.

2. Risk factors known to be associated with CTX-M-EC acquisition will be identified from the post-travel questionnaires completed by volunteers.

The molecular dynamics of acquisition of ctx-m-producing *E. coli* and relationship to sensitive *E. coli* (CHAPTER 4)

1. Acquisition of CTX-M-*E. coli* is due to multiple acquisition events which leads to colonisation with multiple *E. coli* clones after travel
2. CTX-M-EC are maintained in the faecal microbiome after return to the UK
3. CTX-M-EC do not displace non-CTX-M *E. coli* which were present before travel
4. Non-CTX-M strains persist as a minority population in the faecal microbiome, despite CTX-M-EC acquisition
5. CTX-M-EC are usually multi-drug resistant

Plasmid identification and analysis (CHAPTER 5)

1. The $bla_{CTX\text{-}M}$ genes from the CTX-M-EC colonising the faecal microbiome in returning travellers are primarily plasmid mediated, rather than chromosomal in origin;
2. Travel acquired $bla_{CTX\text{-}M}$-containing plasmids are more closely related to plasmids previously identified from South Asia, than to plasmids of UK-origin;
3. The genetic context of $bla_{CTX\text{-}M}$ will be reflective of non-UK-type genetic contexts;
4. The ISECp1-CTX-M spacer sequence will vary according to region of origin of the CTX-M-EC isolate;

5. The CTX-M-bearing plasmids in post travel *E. coli* strains will be transferrable by conjugation.

Chapter 2: Epidemiology of CTX-M beta-lactamases: temporal and geographical shifts in genotype

2.1 Introduction

Extended spectrum beta lactamases (ESBLs) hydrolyse the beta lactam ring of penicillins and third-generation cephalosporins (3GC) and are mainly found in Enterobacteriaceae, especially in *E. coli*. *E. coli* are part of the normal bowel flora in humans, but can also be pathogenic, causing urinary tract infections and bloodstream infections (Davies, 2013).

The largest group of ESBLs are CTX-M, first identified in Germany, (Bauernfeind, Grimm and Schweighart, 1990) France, (Bernard *et al.*, 1992) and South America (Bauernfeind *et al.*, 1992). They have become globally disseminated, with CTX-M-15 and CTX-M-14 being the predominant genotypes. CTX-M ESBLs have increased in prevalence since 2000, (Woerther *et al.*, 2013) and this presents huge challenges to healthcare, with restricted options to treat infections caused by CTX-M-producing bacteria. This has led to increased use of carbapenems, (Laxminarayan *et al.*, 2013) leading to the emergence and spread of carbapenemase-producing Enterobacteriaceae (Hawkey, 2015). Developing countries with low levels of sanitation provide opportunities for the transfer of antimicrobial resistance (AMR) genes in Enterobacteriaceae, including $bla_{\text{CTX-M}}$, between humans, animals and the natural environment (Wellington *et al.*, 2013; Pehrsson *et al.*, 2016).

Understanding the global epidemiology of ESBL-producing bacteria is essential if outbreaks of strains with novel ESBL genotypes are to be detected early and controlled. Although reviews of CTX-M-producing Enterobacteriaceae have been published in the past (Cantón and Coque, 2006; Hawkey and Jones, 2009; Zhao and Hu, 2012), no studies were identified describing the temporal trends in *bla*$_{\text{CTX-M}}$ epidemiology. Therefore, a fresh analysis of the literature in this area was undertaken, with identification of the key drivers leading to the change in global CTX-M epidemiology.

2.2 Hypotheses

1. Plasmids play a key role in the dissemination of *bla*CTX-M genes. Epidemic plasmids are responsible for the horizontal gene transfer of *bla*CTX-M genes.
2. Internationally successful *E. coli* clonal lineages have also led to the global dissemination of *bla*CTX-M
3. A number of anthropogenic factors drive the spread of CTX-M-producing Enterobacteriaceae. These factors can be subdivided:
    a. The role of food animals carrying CTX-M determinants and the overuse of antibiotics in food animals
    b. Poor sanitation in the developing world leads to the contamination of the environment and drinking water supply with human faecal waste
    c. The natural environment which contains human and animal strains of Enterobacteriaceae, acts as a reservoir of *bla*CTX-M genes, and provides the opportunity for the emergence of novel genotypes.

2.3 Methods

2.3.1 Search strategy and selection criteria

The NCBI PubMed database was searched without restrictions using the following terms: "CTX-M" and "extended spectrum beta lactamase (ESBL)" plus "Europe," "North America," "South America," "Africa," "Australasia," "Asia," or "South East Asia," as well as individual country names. These searches generated 2735 papers. Article inclusion criteria: (1) human Enterobacteriaceae isolates from community or hospital origin: these were from studies reporting infecting strains or from faecal carriage studies; (2) isolates must have been identified using suitable screening media; (3) collection dates and location of sampling; (4) *bla*$_{\text{CTX-M}}$ genotyping or multiplex PCR grouping data must be provided. Articles were screened by reading abstracts. Using the inclusion criteria, we identified 220 English articles for review, which were also checked for additional references not captured in original searches.

2.3.2 Assessing changing trends in CTX-M-producers over time

Based on published epidemiological data, and to allow a meaningful display of temporal changes in *bla*CTX-M epidemiology, three time periods were chosen: pre-2005; 2005-2008; and 2009-present. This allows a straightforward visual comparison of *bla*CTX-M genotypes between time periods. As well as assessing the changing trends in human isolates, a sub-analysis considered trends in animal isolates from China using the same time periods. Linear regression plots for the frequency of ESBL faecal carriage over time were plotted for each WHO region (Figure 2.1) and for developing countries (Figure 2.2).

2.3.3 Investigating relationship between ESBL prevalence and access to basic sanitation

Data from the Study for Monitoring Antimicrobial Resistance Trends (SMART) 2008-2009 (Hawser *et al.*, 2010, 2012; Chen *et al.*, 2011), and WHO data on 'access to improved sanitation facilities during the same time period' (WHO, 2016), was used to assess the relationship between ESBL prevalence and sanitation access. Improved sanitation facilities are defined as access to a functioning latrine (WHO, 2016).

2.4 Results

2.4.1 Trends in global *bla*$_{\text{CTX-M}}$ epidemiology

ESBL prevalence increased in all WHO geographical regions, but these upward trends are only statistically significant for Europe (R^2= 0.429, p=0.04) (Figure 2.1). A linear regression analysis was of global community ESBL rates over time was undertaken which showed a statistically significant rise in community ESBL rates (R^2= 0.22, p<0.005), supporting previous analyses (Woerther *et al.*, 2013; Karanika *et al.*, 2016). In particular, there is a strong upward trend in ESBL rates when this analysis is undertaken for developing countries only (R^2=0.814, p=0.0004, Figure 2.2).

The global picture of CTX-M variants is a complex one, but it is clear that the proportion of *bla*$_{\text{CTX-M-15}}$ has increased over time in most countries and is dominant in most regions (Figure 2.3). Exceptions are China, South East Asia, South Korea, Japan and Spain, where group 9

variants (especially CTX-M-14) are dominant, and South America, where *bla*$_{\text{CTX-M-2}}$ is still an important genotype (Figure 2.3: d, e & f).

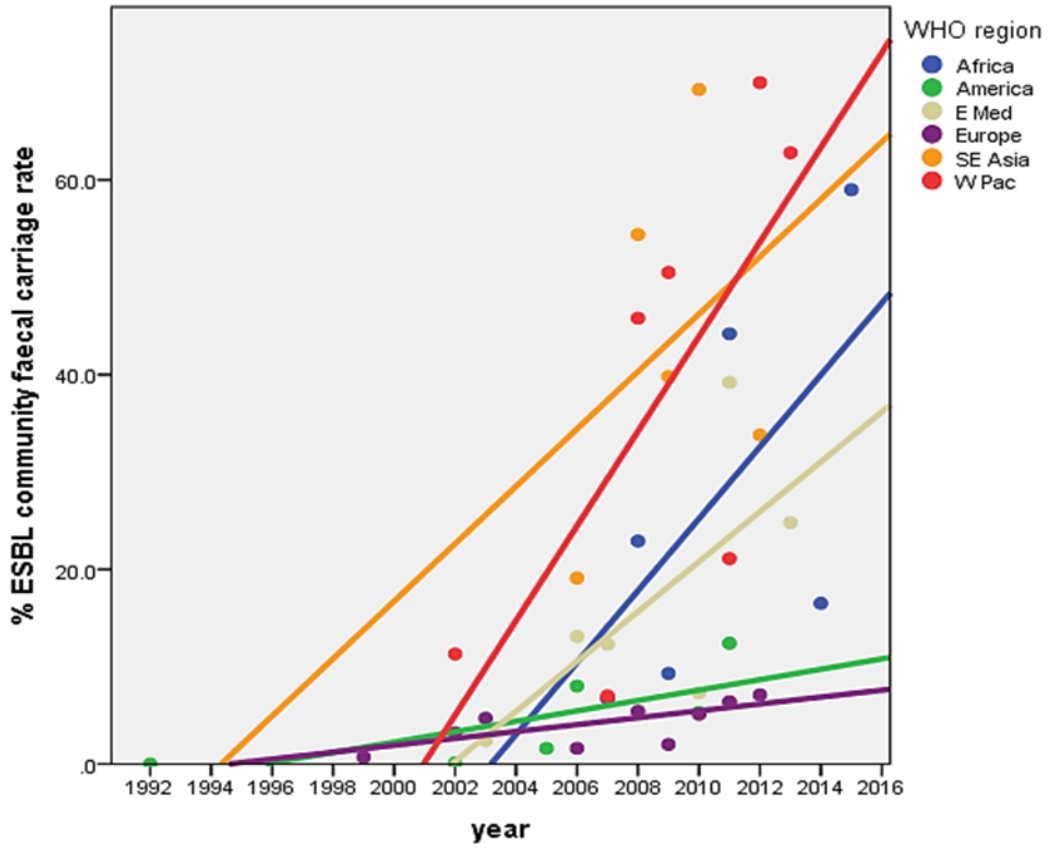


Figure 2.1 Prevalence rate (%) of community faecal ESBL carriage by World Health Organisation (WHO) region. Where there was >1 study per year within a region, the ESBL frequency and denominator values were added for that year, producing one data point (%) per year for each region. Europe was the only region showing a statistically significant rise in ESBL rates by linear regression: R^2= 0.429, p=0.04.

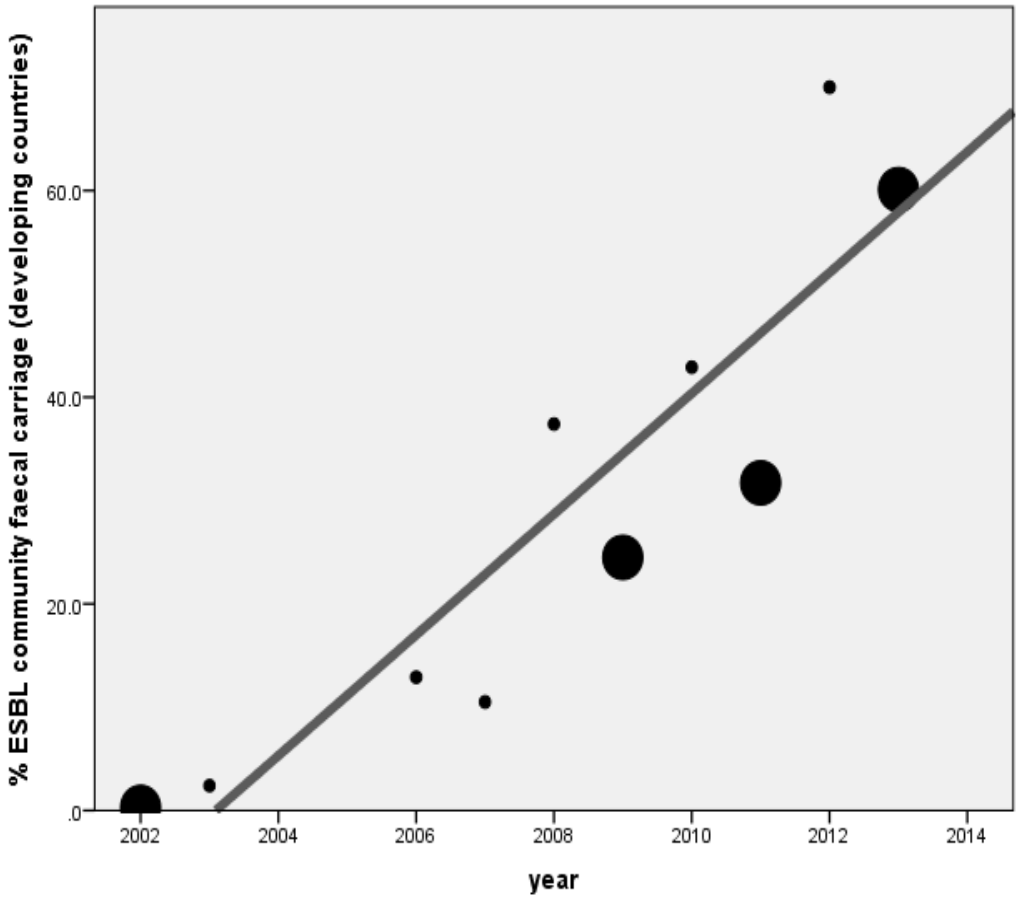


Figure 2.2 Prevalence (%) community faecal ESBL carriage for developing countries only. (Developing countries here are defined as all countries excluding North America and WHO region: Europe). Each data point is the mean of global community faecal carriage rates for that year for developing countries. Large circles: denominator of ESBL data>1000 isolates, small circles <1000, (note: no given year had <250 isolates). R^2=0.814, p=0.0004.

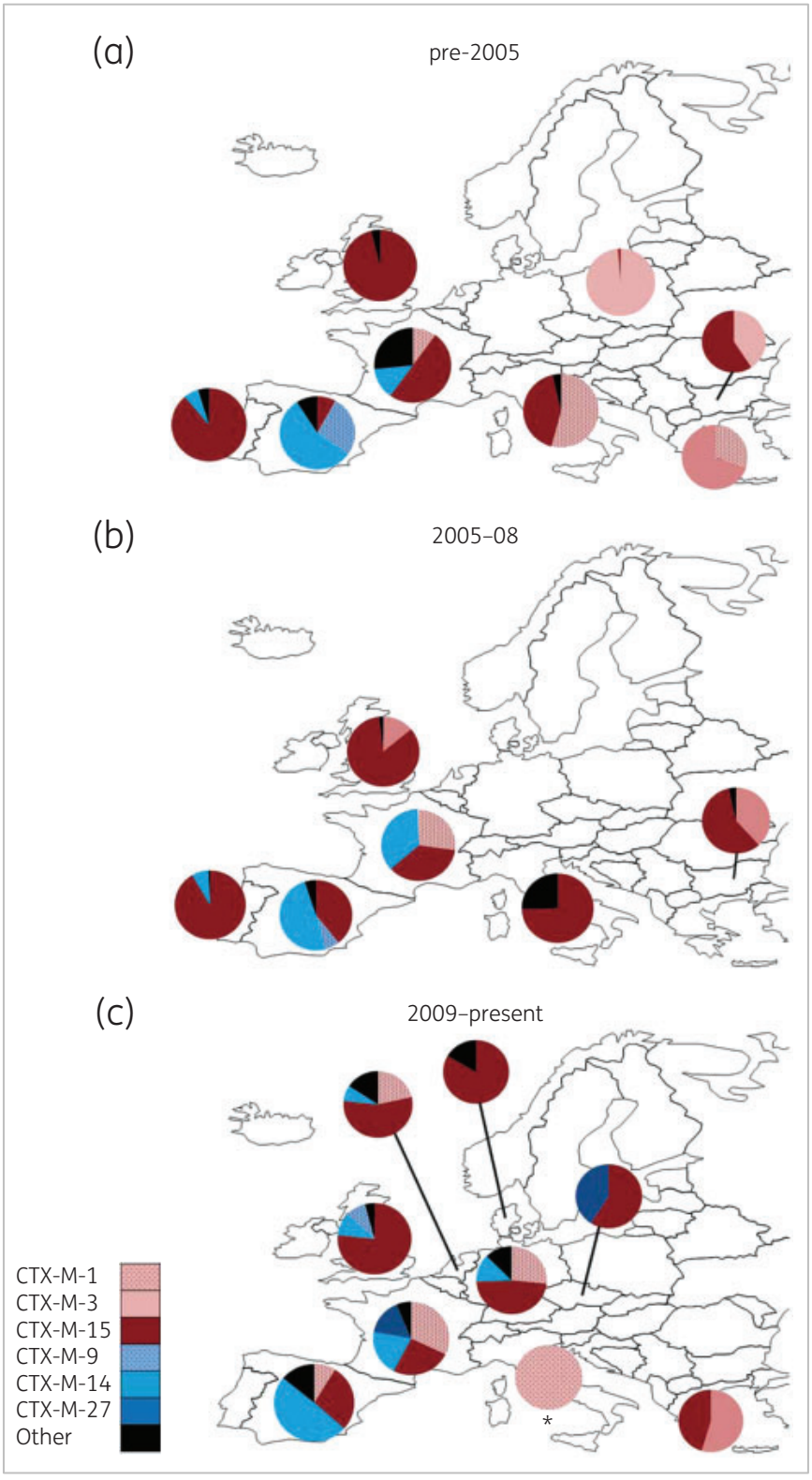


Figure 2.3 a,b,c **CTX-M trends over 3 time periods for Europe;** (data include both hospital and community isolates).
*indicates that for these areas, only CTX-M grouping was done, and these were not subdivided into genotypes. Note: many countries have no published data for certain time periods. Colour shades represent CTX-M groups: red, group 1; blue, group 9.

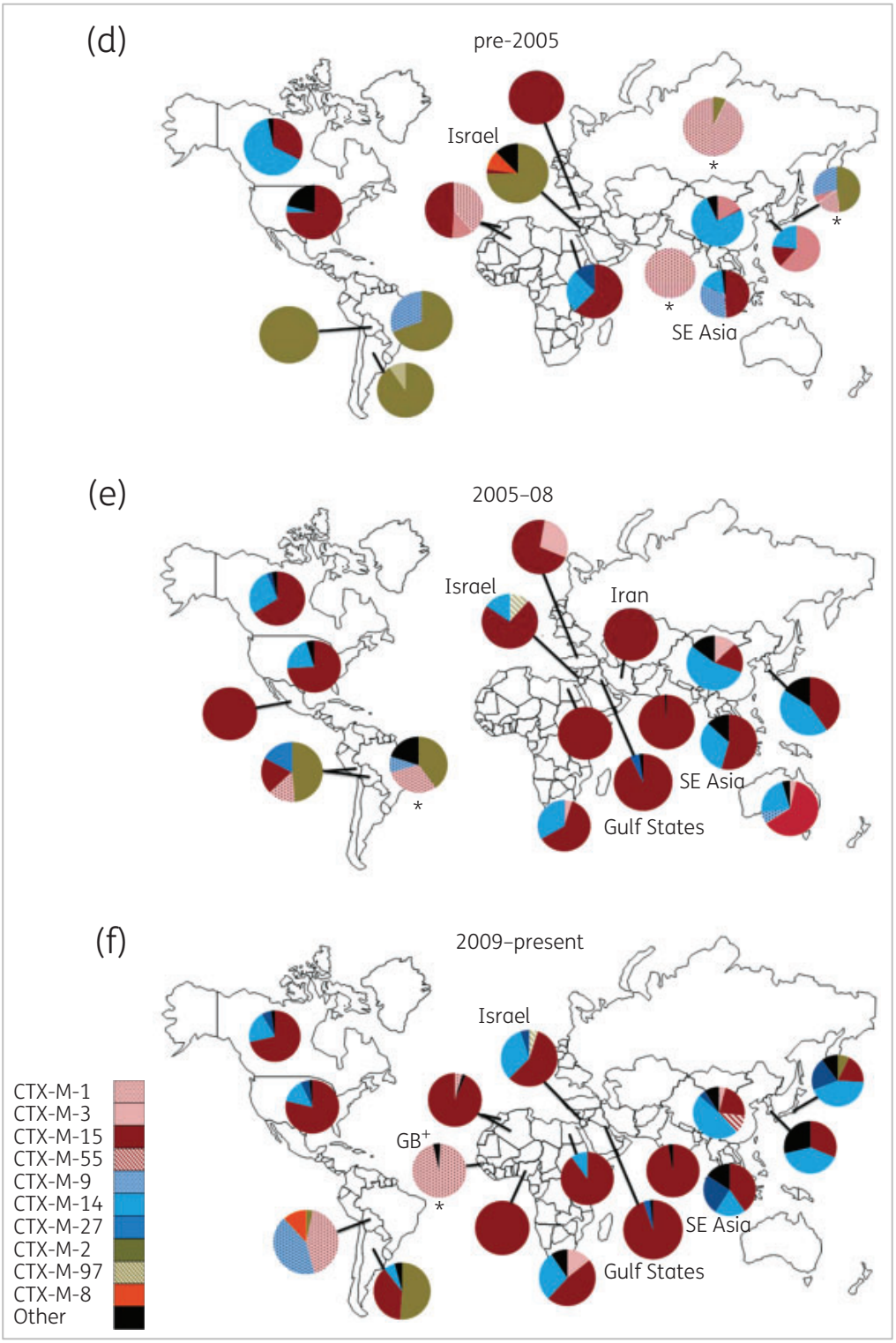


Figure 2.3 d,e,f. **CTX-M trends over 3 time periods for rest of the world. (data include both hospital and community isolates).** *indicates that for these areas, only CTX-M grouping was done, and these were not subdivided into genotypes. South-East (SE) Asia includes Cambodia, Indonesia, Malaysia, The Philippines, Taiwan and Vietnam. Gulf States include Kuwait, Saudi Arabia and United Arab Emirates. Data for Australia and New Zealand are combined. GB, Guinea-Bissau. Colour shades represent CTX-M groups: red, group 1; green, group 2; orange, group 8/25; blue, group 9.

2.4.2 Diversity of *bla*$_{CTX-M}$ genotypes in food-animal isolates in China

Due to the plethora of data on *bla*$_{CTX-M}$ genotypes from food animal-derived *E. coli* in China (Ma *et al.*, 2012; Rao *et al.*, 2014; Xia *et al.*, 2014; S. Li *et al.*, 2015), this country has been used as an example of the trends in *bla*$_{CTX-M}$ genotypes in food animals (Figure 2.4). The trend over time is an early complete dominance of group 9 *bla*$_{CTX-M}$ genotypes, especially *bla*$_{CTX-M-14}$ (Figure 2.4). Since 2009, the rise of group 1 *bla*$_{CTX-M}$ has occurred, especially *bla*$_{CTX-M-55}$ which has begun to replace group 9 enzymes (Figure 2.4).

2.4.3 Sanitation

The relationship between access to improved sanitation facilities and ESBL community carriage by country was determined (Figure 2.5). A linear regression analysis showed a modest but significant association between ESBL carriage and access to basic sanitation with some exceptions (R^2=0.169, p=0.03) (Figure 2.5).

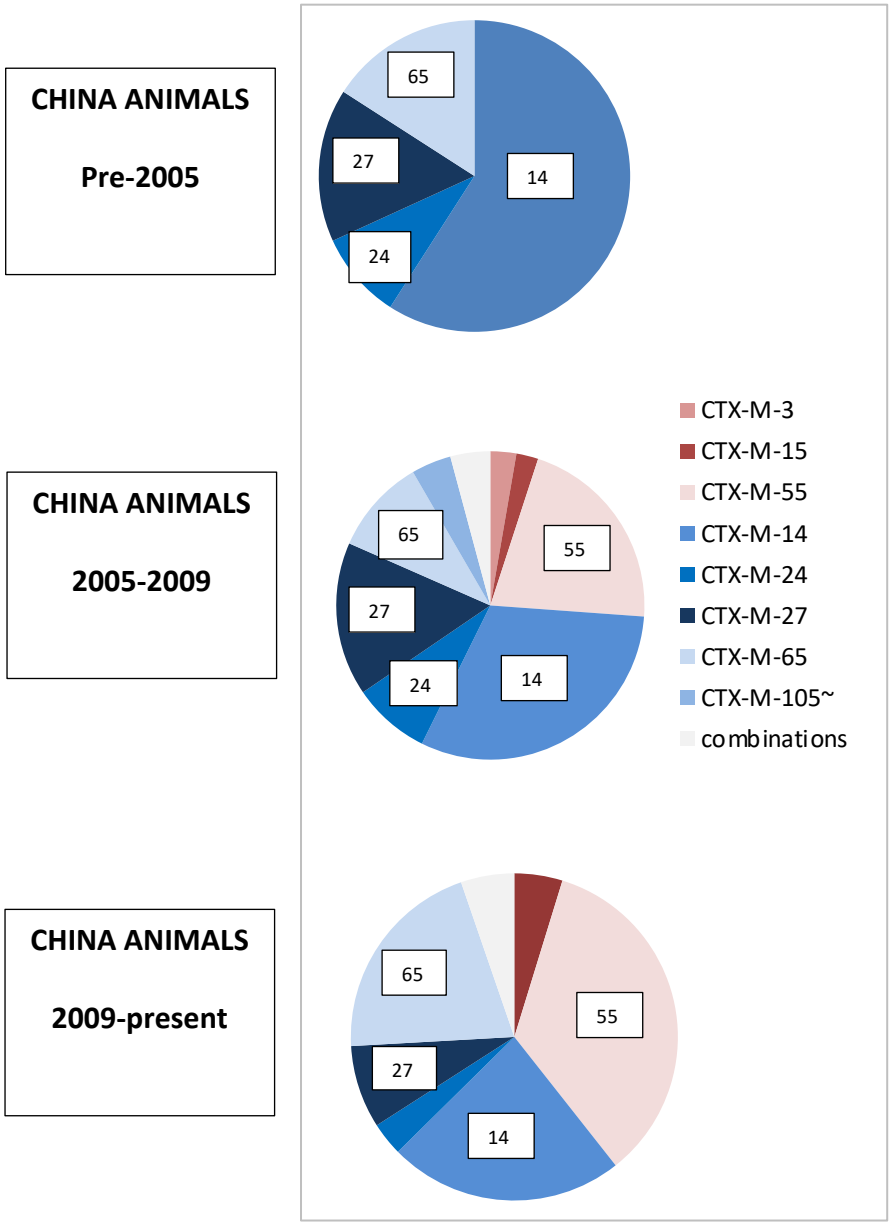


Figure 2.4 Epidemiology of *bla*$_{CTX-M}$ in isolates from food animals in China. Genotypes are superimposed on major pie segments

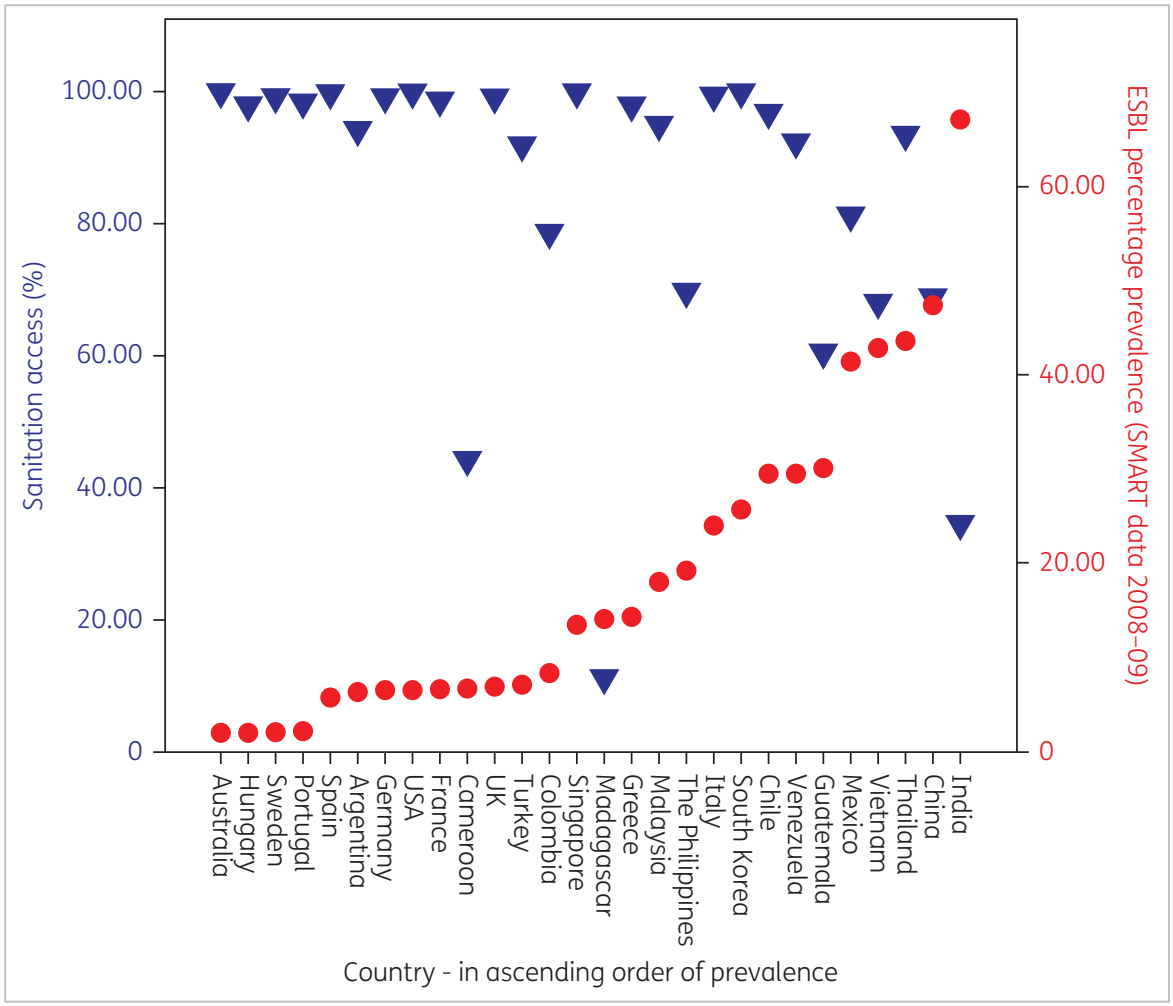


Figure 2.5 Access to basic sanitation and ESBL prevalence. Sanitation access is access to improved sanitation facilities, 2008–09, as defined by WHO/UNICEF (data.worldbank.org/indicator/SH.STA.ACSN).

2.5 Discussion

The investigation of ESBL global epidemiology outlined in this chapter has focussed on the temporal and regional changes in *bla*$_{CTX-M}$ (figure 2.3). Several interlinked factors can be used to account for these global changes: (1) plasmids and horizontal gene transfer; (2) successful *E. coli* clones; (3) Food animals; (4) The natural environment; (5) Human migration, and (6) access to basic sanitation.

2.5.1 The role of epidemic plasmids and other mobile genetic elements

The role of a plasmid in the mobilisation of a beta-lactam gene was first described in relation to transfer of multiple drug resistance phenotypes between *E. coli* and *Shigella* spp. by a Japanese research group (Watanabe and Fukasawa, 1961). The first beta-lactamase in *E. coli* was identified from the blood culture of a Greek patient named Temoneira, hence the enzyme was named TEM, which was transferrable via plasmid conjugation (Datta and Richmond, 1966).

The horizontal transfer of *bla*$_{CTX-M}$ genes on conjugative plasmids is fundamental to their evolution and global spread (Carattoli, 2013). Plasmids in Enterobacteriaceae tend to be well adapted to their host, often encoding plasmid addiction systems such as post segregational killing systems, which promote plasmid maintenance in progeny, even in the absence of antibiotic selective pressure (Carattoli, 2009). This helps to explain increased community ESBL carriage globally (figure 2.1). Furthermore, indistinguishable plasmids found across

different strains in Enterobacteriaceae are referred to as “epidemic plasmids” (Carattoli, 2009) (see Chapter 5).

In addition to the role of plasmids, other mobile genetic elements, such as insertion sequences, transposons, and integrons, play a large part in the dissemination of bacterial resistance genes, including *bla*$_{CTX-M}$ genes (Toleman and Walsh, 2011). The role of plasmids and these other mobile genetic elements in *bla*$_{CTX-M}$ dissemination, will be outlined in detail later in this thesis (Chapter 5).

2.5.2 The role of *E. coli* clonal lineage ST131

The clonal spread of virulent strains such as uropathogenic *E.coli* B2,025:H4-ST131, which commonly carries *bla*$_{CTX-M-15}$, play an important role in the global dissemination of these genes (Nicolas-Chanoine et al. 2014, Woodford et al. 2011). The most widespread ST131 sub-clone is *H*30-Rx, which has a propensity for carriage of *bla*$_{CTX-M-15}$ (Price, Johnson and Aziz, 2013).

Worldwide surveys in 2008 led to the discovery of the international ESBL *E.coli* clone ST131 which belongs to phylogenetic group B2, serotype O25b:H4 (Coque *et al.*, 2008; Nicolas-Chanoine *et al.*, 2008). ST131 has been found mostly in human E.coli isolates, but also in animals (Platell *et al.*, 2011) and the environment (Zurfluh *et al.*, 2013; Amos *et al.*, 2014). Successful in Western Europe and North America (Banerjee and Johnson, 2014), ST31 has only been found occasionally in India (Hussain *et al.*, 2012; Roy *et al.*, 2015), and China (Zhong et al. 2015; Wang et al. 2016; Xia et al. 2014). The most common *bla*$_{CTX-M}$ genotype in

most regions of the world is $bla_{CTX\text{-}M\text{-}15}$ (Figure 2.3), and it is reasonable to suggest that most of the CTX-M-15-producing *E. coli* isolates in the world are ST131, however there is a notable lack of MLST data to support this claim, especially in developing countries.

Due to its relatively low prevalence in non-human isolates, ST131 probably originated from human sources (Banerjee and Johnson, 2014). The most common ST31 lineage, H30, has acquired fluoroquinolone resistance, followed by $bla_{CTX\text{-}M\text{-}15}$ acquisition on a plasmid; both are associated with the sub-lineage H30Rx (Price, Johnson and Aziz, 2013). Recent sequence analysis of global ST131 isolates suggests that H30Rx originated in North America around 1991 (Stoesser, et al 2016). The capture of fluoroquinolone resistance and $bla_{CTX\text{-}M\text{-}15}$ by ST131 was a highly evolutionarily advantageous event given the high rates of use of fluoroquinolones and cephalosporins in the USA at this time (Stoesser, et al 2016). It seems likely that human carriage of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$ to North America from India and China respectively occurred, then leading to the capture of these genotypes by ST131 (onto pre-existing plasmids in ST131 strains, via IS26-mediated transposition as discussed above), and clonal dissemination thereafter. Presumably the spread of ST131 E. coli out of North America was a later event. However, it must be noted that this hypothesis is based on the assumption that because $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}15}$ were first described in China and India respectively, that these countries were also the origin of these variants.

Importation of the successful H30Rx sub-clone via human migration has probably led to the dominance of ST131 *E.coli* with $bla_{CTX\text{-}M\text{-}15}$ in many regions of the world such as Canada (Pitout *et al.*, 2009; Peirano *et al.*, 2012), Japan (Osawa *et al.*, 2015), Israel (Karfunkel *et al.*,

2013), and there is evidence of worldwide dissemination of H30Rx including in India and China (Peirano *et al.*, 2014). Human migration is likely to be the cause of the worldwide dissemination of ST131 and has probably led to the displacement of existing genotypes with $bla_{CTX\text{-}M\text{-}15}$ in other regions, especially South America, and much of Western Europe (figure 2.3). ST131 is also known to carry bla-CTX-M variants from all four main CTX-M groups, and notably is associated with $bla_{CTX\text{-}M\text{-}14}$ (particularly in Canada, China, Japan and Spain) (Nicolas-Chanoine, Bertrand and Madec, 2014).

The *E.coli* ST131 clonal group is associated with a host of specific virulence genes which, combined with antimicrobial resistance genes has made this a uniquely successful extra-intestinal pathogenic *E.coli* (ExPEC) strain (Johnson *et al.*, 2010).

2.5.3 Role of food animals in the spread of $bla_{CTX\text{-}M}$

The use of antimicrobials in food animals provides selective pressure for the propagation of AMR bacteria in animals which are often relevant human pathogens (Robinson *et al.*, 2016). Variants of $bla_{CTX\text{-}M}$ often colonise the gut in farmed birds, mammals (Stokes *et al.*, 2012; Zheng *et al.*, 2012), as well as raw meat for human consumption (Doi *et al.*, 2010; Overdevest *et al.*, 2011).

There is evidence for the transmission of ESBL-producing E.coli between animals and humans, (de Been *et al.*, 2014; Lazarus *et al.*, 2015), and data from the UK suggests animal *E.coli* strains probably acquired the bla-CTX-M plasmid from human strains (Hunter *et al.*, 2010). A systematic review showed that there is a role for both bacterial clonal transmission

and horizontal gene transfer of ESBL and ampC genes between human and food animal reservoirs. Transmission of ESBL-producers between poultry sources and human sources appears to be more common than transmission from other food animals (Lazarus *et al.*, 2015).

Epidemic plasmid spread among animal and human *E.coli* strains has been found in The Netherlands (de Been *et al.*, 2014), China (Ho *et al.*, 2012) and in the UK (Stokes *et al.*, 2012). In The Netherlands, horizontal gene transfer between human and animal strains appears to have a greater role than the clonal spread of *E. coli* strains between these niches. Assembled plasmids in genetically diverse human and animal strains had almost identical plasmid backbones (de Been *et al.*, 2014). Moreover, further evidence suggesting the role of plasmid spread between human and animal strains comes from Bolivia, where two plasmids from *Klebsiella pneumoniae* encoding $bla_{\text{CTX-M-65}}$ (a rare genotype in South America), isolated from clinical samples, were found to be almost identical to the *E.coli* plasmid pHN7A8 (Sennati *et al.*, 2016), which had previously been isolated from a dog in China in 2008 (He *et al.*, 2013).

The potential spread of $bla_{\text{CTX-M}}$ between animals and humans via the food chain (Overdevest *et al.*, 2011; de Been *et al.*, 2014), through animal handling (Nakane, Kawamura and Goto, 2016), and from animals to the environment (Ma *et al.*, 2012; S. Li *et al.*, 2015) is made worse by the high prevalence of unregulated wet food markets in the developing world, for example in China (P. L. Ho *et al.*, 2011). This microbial habitat, combined with heavy use of antibiotics in food animal production in China (Zhang *et al.*, 2015), provide the perfect conditions for horizontal gene transfer from animal to human strains. This dynamic

evolutionary situation explains why there is a tremendous diversity of *bla*$_{CTX-M}$ genotypes isolated from food animals in China (figure 2.4). The prevalence of *bla*$_{CTX-M-55}$ in China has grown significantly in recent years in both animal (Rao *et al.*, 2014) and human populations (Xia *et al.*, 2014), and it has been suggested that *bla*$_{CTX-M-55}$ in human isolates arose from food animal sources (Zhang *et al.*, 2014). Isolates of human and food animal origin share dominant *bla*$_{CTX-M}$ genotypes (figure 2.3 and 2.4), suggesting that there is clonal or horizontal exchange of *bla*$_{CTX-M}$ between these settings. Due to rising demands, worldwide antibiotic use in food animals is projected to rise by 67% by 2030, therefore the role of food animals as a source of AMR genes will become more prominent (Van Boeckel *et al.*, 2015).

2.5.4 Role of the natural environment in dissemination of ESBLs

The natural environment as a reservoir of ESBL genes is inextricably linked to human and animal reservoirs (Wellington *et al.*, 2013; Woerther *et al.*, 2013). The origin of CTX-M enzymes can be traced back to *Kluyvera* spp, which normally inhabit the rhizosphere, so environmental sources play an important role in the dissemination of *bla*$_{CTX-M}$.
ESBL-producing bacteria have been found in sewage (Yang *et al.*, 2012), rivers downstream of wastewater treatment facilities (Amos *et al.*, 2014), urban freshwater (Dhanji, Murphy, *et al.*, 2011; Bajaj *et al.*, 2015; Azam, Jan and Haq, 2016) and marine environments (Maravić *et al.*, 2015). Furthermore, resistant organisms have been found in several agricultural settings (Hammerum *et al.*, 2014),(Randall *et al.*, 2014),(Laube *et al.*, 2014).

Pollution of the environment with Enterobacteriaceae from human and animal waste is an issue in developing and developed countries. However, the poor standards of sanitation, and

contamination of drinking water supplies in the developing world allows increased cycling of CTX-M-producing *E.coli* between humans and the environment, (Hawkey, 2015). This explains our finding of a significant upward trend in ESBL carriage rates in developing countries (figure 2.2), notably in India, where ESBL carriage rates are amongst the highest in the world (Chen *et al.*, 2011). Moreover, the environmental-human exchange of AMR genes is highly relevant to the spread of other faecally-carried genes such as $bla_{\text{NDM-1}}$ (Walsh *et al.*, 2011).

The presence of resistant genes in an environment is closely related to anthropogenic activities. For example, the prevalence of class I integrons carrying resistance genes in sewage sludge, pig slurry and textile mill effluent is significantly higher than levels found in fallowed soil. The common practice of using animal manure as fertiliser creates opportunities for species of Enterobacteriaceae to capture novel resistance genes on mobile genetic elements from soil bacteria (Wellington *et al.*, 2013). More recently, a metagenomics approach was used to assess the AMR gene content across human, environmental and animal microbiomes in two developing regions in South America, and showed that mobile genetic elements played a significant role in the transfer of ESBLs (notably $bla_{\text{TEM-1}}$) between different resistomes (Pehrsson *et al.*, 2016).

Environmental contamination with fluoroquinolones is especially worrisome, as these are known to persist in rivers and soil, and have the ability to select bacteria bearing class I integrons which carry resistance cassettes, including *qnr* genes and $bla_{\text{CTX-M}}$ genes (Wellington *et al.*, 2013). In India, high levels of ciprofloxacin in the waste water effluents of

pharmaceutical factories and in nearby sources of drinking water is a serious issue which is likely to be a major contributory factor in the emergence and spread of *bla*$_{CTX-M}$ in human populations and in the environment (Fick *et al.*, 2009).

2.5.5 Role of poor sanitation in developing countries

There is increasing community prevalence of ESBL-producing Enterobacteriaceae worldwide (figure 2.1), especially CTX-M-15, which is reflected by much higher faecal carriage rates in Asia compared to Europe and North America (Woerther *et al.*, 2013). Individuals with ethnic origins in the Middle East or South Asia have significantly higher carriage rates of CTX-M-15 producing *E.coli* compared with Europeans, which is likely due to the high frequency of travel to these areas amongst particular ethnic groups (Wickramasinghe *et al.*, 2012). Prospective cohort studies have shown that travellers from Western countries visiting countries with high prevalence of ESBL producing bacteria are likely to become asymptomatic carriers: 75% of those travelling to Southern Asia acquired ESBL-producing organisms (Arcilla *et al.*, 2017).

Access to improved sanitation facilities as defined by WHO/UNICEF is an important indicator of social and economic development, and is important in reducing the spread of diarrhoeal diseases (http://data.worldbank.org/indicator/SH.STA.ACSN). It is likely that such access is also paramount in controlling the spread of ESBLs. Improving levels of sanitation worldwide was identified as a key intervention in preventing acquisition of resistant bacteria (O'Neil, 2016).

Figure 2.5 shows that there is an association between access to basic sanitation and rates of ESBL-producing Enterobacteriaceae by country. However, it must be noted that confounding factors could easily be implicated in this relationship. For example, more developed countries such as the UK, with high levels of access to basic sanitation, tend to have good standards of human sewage treatment, regulations limiting environmental pollution in industry and agriculture, and are good at limiting the use of antibiotics in humans and animals. All these factors will contribute to lower levels of ESBL-producing organisms.

India is likely to be where *bla*$_{\text{CTX-M-15}}$ originated (Ensor *et al.*, 2006) and is the most common ESBL worldwide. Despite gradual improvements in India, access to basic sanitation still falls below 40% (figure 2.5). Poor access to latrines in India, combined with high population density and increasing human migration, have made this region a key epicentre of ESBL evolution, and a source for the worldwide spread of *bla*$_{\text{CTX-M-15}}$.

2.5.6 Prominent global changes in CTX-M epidemiology: the fall of *bla*$_{\text{CTX-M-2}}$ and the emergence of *bla*$_{\text{CTX-M-27}}$

The distribution of CTX-M variants in particular geographical regions is not always easily attributed to human migration. Given historical and cultural links between Spain/Portugal with South America, it follows that CTX-M variants in these regions should be similar, due to frequent migration between these areas. However, where *bla*$_{\text{CTX-M-2}}$ has been the main genotype in South America until the recent spread of *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-2}}$ has never gained a foothold in Spain or Portugal (Livermore *et al.*, 2006). One explanation for this phenomenon

is that *bla*$_{CTX\text{-}M\text{-}2}$ evolved in South America, was probably imported to Western Europe on multiple occasions, but never had the successful evolutionary characteristics to compete with the dominant genotypes in Europe. Outside of South America, the only regions showing a predominance of *bla*$_{CTX\text{-}M\text{-}2}$, before 2004, were Japan (Yagi *et al.*, 2000) and Israel (Karfunkel *et al.*, 2013). It is clear that *bla*$_{CTX\text{-}M\text{-}2}$ is somehow evolutionarily 'less fit' than its counterparts *bla*$_{CTX\text{-}M\text{-}14}$ and *bla*$_{CTX\text{-}M\text{-}15}$, as these two genotypes have started to overtake *bla*$_{CTX\text{-}M\text{-}2}$ in all regions where it was previously the main CTX-M variant (see figure 2.3).

There is increasing evidence to suggest that *bla*$_{CTX\text{-}M\text{-}27}$, a single nucleotide variant of *bla*$_{CTX\text{-}M\text{-}14}$, has begun to out-compete other *bla*$_{CTX\text{-}M}$ genotypes globally (figure 2.3). First isolated from a clinical *E.coli* in France (Bonnet *et al.*, 2003), *bla*$_{CTX\text{-}M\text{-}27}$ is now found worldwide, recently gaining ground in Japan, China, South East Asia, North America, and in Europe (Doumith *et al.*, 2012; Castanheira *et al.*, 2013; Denisuik *et al.*, 2013; Biedenbach *et al.*, 2014; Micenková *et al.*, 2014; Matsumura *et al.*, 2015; Vlieghe *et al.*, 2015; Zhong *et al.*, 2015; Birgy *et al.*, 2016) (see figure 2.3). The cause of this rise is unclear: CTX-M-27 has a higher MIC to ceftazidime compared with CTX-M-14, so use of ceftazidime would theoretically select for *bla*$_{CTX\text{-}M\text{-}27}$ (Kuroda *et al.*, 2012). *E.coli* clones producing CTX-M-27 may also be more transmissible in a nosocomial environment, when compared to CTX-M-15-producers (Adler *et al.*, 2012).

As discussed above, there is genetic exchange and/or clonal exchange of *E. coli* of animal or human origin between humans, animals and the environment. The presence of *bla*$_{CTX\text{-}M\text{-}27}$ in food animal isolates from China in all three time periods suggests a stable reservoir for

bla$_{CTX\text{-}M\text{-}27}$ in this ecological niche (figure 2.4). Indeed, *bla*$_{CTX\text{-}M\text{-}27}$ identified from E.coli from food animals in a multicentre Chinese study represented 12% of *bla*-CTX-M variants detected (notably in ducks) (Zheng *et al.*, 2012), and *bla*$_{CTX\text{-}M\text{-}27}$ also dominates ESBL-producing *Salmonella enterica* serotypes in China (Zhang *et al.*, 2016). In Vietnam as in China, where close proximity of animals and humans is common, *bla*$_{CTX\text{-}M\text{-}27}$ is the most common *bla*$_{CTX\text{-}M}$ genotype (Biedenbach *et al.*, 2014), which could be due to human transmission from animal sources.

Clonal spread of *E.coli* ST131 in humans has certainly played a role in the dissemination of *bla*$_{CTX\text{-}M\text{-}27}$ in Japan (Yano *et al.*, 2013; Osawa *et al.*, 2015), China (Zhong *et al.*, 2015), France (Blanc *et al.*, 2014), Portugal (Rodrigues *et al.*, 2016), Germany (Valenza *et al.*, 2015) ,and the Czech Republic (Micenková *et al.*, 2014). Moreover, *E.coli* ST131 isolates producing CTX-M-27 have been reported from companion animals in Japan (Harada, Nakai and Kataoka, 2012), from wild birds in Czech Republic (Tausova *et al.*, 2012), and from freshwater in Switzerland (Zurfluh *et al.*, 2013).

However, ST131 CTX-M-27-producing isolates from South Korea and Japan did not belong to the H30Rx sub-clone (Matsumura *et al.*, 2015; Kim *et al.*, 2016). Rather, Matsumura and colleagues found that *bla*$_{CTX\text{-}M\text{-}27}$ isolates were significantly associated with the H30R sub-clone (Matsumura *et al.*, 2015). They also suggested that although *bla*$_{CTX\text{-}M\text{-}27}$ is a single nucleotide variant of *bla*$_{CTX\text{-}M\text{-}14}$, *bla*$_{CTX\text{-}M\text{-}27}$ is unlikely to have evolved by point mutation from *bla*$_{CTX\text{-}M\text{-}14}$ in Japan, as the genetic surroundings and virulence profiles of *bla*$_{CTX\text{-}M\text{-}27}$ versus *bla*$_{CTX\text{-}M\text{-}14}$ are quite different (Matsumura *et al.*, 2015). This suggests that separate

importations to Japan and/or separate *bla*-CTX-M capture events to *E.coli* clonal lineages occurred for $bla_{CTX\text{-}M\text{-}27}$ and $bla_{CTX\text{-}M\text{-}14}$. In contrast, it remains a possibility that $bla_{CTX\text{-}M\text{-}27}$ did evolve from $bla_{CTX\text{-}M\text{-}14}$ by point mutation in Japan, as the first cluster of CTX-M-27-producing isolates in the world was described in Japan (Kuroda *et al.*, 2012). It is possible that the original $bla_{CTX\text{-}M\text{-}14}$ progenitor has since been subject to out-competition and/or genetic rearrangement which might explain why it has not been detected in the more recent studies outlined above.

The role of horizontal gene transfer in the spread of $bla_{CTX\text{-}M\text{-}27}$ has very poor coverage in the literature. However, interestingly, published data suggests that in animal strains, $bla_{CTX\text{-}M\text{-}27}$ is associated with a wide range of plasmid replicons in transconjugants including N, FIB, FII, I1, HI2, A/C, and P (Ma *et al.*, 2012; Jiang *et al.*, 2014; Zhang *et al.*, 2016), whereas in studies with human isolates, $bla_{CTX\text{-}M\text{-}27}$ has been found in E.coli ST131 strains associated with with F1A, FIB and FII replicons only (Kim *et al.*, 2016; Nakane, Kawamura and Goto, 2016). Thus, one could speculate that $bla_{CTX\text{-}M\text{-}27}$ has undergone transposition onto a IncF plasmid residing in *E.coli* ST131, similar to the IS26 mediated capture of $bla_{CTX\text{-}M\text{-}15}$ described recently (Johnson *et al.*, 2016).

Understanding why certain $bla_{CTX\text{-}M}$ genotypes are more successful (e.g. $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}14}$, and $bla_{CTX\text{-}M\text{-}27}$) than others (e.g. $bla_{CTX\text{-}M\text{-}2}$), will support the development of more effective infection prevention and control policies.

2.6 Conclusion

Infections with CTX-M-producing Enterobacteriaceae are of huge clinical importance, as increasing rates of ESBL-producers drive carbapenem prescribing, which in turn promotes the spread of potentially untreatable carbapenemase-producing Enterobacteriaceae (Geyer *et al.*, 2015). Dealing with this situation requires constant monitoring of the global epidemiology of CTX-M genotypes, allowing early anticipation of emerging ESBL genes. Moreover, improving the global surveillance of AMR has been identified as a key intervention in addressing the rise of resistant bacteria (O'Neil, 2016).

The data presented in this chapter has several limitations. Notably, in the early surveys of $bla_{\text{CTX-M}}$, sample sizes were often small, and several studies, undertook only CTX-M grouping PCR. Furthermore, many studies do not report on the clonal relatedness of the strains they included, leading to bias by outbreaks. There is publication bias, e.g. in China, many studies describe CTX-M prevalence, whereas in Africa there are few studies. Therefore, the limited data from under-resourced countries is likely to represent the 'tip of the iceberg' in terms of percentage ESBL prevalence in these regions, leading to late detection of new genes. WHO/UNICEF data on access to improved sanitation facilities was utilised, which is defined as "one that hygienically separates human excreta from human contact."(WHO, 2016) This includes pit latrines and flushing latrines, and it must be noted that the fate of this human waste is not recorded in this data and therefore the presence or effectiveness of onward sewage processing is not recorded. Therefore human exposure to human sewage (and therefore ESBL-producing bacteria) in many underdeveloped regions is likely to occur, even

if access to improved sanitation facilities is high, and such exposures are likely even with high standards of sewage treatment (Amos *et al.*, 2014).

This chapter has shown that CTX-M community faecal carriage rates are rising (Figure 2.1 and 2.2), and this is more pronounced in developing countries, supporting previous studies (Woerther *et al.*, 2010; Karanika *et al.*, 2016). Therefore, there exists a vast human reservoir of $bla_{CTX\text{-}M}$-producing strains, which provide a source of bacteria which often go onto cause antimicrobial-resistant infections. Equivalent ESBL reservoirs are present in the environment and in food producing animals, which constantly exchange clones and mobile genetic elements with the human reservoir. Transfer of $bla_{CTX\text{-}M}$ thus occurs by the spread of clonal lineages, epidemic plasmid spread, or via the movement of smaller genetic elements by transposition or transduction. Although these processes are usually studied in isolation, they do not occur in isolation. Rather, the concept of the "selfish gene" (Dawkins, 1976) should be applied: each individual $bla_{CTX\text{-}M}$ gene is opportunistic, using every possible mechanism to propagate itself. Therefore, the global success of $bla_{CTX\text{-}M}$ is due to a combination of all the mechanisms discussed above.

There is little doubt that a combination of human factors has increased the propagation of $bla_{CTX\text{-}M}$. Antibiotic overuse in humans and animals, increased global migration and population density, and contamination of the food chain and environment with human and animal waste promote AMR (Holmes *et al.*, 2016). The evolution of highly successful lineages of CTX-M-producing bacteria is a critical factor. We have described the contribution of poor access to basic sanitation facilities in some countries as a risk factor for higher ESBL carriage.

Poor sanitation access in these countries, combined with poor standards of animal husbandry, unregulated wet food markets, and antibiotic overuse in humans and animals is dangerous, and provides opportunities for the evolution of novel AMR genes.

Where possible, attempts must be made to mitigate these risks through preventative strategies, such as improving access to latrines in developing countries, better antimicrobial stewardship in humans and animals, environmental controls, and targeted evidence based ESBL screening strategies (O'Neil, 2016).

The data presented in this chapter is not only of relevance to ESBL-producing Enterobacteriaceae. Bacteria carrying *mcr-1* and/or carbapenemase genes are found in similar habitats to Enterobacteriaceae producing CTX-M, such as the human gut. Therefore, explanations for the trends observed in $bla_{\text{CTX-M}}$ epidemiology can be applied to these other faecally-carried AMR genes.

This chapter has provided an up to date review and analysis of the global epidemiology of the $bla_{\text{CTX-M}}$ family, demonstrating a continued global rise in ESBL incidence. This has led to an inevitable rise in carbapenem use in humans with an associated rise in resistance. There have been dramatic regional shifts in the epidemiology of CTX-M-producing Enterobacteriaceae; but also remarkable stability in many regions. Both changes and stability in $bla_{\text{CTX-M}}$ genotypes in specific regions of the world seem to favour the highly successful $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ genes. Understanding global temporal trends in these

genotypes will help develop hypotheses as to why these changes occur, supporting strategies for reducing the spread of AMR in gram negative bacteria.

Chapter 3: Volunteer recruitment, demographics, and CTX-M-producing *E. coli* acquisition

3.1 Introduction

The global prevalence of CTX-M-producing *E. coli* (CTX-M-EC) is rising, thereby increasing the use of carbapenem antibiotics, and therefore acting as a driver for the emergence of carbapenemase-producing Enterobacteriaceae (CPE) (Hawkey, 2015). Anthropogenic factors such as low levels of basic sanitation, poor standards of animal husbandry in agriculture, and the overuse of antibiotics in animals and humans, all contribute to high prevalence of CTX-M-EC in developing countries, especially in South Asia. (Bevan, Jones and Hawkey, 2017).

International travel plays a key role in the spread of antibiotic resistant bacteria carried in the human gut (Nordahl Petersen *et al.*, 2015), and the acquisition of CTX-M-EC by travellers who visit countries with high levels of CTX-M- producers in the environment, is a well-recognised phenomenon (Chapter 1, Section 1.7). In particular, travel to South Asia, which includes densely populated countries such as India and Bangladesh, presents the highest risk for acquisition of CTX-M-producing Enterobacteriaceae (Arcilla *et al.*, 2017).

Twenty-two studies investigating the acquisition of CTX-M-producing bacteria by travellers have been published, but none prospectively recruited volunteers from the UK (Chapter 1, Table 1.1). The work presented in this thesis is therefore the first study to assess CTX-M

acquisition in travellers returning to the UK. The following chapter will outline volunteer recruitment and demographics, post travel survey data, and CTX-M-EC acquisition rate.

3.2 Hypotheses

1. The rate of acquisition of CTX-M-EC will be similar to previous studies with volunteers travelling from developed countries to South Asia.
2. Risk factors known to be associated with CTX-M-EC acquisition will be identified from the post-travel questionnaires completed by volunteers.

3.3 Methods

3.1 Study Design and Ethical Approval

The traveller study methodology was conceived after discussion and approval by South Birmingham Research Ethics Committee (REC) and presentation to the patient forum group at Heart of England NHS Foundation Trust. The REC-approved study documents which included advertisements, participant information sheets, consent forms, and questionnaires, are provided in appendix 2. A power calculation was not undertaken at the outset because the frequency of transfer of ESBL-EC plasmids in-vivo is unknown. Therefore, the number of volunteer isolates required to detect such an event could not be determined.

3.2 Recruitment and Inclusion/Exclusion Criteria

Healthy volunteers were recruited from the student and staff members of the University of Birmingham and Heart of England NHS Foundation Trust. The study was advertised using posters and entries in weekly departmental e-newsletters. Targeted recruitment, through group emails, and announcements before lectures, was aimed at medical elective students planning overseas travel. A Twitter campaign using University of Birmingham twitter account was also undertaken. Individuals planning travel to South Asia, who were interested in becoming study participants, contacted ERB by email or telephone, at which point they were excluded from taking part if they reported previous travel to South Asia in the previous three months, or if they had significant long-term chronic disease. For the purposes of this study, South Asia included India, Pakistan, Bangladesh, or Sri Lanka. Long term chronic disease which would lead to exclusion from the study included diabetes mellitus, inflammatory bowel disease, cancer, any immunosuppression or any other long-term systemic illness. Children or adults lacking mental capacity were also ineligible to take part in the study. Providing they did not meet any exclusion criteria, the participant information sheet (appendix 2) was then sent to the individual by email for consideration. A meeting was then arranged with the participant to provide further information if required, and to sign the study consent form and to complete the pre-travel questionnaire (appendix 2).

3.3 Laboratory Methodology: Sample Collection, Culture and Identification

After giving consent to take part in the study, volunteers were given stool sample collection containers and transparent specimen bags. Study participants were allocated a number to identify them and allow pseudo-anonymization, and each sample was given a unique

number which would allow determination of the sample type (pre or post travel and sample number). Volunteers were asked to provide at least 5g of sample (filling container half way) and were asked to provide a specimen as close to the time of sample submission as possible. Volunteers were then met in person by ERB, and samples were stored for no more than 24h hours at 4°C.

The following culture-based ESBL isolation strategies were then undertaken: (1) A sterile swab was used to inoculate a faecal sample onto oxoid ESBL brilliance agar which was incubated at 37°C for 48 hours. Plates were checked and moved to 4°C if growth was seen at 24 hours; (2) Additional chromogenic agar (oxoid UTI brilliance agar) supplemented with a cefpodoxime disc (10µg/ml) was inoculated with serial dilutions of stool culture (x10, x100, x1000, x10000) to allow isolation of single colonies after 24 hours incubation at 37°C; (3) Brain heart infusion (BHI) broth supplemented with a cefpodoxime disc (10µg/ml) was also inoculated with a sterile swab loaded with faeces, incubated at 37°C for 24 hours. A 10µl loop of the overnight broth was then sub-cultured onto a fresh UTI chromogenic agar plate supplemented with a cefpodoxime disc and incubated for a further 24 hours. Resistance to cefpodoxime is a widely used screening tool for ESBL producers in clinical laboratories (Gibb and Crichton, 2000).

Putative ESBL-EC strains were identified as blue or pink colonies on ESBL agar, and pink or clear colonies within the cefpodoxime disc zone (or outside the zone in pre-travel samples) on UTI media before and after broth enrichment. After culture, faecal samples were stored at -80°C.

Each colony pick (appendix 1) subculture was confirmed to be *E.coli* using matrix-assisted laser desorption ionisation time-of-flight mass spectroscopy (MALDI-TOF MS). A bacterial colony was touched with a sterile toothpick which was then used to produce a smear on the polished MALDI-TOF MS target. A solution of matrix was applied to each smear on the target and allowed to dry. MALDI-TOF MS organism scores (based on degree of match to the organism mass spectral database) of >2.0 provided identification to species level. If the score was <2.0, the MALDI-TOF MS method was repeated for that isolate. After identification, isolates were stored at -80°C on protect beads. Sweeps from ESBL agar and UTI media were also taken and saved, as a representation of the 3GC-resistant population cultured.

3.4 Identification of ESBL genes

Primers for multiplex PCR were used to identify the three most common CTX-M groups: Group 1, group 2 and group 9 (Dallenne *et al.*, 2010). PCRs were only carried out on isolates which had either grown on ESBL agar, or from within the zones of cefpodoxime on non-selective UTI agar. Positive control organisms for each CTX-M group were utilised which had been previously confirmed as encoding the specific $bla_{\text{CTX-M}}$ group after Sanger sequencing.

3.4.1 CTX-M grouping PCR

Crude cell lysates for PCR reactions were obtained by suspending a colony in 200µl in molecular grade water (mH_20) and heating to 95°C for 15 minutes. After this point the PCR constituents were prepared on ice. The total volume for each PCR reaction constituted: 4µl multiplexed primers (4 x 0.5µl forward, 4x 0.5µl reverse), 12.5µl mytaq redmix, 3µl cell

lysate, and 5.5µl mH20. Reactions were run on a Veriti PCR thermal cycler using the following conditions: 95°C for 1 min, followed by 30 cycles of: 95°C 15 sec, 58°C 15 sec, and 72°C 10 sec.

3.4.2 Agarose gel electrophoresis

Agarose gel (1%) was prepared by suspending 1g agarose in 100mls in 0.5M tris-borate EDTA (TBE) buffer and boiling until all agarose was dissolved. The molten agarose was allowed to cool for 3 minutes before adding 5µl Midori Green (0.05µl/ml). The agarose was mixed briefly and then allowed to set in a gel tray with comb. PCR products were loaded directly into the wells of the agarose gel (12µl per well) and a 1kb DNA sizing ladder was added at the start and end of the test samples. Gels were run at 150V for 40 minutes and images were viewed and edited using a UV trans-illuminator and GeneSys software. Remaining PCR products were stored at -20°C.

3.4 Results

3.4.1 Recruitment of volunteers

3.4.1.1 Participant characteristics

Eighty individuals responded to study advertisements. Respondents were sent an email providing further information about the study, including the Participant Information Sheet (PIS) (appendix 2). Subsequently, 23/80 (28.8%) did not respond after the PIS was provided (Table 3.1).

3.4.1.2 Volunteer drop-outs

Twenty-three volunteers provided informed consent to take part in the study (Table 3.1). After consent was provided, each volunteer was allocated a number in chronological order of recruitment. Four volunteers (17%) withdrew from the study before providing samples (Figure 3.1). Of the volunteers who dropped out, 3/4 provided written informed consent to take part, but failed to respond after repeated reasonable efforts to contact them after the initial meeting (volunteers 2, 11, and 14). These included emails and answerphone messages. The remaining participant dropped out after his trip to Bangladesh was cancelled (volunteer 13).

Table 3.1 Fate of 80 initial expressions of interest in enrolling in the study

Outcome after initial Enquiry and provision of Participant Information Sheet*	**Frequency (%)**
No response to follow up email	38 (47.5)
Previous travel to Indian subcontinent within preceding 4 months	7 (8.8)
Exclusion due to medical history	3 (3.8)
Unwilling to provide samples	7 (8.8)
Unable to deliver samples	2 (2.5)
Provided Informed Consent	**23 (28.8)**
TOTAL	80 (100.0)

*80 potential participants contacted ERB, these are the outcomes after more information was provided.

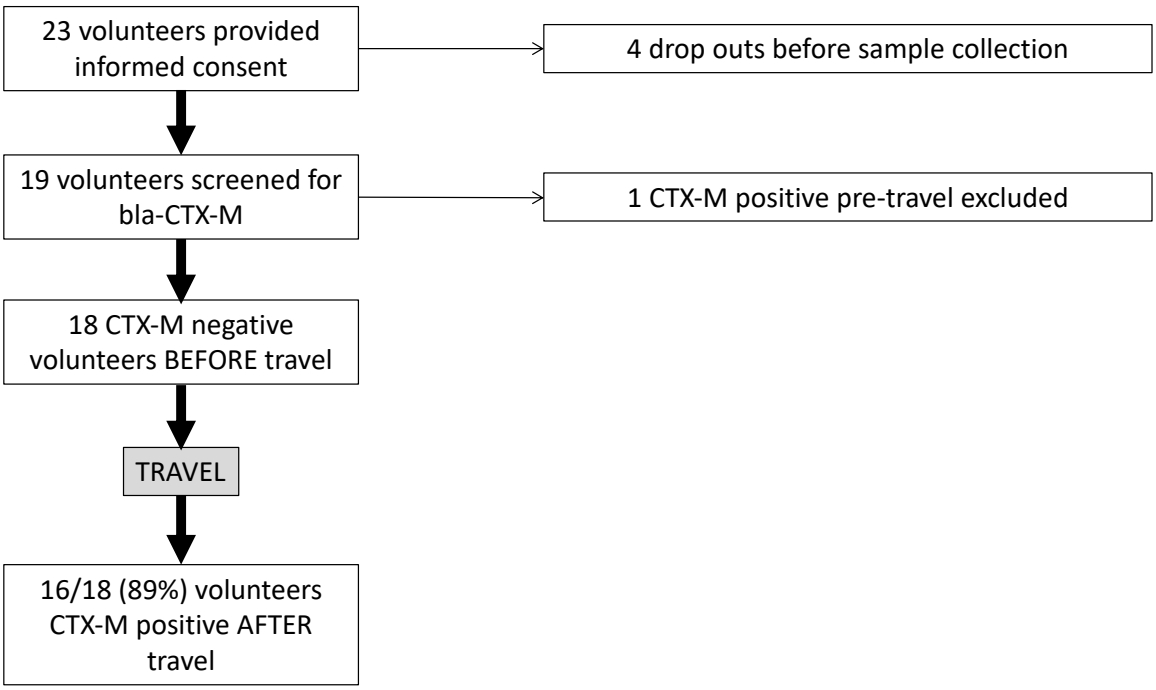


Figure 3.1. CTX-M positivity rate and fate of twenty-three volunteers who provided informed consent to take part in the study

3.4.2 Volunteer demographics

The participant demographic data for volunteers who provided pre and post travel faecal samples is displayed in Table 3.2**.** The mean and median age of volunteers was 35 and 30 years, respectively. The youngest volunteer was 21, and the oldest, 67. 10/19 were male; 9/19 female. In response to the volunteer pre-travel questionnaire (appendix 2). With respect to ethnicity, 58% (11/19) identified as 'White/British', while 32% (6/19) identified as 'Indian' or 'British Pakistani.' Ethnicity was self-reported and in accordance with the UK Office for National Statistics (ONS).

All participants reported a travel history outside the UK in the previous 2 years (Table 3.2). In 13/19 (68%) of volunteers, locations of previous travel included countries with a significant risk (>18% risk) of ESBL-EC acquisition post travel, as reported by Arcilla and colleagues (Arcilla *et al.*, 2017). 5/19 had travelled to India in the past 2 years which carries a very high risk of acquisition: up to 89% (Arcilla *et al.*, 2017)(Table 3.2).

In terms of past medical history, those with significant chronic systemic illnesses, were excluded from the study as outlined above. Most of the volunteers had no previous medical history nor did they take any medications. We did include 4 participants who had relatively minor, controlled or treated conditions which included hay-fever, hypertension, kidney stones, asthma and previously treated tuberculosis (Table 2).

Table 3.2. Pre-travel volunteer demographic data

Volunteer	Age	Sex	Ethnicity*	2 year travel history prior to entry into study^	Medical history/medications
1	38	M	Indian	USA	Nil
3	22	M	White British	Australia, Italy, Spain	Nil
4	23	M	White British	Spain, Croatia, Italy, USA, Malta, Australia, Bulgaria, Iceland	Hayfever/on desensitisation treatment
5	67	F	White British	Spain, **Cuba, Costa Rica**, France, USA, **Burma**, **Mexico**	Nil
6	31	M	Mxed British	Greece, **India**, **Morocco**, Lithuania, Netherlands, Germany	Nil
7	22	M	White British	Greece	Nil
8	53	M	Indian	**India** (Jan 2014), USA, **China** (2014), Italy, Slovenia, France	Hypertension, kidney stones
9	30	F	White British	Portugal, Italy, Poland, Greece, **Vietnam**, **Cambodia**, **Thailand** (Dec 2013-Jan 2014	Nil
10	29	M	White British	Portugal, Italy, Poland, Greece, **Vietnam**, **Cambodia**, **Thailand** (Dec 2013-Jan 2014	Nil
12	29	M	White British	USA, **Guinea** (Mar 2015), Albania/Greece, Ukraine	Nil
15	43	F	Italian	**India**, **Kenya**, Portugal, Italy	Nil
16	21	M	White British	Spain, Greece	mild asthma
17	53	F	Indian	USA, Japan, **Vietnam**/**Cambodia** (June 2015), **China** (July 2016), **Turkey**	Nil
18	23	F	White British	Spain, Portugal, **Vietnam**, **Cambodia**	Nil
19	22	F	White British	**Cambodia**, **Vietnam**, New Zealand, France, Spain, Croatia, Italy	Nil
20	34	F	Indian	**India**, 2015	Previous TB, treated
21	29	F	British Pakistani	**Morocco**, 2015	Nil
22	56	F	White British	USA, Belgium, Spain	Nil
23	34	M	Indian	**India**, 4 weeks, 1 year previous	Nil

*based on ONS classification; ^Countries in bold are high risk for acquisition (>18%); those underlined have a very high risk of acquisition of ESBL-E (75%) (Arcilla *et al* 2017).

3.4.3 Volunteer travel-related meta-data

Volunteers completed a post travel survey on return to the UK (appendix 2). The primary destinations visited by volunteers were India (68%; 13/19;) and Sri Lanka (31%; 6/19), and the mean travel duration was 27 days (median 21 days). A number of factors were reported by volunteers which are known to be risk factors for acquisition of ESBL-producing Enterobacteriaceae (ESBL-E) (Arcilla *et al.*, 2017; Peirano *et al.*, 2017). These included symptoms of diarrhoea during the trip (63%; 12/19), taking antibiotics (26%; 5/19), staying in a hostel or guesthouse/B&B (47%; 9/19), and consumption of food from street vendors (36%; 7/19). Moreover, 18/19 volunteers travelled for non-business reasons, which is also a risk factor for ESBL-EC acquisition (Peirano *et al.*, 2017) (Table 3.3).

3.4.4 Pre-travel sample collection

After screening volunteer stool samples using selective culture and PCR, 18/19 were $bla_{\text{CTX-M}}$ negative before travel (Table 3.4). Volunteer 23, who had travelled to India almost exactly 4 months prior to his recruitment to the study, was found to be carrying CTX-M-producing *E. coli* (Group 1 CTX-M) (Table 3.4). This was not an unexpected finding, but as volunteer 23 was on the 'borderline' of exclusion criteria, a decision was made to go ahead and recruit this individual and check for pre-travel CTX-M-EC.

Table 3.3. Travel related demographic data

Volunteer	Destination	Medical elective?	Co-travellers	Duration	Accommodation	Diet	Illnesses while away	Antibiotic use
1	India and UAE (transit only)	N/A	0	21	family home	food at hotels, home and street market, tap water	none	amoxicillin 5/7 for CAP same week as sample 1e submission
3	India	Mumbai	1	45	private homes, hotels, hostels, houseboat	restaurants, street food, bottled and spring water	Severe D&V	Omidazole/olfloxacin, 5 days
4	Sri Lanka	Columbo	0	30	hostel	restaurants, bottled water only	48hrs D&V	None
5	Uzbekistan and India*	N/A	1	14	hotels	bottled water only	D&V	amoxicillin in Uzbek.
6	India	N/A	1	10	hotel and family home	restaurants, UV filtered, and bottled water	vomiting <24 hrs	none
7	India	N/A	20	14	hotel	restaurants, bottled water	mild diarrhoea	none
8	India and Qatar (3 days)	N/A	1	23	home, hotels	restaurants, bottled water	diarrhoea	Norfloxacin/Tinidazole 400/300 BD 3 days for diarrhoea
9	Sri Lanka and India (transit only)	N/A	1	21	B&B, hotels	restaurants, bottled water	D&V	none
10	Sri Lanka and India (transit only)	N/A	1	21	B&B, hotels	restaurants, bottled water	none	none
12	India	N/A	1	21	B&B, hotels	restaurants, street food, bottled water	mild diarrhoea	none
15	India, Italy, Greece^	N/A	1	35	hotels	restaurants, bottled water	mild diarrhoea	none
16	India	Bangalore	3	56	hotels, hostels, tent	hotels, street food, bottled water with ice	D&V	none

Volunteers who travelled together (2 pairs) are boxed in dark grey. Risk factors associated with CTX-M acquisition are underlined.

Table 3.3. Continued.

Volunteer	Destination	Medical elective post	Co-travellers	Duration	Accommodation	Diet	Illnesses while away	Antibiotic use
17	India and Italy	N/A	3	10	hotels	restaurants, bottled water	none	none
18	Sri Lanka and China	Galle	1 (vol 19)	56	private homes, hotels, hostels	restaurants, street food, bottled water	none	none
19	Sri Lanka and China	Galle	1 (vol 18)	56	private homes, hotels, hostels	restaurants, street food, bottled water	Chest infection, no admission	Yes, unsure name of drug
20	India	N/A	1	21	family home	tap water	mild diarrhoea	amoxicillin for LRTI in UK, 4 days before travel
21	Pakistan and Turkey	N/A	4	21	family home	bottled and tap water	diarrhoea	none
22	Sri Lanka	N/A	1	18	hotels. Guesthouses	markets, bottled and tap water	mild cold	none
23	India		1	30	home	home food, street food, bottled water	none	none

Volunteers who travelled together (2 pairs) are boxed in dark grey. Risk factors associated with CTX-M acquisition are underlined.

Table 3.4 . Outcomes for 18 volunteers before vs. after travel.

Volunteer	Destination(s)*	Pre-travel CTX-M	Post travel CTX-M	CTX-M genotype	Risk factors	Duration of carriage
1	India	No	Yes	15	NBT, SV	1-3 weeks
3	India	No	Yes	15	Acc, NBT, Abx, SV	*> 9 months*
4	SL	No	Yes	14 and 15	Acc, NBT, G	*> 5 months*
5	India	No	Yes	15	NBT, G, Abx	*> 12 months*
6	India	No	Yes	15	NBT, G	6 weeks
7	India	No	Yes	15	NBT, G	2-4 months
8	India	No	Yes	15	NBT, G, Abx	*> 11 months*
9	SL & India	No	Yes	15	Acc, NBT, G	*> 7 months*
10	SL & India	No	No	N/A	Acc, NBT, G	N/A
12	India	No	Yes	15	Acc, NBT, G, SV	2-4 months
15	India	No	yes	15	G	*>1 week*
16	India	No	yes	14 and 15	Acc, NBT, G, SV	*>6 months*
17	India	No	Yes	15	NBT	3-6 weeks
18	SL & China	No	No	N/A	Acc, NBT, G, SV	N/A
19	SL & China	No	Yes	14	Acc, NBT, Abx, SV	*>14 days*
20	India	No	Yes	15	NBT, G	3-4 months
21	Pakistan	No	Yes	15	NBT, G	1-2 weeks
22	SL	No	Yes	15	Acc, NBT	1-3 weeks

SL, Sri Lanka. Light shading: lack of CTX-M by culture, PCR and WGS. Dark shading: $bla_{\text{CTX-M}}$ detected. Risk factors for acquisition: Acc, accommodation was hostel, B&B, or guesthouse; NBT, non-business travel; G, gastroenteritis symptoms; SV, street vendor food consumption; Abx, antibiotic consumption during travel. Duration of carriage is marked in red and italicized where a 'CTX-M free' follow-up faecal sample could not be obtained. *Other destinations during the same tip with a low $bla_{\text{CTX-M}}$ community carriage prevalence are not included in this column e.g. Countries in Europe.

3.4.5 Travellers to South Asia have a high CTX-M-EC acquisition rate

3.4.5.1 Results of selective stool culture and CTX-M grouping PCRs

Putative *E. coli* colonies were picked from selective chromogenic media (Figure 3.2) and identified using MALDI-TOF. ESBL oxoid agar has a shelf life of only 14 days, therefore on receipt, each pack was checked with a positive (*K. pneumoniae* producing ATCC 700603), and a negative control bacterium (*E. coli* ATCC 10798).

CTX-M-grouping PCRs were used to confirm the presence of $bla_{\text{CTX-M}}$ genes for colonies growing on ESBL-screening plates. Figure 3.3 is a representative example of $bla_{\text{CTX-M}}$ grouping PCR after agarose gel electrophoresis. In this example, four isolates from a volunteer 1 post travel faecal sample (sample 1e) are displayed which did not contain CTX-M-EC. In addition, four isolates each from volunteer 3 (sample 3e, post travel) and volunteer 4 (sample 4e, post travel) are displayed, all of which have a band at 688bp which is consistent with the presence of group 1 CTX-M (Figure 3.3). Ultimately, WGS was used to confirm the resistance mechanism responsible for the phenotype detected using the selective culture of faecal samples (Chapter 4).

The culture-based screening approach yielded 236 putative ESBL-producing *E. coli* isolates from 17 volunteers (16 post travel, 1 pre-travel) (Figure 3.4). After WGS and identification of resistance genes, 86% (203/236) of culture positive *E. coli* were CTX-M grouping PCR positive, with 174 isolates confirmed as carrying $bla_{\text{CTX-M-15}}$, and 14 isolates carrying $bla_{\text{CTX-M-14}}$.

In the case of 8 isolates from 2 volunteers, CTX-M grouping PCR was positive, but analysis of draft WGS assemblies did not reveal any *bla*$_{\text{CTX-M}}$ genes (Figure 3.4). These isolates were 3 ST10s from volunteer 7, 3 ST648s from volunteer 7, and 2 isolates from volunteer 20 with no ST assigned. It is likely that plasmid loss occurred, after the initial PCR, as repeat grouping PCRs on the same isolates were negative for *bla*$_{\text{CTX-M}}$ genes. An alternative explanation is that contamination occurred on the first occasion PCR was done for these isolates, resulting in false positives.

In the case of 14% (33/236) of culture positive isolates on selective media which were grouping PCR negative, WGS was used to determine the resistance genes leading to growth on cephalosporin-contianing agar. Seventeen isolates were AmpC enzyme producers, 2 were *bla*-SHV-12-producers, and 14 had no CTX-M or AmpC genes, after the analysis of WGS assemblies using abricate (Seemann, 2017a) (Figure 3.4)**.**

A B

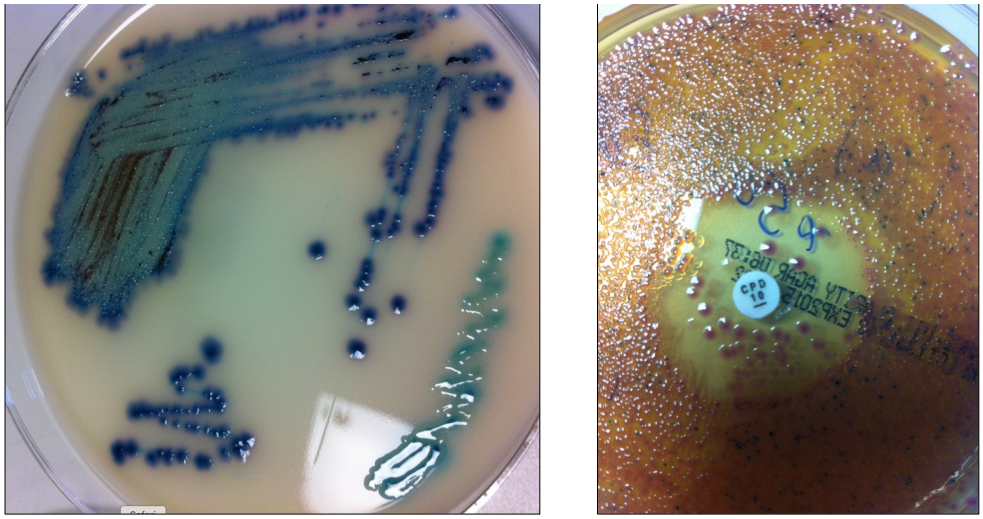


Figure 3.2. Representative examples of ESBL screening media. **A**: ESBL chromogenic media with faecal sample 3e with positive control visible in green (*Klebsiella pneumoniae* ATCC 700603) and **B**: Chromogenic UTI media plus addition of cefpodoxime disc, also sample 3e cultured with putative ESBL-*E. coli* (pink colonies) within the cefpodoxime disc zone.

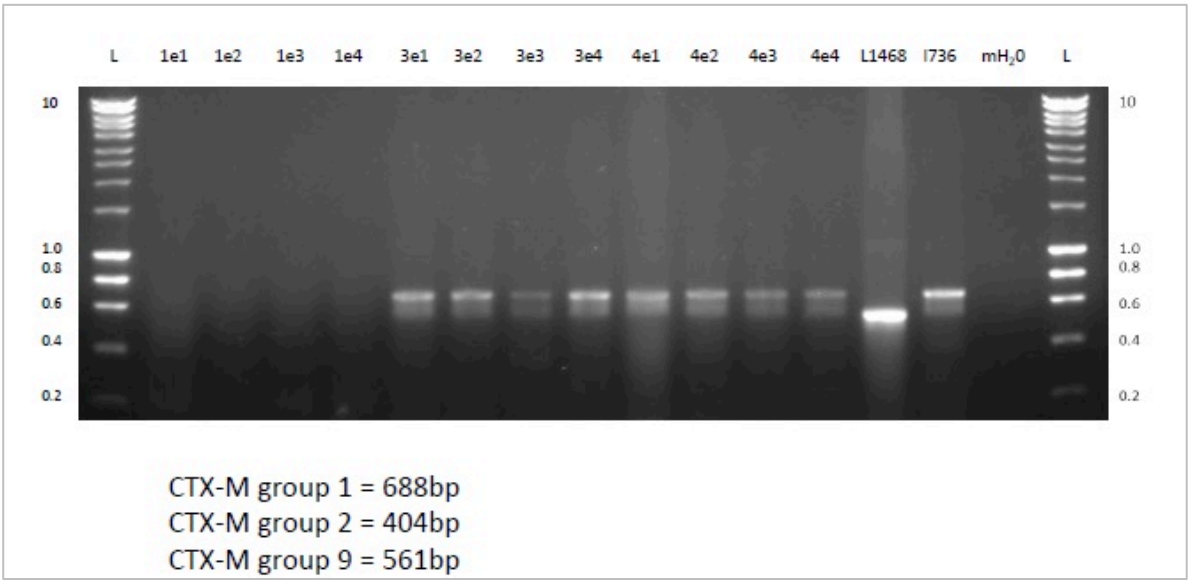


Figure 3.3. Representative example of CTX-M grouping PCR products after agarose gel electrophoresis. L: 1kB ladder. I736: group 1 (*bla*$_{CTX\text{-}M\text{-}15}$) positive control. L1468: group 9 (*bla*$_{CTX\text{-}M\text{-}14}$ positive control).

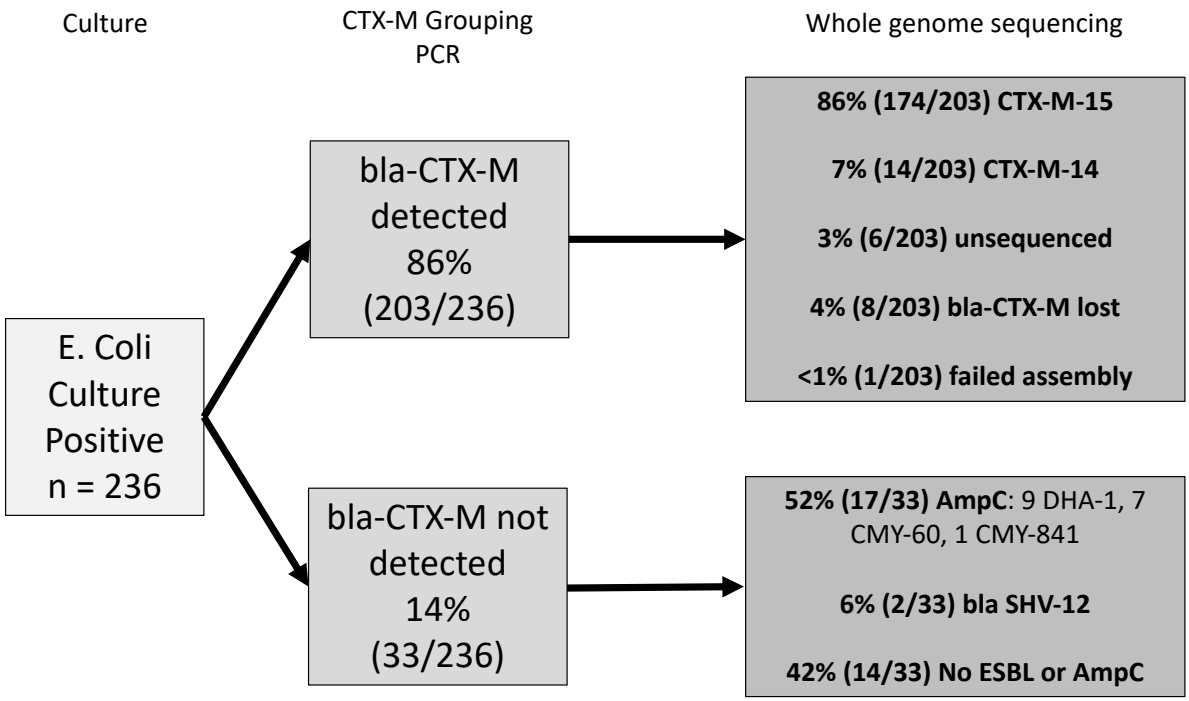


Figure 3.4. Fate of 3rd-generation cephalosporin-resistant isolates after culture, CTX-M-grouping PCR, and WGS.

3.4.5.2 *bla*$_{CTX-M}$ acquisition rate

After travel, *bla*$_{CTX-M}$ had been acquired by 16/18 (89%) volunteers (Figure 3.1). Most volunteers acquired CTX-M-15-producing *E. coli* after travel to India, (75%; 12/16), while the remaining 4 volunteers who acquired CTX-M-EC travelled to Sri Lanka (volunteers 4 and 22), Sri Lanka and China (volunteer 19), and Pakistan (volunteer 21) (Table 3.4). Two volunteers acquired *E. coli* producing both CTX-M-14 and CTX-M-15, and one volunteer acquired only CTX-M-14 (Table 3.4). All 18 volunteers had one or more of the following risk factors for CTX-M-EC acquisition: staying at a hostel, B&B, or guesthouse; travel for non-business reasons; symptoms of gastroenteritis during travel; consuming food from a street vendor; and/or antibiotic consumption during travel (Table 3.4). This study did not set out to, or achieve, sufficient study volunteer numbers to carry out an analysis as to whether any risk factor was statistically linked to CTX-M-EC Acquisition.

3.5 Discussion

3.5.1 Recruitment of volunteers

This is the first prospective study recruiting volunteers from the UK to determine the rate of acquisition of CTX-M-EC in the faecal microbiome after travel. Eighty individuals responded to our study advertisements, but only 28.8%, (23/80) were recruited to the study. Nearly half of the initial advertisement respondents (47.5%; 38/80) failed to reply to ERB, after provision of the participant information sheet (PIS) (appendix 2) via email. The main explanation for this poor response after full details of the study were provided is that the initial advertisement (appendix 2), does not state that volunteers are required to provide faecal samples, whereas the PIS clearly states the requirements of multiple faecal samples at several time points, without any financial reimbursement. The population targeted by the advertising campaign are full time professionals or students working in the NHS or at the University of Birmingham, and therefore it can be appreciated that these individuals may not be expected to have enough time to devote to providing and delivering faecal samples to ERB.

Lecky and colleagues described the barriers facing successfully recruiting healthy volunteers for faecal sample collection in the UK (Lecky *et al.*, 2017). Lecky et al reported from a large population based general practice faecal collection study, where of 58337 contacted persons, only 3.9% returned a questionnaire and stool sample, but their sample return rate for interested individuals was 67.7% (Lecky *et al.*, 2017). Therefore, our success rate of 28.8% (23/80) does not compare favourably with the paper by Lecky et al, although the

information provided on our advertisements did not state that faecal sample collection was required, which is likely to be an important driver for non-participation (McNulty *et al.*, 2018). Although, beyond the resources available for the present study, a small gift voucher incentive (e.g. £5) would be a potential motivating factor for recruitment, particularly among the university student population (Lecky *et al.*, 2017).

Our attrition rate of 4/23 (17%) after volunteer consent and recruitment is lower than the reported rate of 33.3% in the aforementioned study, (Lecky *et al.*, 2017). This may reflect the young, relatively enthusiastic group of volunteers, who were contacted by email and text message as reminders to provide samples. An improvement on this approach may have been more acceptable to provide volunteers with a larger 'whole stool' collection pot, rather than the 20ml stool container with mini-scoop, therefore providing a less unpleasant faecal collection method. In 3/4 cases, the volunteer drop-outs did not contact ERB stating a wish to withdraw from the study. Withdrawal was implied through lack of response to multiple emails and text messages to determine the wishes of the volunteer in question.

3.5.2 Volunteer demographics

Ethnicity is an important factor to illicit in prospective traveller studies of this kind, because individuals of Middle Eastern or South Asian (MESA) origin, are known to have a higher rate of ESBL-EC compared to individuals of European origin, as evidenced by a study from Birmingham, UK (Wickramasinghe *et al.*, 2012). In general practice populations, the MESA group had 22.8% ESBL-EC faecal carriage compared with 8.1% carriage in the European group. In the present study, however, we did not exclude MESA-origin participants. This

would be ethically questionable, and furthermore, individuals of South Asian origin make up a large population of Birmingham, and 32% of volunteers in our study.

A potential limitation of the study is a degree of selection bias in that our study population is not representative of the population as a whole. The study population are young (mean age 35) and given the recruitment sites – well educated – all volunteers in this study were at least previous recipients of a bachelor's degree or were current undergraduate students. Recruiting a more representative sample including different age groups, socioeconomic groups, and ethnicities would require extensive resources outside the capabilities of our study. Nevertheless, our main study outcomes are broadly in concordance with other large traveller studies, which will be described in due course.

3.5.3 Sample collection

3.5.3.1 Pre-travel participant exclusion

It can be seen that our study exclusion criteria were effective in ruling out participants based on a previous travel history to the Indian subcontinent, as only 1/19 volunteers carried CTX-M-EC before travel. This person had travelled to India 4 months prior to the pre-travel sample submission, which is the likely source of the CTX-M-EC detected. In the UK, previous travel to high endemicity regions is a risk factor for ESBL faecal carriage (McNulty *et al.*, 2018).

3.5.3.2 Faecal sample collection

A separate sterile swab loaded with faeces was used to inoculate culture plates and BHI broth for enrichment. Sterile swabs were confirmed to carry approximately 0.5g of faecal material, however, this varies according to the water content of the sample. Therefore, estimating the *E. coli* cfu/ml in any faecal sample was not possible. In addition, a previous study suggests that stool consistency is related to gut composition and microbial richness (Vandeputte *et al.*, 2015). Stool consistency was not recorded in the present study, and this is difficult to assess once samples are compressed in a 10ml faecal sample container. Therefore, no attempt was made to estimate the *E. coli* abundance (cfu/ml) in this study.

3.5.4 Volunteer post travel- related meta-data

Arcilla and colleagues identified several risk factors for ESBL-E acquisition using multi-variable logistic regression. These included antibiotic consumption during travel, travellers' diarrhoea, consumption of food from street vendors, and in South Asia, eating food daily at a hostel or guesthouse (Arcilla *et al.*, 2017).

Travelling for 'non-business' reasons (95%; 18/19) has also been identified as a risk factor for ESBL-EC acquisition (Peirano *et al.*, 2017). Although not known to be a risk factor for CTX-M-E acquisition, 5/19 volunteers were medical students who spent time during their trip working in hospitals. There is therefore a possibility that these five individuals may have acquired ESBL-E from the hospital environment (Table 3.3).

3.5.5 Travellers to South Asia have a high CTX-M-EC acquisition rate

This study has demonstrated an ESBL-EC acquisition rate of 89% (Figure 3.1 and Table3.4), which is in concordance with previous studies. The largest traveller study to date which included 2001 healthy volunteers (the COMBAT study) found that the country visited with the highest ESBL-EC acquisition rate was India: 89% (70/79) of those who travelled from the Netherlands to India acquired ESBL-producing *E. coli* (Arcilla *et al.*, 2017). Other traveller studies have also shown high acquisition rates of ESBL-producing Enterobacteriaceae in those who visit the Indian subcontinent: prospective studies report acquisition rates of 73% (66/90 participants) (Peirano *et al.*, 2017); 49% (30/61) (Vading *et al.*, 2016); 85% (53/62) (Ruppé *et al.*, 2015); 46% (28/61) (Kantele *et al.*, 2015); and 87% (59/68) (Kuenzli *et al.*, 2014). After WGS and bioinformatic screening of contigs, none of our post travel isolates contained carbapenemase genes. This is in keeping with the COMBAT study which reported the rate of CPE acquisition in returning travellers as <1% (Arcilla *et al.*, 2017).

Specific *bla*$_{\text{CTX-M}}$ genotypes are often strongly associated with specific regions, in particular, CTX-M-producers in India are almost entirely comprised of *bla*$_{\text{CTX-M-15}}$, whereas in China, *bla*$_{\text{CTX-M-14}}$ is the predominant genotype (Bevan, Jones and Hawkey, 2017). Therefore, the high acquisition rate of CTX-M-15-producing *E. coli* is unsurprising, as most volunteers travelled to India. Volunteers 4 and 19 who acquired *bla*$_{\text{CTX-M-14}}$ travelled to China and/or Sri Lanka, where *bla*$_{\text{CTX-M-14}}$- producing *E. coli* are common (Hawkey, 2008; Tillekeratne *et al.*, 2016). However, volunteer 16 only travelled to India, where *bla*$_{\text{CTX-M-14}}$ is a rare genotype, but this individual travelled widely (Kerala, Karnataka, New Delhi, and Uttarakhand), and

also worked in a Bengaluru hospital as a volunteer for 4 weeks, providing opportunities for acquisition of what is an unusual *bla*$_{CTX-M}$ genotype in India.

The results of our selective culture-based screening showed that this was a sensitive method, sometimes detecting isolates with mechanisms conferring 3GC resistance, other than CTX-M production (Figure 3.4). In the case of 14 isolates, we did not detect any ESBL or AmpC genes, which suggests that either these *E. coli* grew on selective media which failed to suppress their growth for some reason, or the isolates in question have an alternative resistance mechanism, which was not detected using the resistance-gene finder.

We used CTX-M grouping PCRs to confirm the presence or absence of *bla*$_{CTX-M}$ in all our isolates which were culture positive on selective media for ESBL-producing *E. coli*. Our approach did have some limitations. The PCR primers used in these experiments did not include group 8/25 CTX-M. The lack of coverage of group 8/25 CTX-M producers in the PCR screening stage was mitigated by the sequencing (WGS) of all culture-positive isolates (putative ESBL producers) which were CTX-M-negative after grouping PCR. Therefore, rarer CTX-M genes or other resistance mechanisms could be detected after WGS. We did not find any *bla*$_{CTX-M}$ genotypes other than CTX-M-14 and CTX-M-15, which is unsurprising, as these are the genotypes prevalent in the countries visited by our volunteers (Tillekeratne *et al.*, 2016; Bevan, Jones and Hawkey, 2017).

3.6 Conclusion

A prospective observational cohort study was undertaken, recruiting persons travelling from the UK to South Asia. Twenty-three participants were recruited and retained for follow-up, exceeding the original target of twenty.

Although there may be a degree of selection bias in terms of the demographic and socio-economic groups recruited, this is a difficult hurdle to overcome – as individuals who make long haul trips to South Asia tend to be well educated and/or have above average disposable income. Furthermore, the acquisition rate of CTX-M-EC, and the CTX-M genotypes in returning travellers, correlated closely with data from previous studies.

This chapter has outlined volunteer recruitment, demographics, and CTX-M-EC acquisition. The following chapters will focus on the dynamics of CTX-M-EC acquisition, with particular focus on the *E. coli* clones acquired after travel (Chapter 4) and characterisation of the $bla_{CTX\text{-}M}$ plasmids (Chapter 5).

Chapter 4: The molecular dynamics of acquisition of CTX-M-producing *E. coli* and relationship to non-CTX-M *E. coli*

4. 1 Introduction

In the present study, a high acquisition rate of CTX-M-producing Enterobacteriaceae was found (89%), for individuals who returned to the UK from South Asia. In a larger cohort of volunteers, Arcilla and colleagues also found that 89% (70/79) individuals returning to The Netherlands from India, also acquired CTX-M-producing Enterobacteriaceae (Arcilla *et al.*, 2017). Acquisition rates by world region, and risk factors for acquisition have been investigated previously (Chapter 1, section 1.7.), however, the dynamics of acquisition of CTX-M-producers and non-producers in travellers is not well understood.

The aim of this chapter is to investigate the acquisition and Loss of CTX-M-producing and non-producing E. coli in the faecal microbiome of travellers to South Asia. Whole genome sequencing (WGS) was used to type strains before and after travel, providing an insight into the changing populations of E. coli, including the role of non-CTX-M, cephalosporin sensitive E. coli, in the in the human faecal microbiome of returning travellers.

3.3 Hypotheses

1. Acquisition of CTX-M-E. coli is due to multiple acquisition events which leads to colonisation with multiple E. coli clones after travel
2. CTX-M-EC are maintained in the faecal microbiome after return to the UK
3. CTX-M-EC do not displace non-CTX-M E. coli which were present before travel
4. Non-CTX-M strains persist as a minority population in the faecal microbiome, despite CTX-M-EC acquisition
5. CTX-M-EC are usually multi-drug resistant

4.3 Methods

The methodology for faecal sample collection, culture, and isolate identification is outlined in chapter 3. PCR was used as a screening method before putative ESBL producers were subjected to WGS. Primers for multiplex PCR were used to identify the three most common CTX-M groups: group 1, group 2, and group 9. PCR was carried out only on isolates which had grown either on ESBL agar or from within the zones of cefpodoxime on nonselective UTI agar. PCR amplimers were visualized using 1% agarose gel electrophoresis, as described in chapter 3.

4.3.1 Tooth-picking experiments

In order to characterise the cefotaxime-sensitive population of *E. coli* within stool samples containing CTX-M-positive post travel *E. coli,* a selection of post-travel stool samples were subjected to tooth-picking experiments. As outlined in chapter 3, Oxoid UTI brilliance agar was used to culture *E.* coli (pink colonies) directly from stool samples. After checking for the presence of $bla_{CTX\text{-}M}$ using PCR, tooth-picking experiments were undertaken. A sterile wire was used to pick individual putative *E. coli* colonies (pink colonies) from the chromogenic plate. Each colony was then lightly 'scored' (2-3mm) onto antibiotic free LB agar, and to LB agar containing cefotaxime (4µg/ml). The wire was flamed between each colony pick. 100 colonies were picked from each faecal sample tested. Isolates which grew on the antibiotic-free plate but failed to grow on the cefotaxime plate were enumerated and sweeps of this cefotaxime-sensitive population were stored at -80^{o}C for each stool sample tested.

4.3.2 Whole genome sequencing

Automated DNA extraction and whole-genome sequencing were undertaken by MicrobesNG at the University of Birmingham. Sequencing was performed using HiSeq 2500 with 250-bp paired-end reads. QC was performed using QUAST (Gurevich *et al.*, 2013). All isolates were assembled using SPAdes (Bankevich *et al.*, 2012) and annotated using Prokka (Seemann, 2014).

4.3.3 Bioinformatic analyses

Downstream bioinformatics analysis after illumina sequencing was undertaken using the Cloud Infrastructure for Microbial Bioinformatics (CLIMB). This is a cloud based computing network for the processing storage and analysis of large genome datasets (Connor *et al.*, 2016). All analysis using illumina reads and contig files was undertaken using command-line based software.

All sequenced E. coli isolates were subjected to analysis, by determining the multi locus sequence type (MLST) (Maiden *et al.*, 1998) and determining the antimicrobial resistance (AMR) genes present in each strain. The E. coli Achtman MLST scheme (enterobase.warwick.ac.uk/species/ecoli/) of each strain was determined by inputting contig files and running the mlst program, (github.com/tseemann/mlst) (Seemann, 2017b). AMR gene sequences were found using each strain contig file using abricate, which uses the AMR databases CARD and Resfinder (github.com/tseemann/abricate) (Seemann, 2017a).

4.3.4 Identifying *E.coli* clones from WGS data

Illumina sequence reads (fq files) and assembled draft genomes (fasta files) were used to determine the phylogenetic relationships of selected strains. In addition to obtaining the MLST of each E. coli strain in the study, a core genome alignment of all strains was also undertaken, using Parsnp, from the harvest suite of bioinformatics tools. Parsnp is useful in aligning large numbers of similar strains using core regions of similarity between strains (Treangen *et al.*, 2014).

Higher resolution SNP typing was undertaken to ascertain the relationship between identical STs (i.e. the number of SNPs between E. coli strains). SNP typing was undertaken using a pipeline of individual programs. Firstly, snippy was used to find SNPs between a chosen reference genome and a set of sequence reads, thus creating a core genome SNP alignment, as described, github.com/tseemann/snippy (Seemann, 2017c). Using the output from snippy, a maximum likihood phylogenetic tree, with bootstrapping, was generated using MEGA (run on Mac OS Sierra), (Kumar, Stecher and Tamura, 2016). A MEGA computation would usually take approximately 4 hours for a phylogeny consisting of 40 E. coli sequences. Finally, SNP distances between each isolate in the phylogeny were determined using snp-dists, which produces an output in the form of a table matrix (github.com/tseemann/snp-dists) (Seemann, 2017d). Outputs were displayed using phandango (Hadfield *et al.*, 2017).

4.3.5 Determining resistance gene content of *E. coli* strains

Resistance gene finder (cge.cbs.dtu.dk/services/ResFinder/) was used to determine the AMR gene content of strains: the draft WGS genomes were run against the ResFinder database (Zankari *et al.*, 2012).

4.4 Results

4.4.1 Travellers to South Asia have a high CTX-M-EC acquisition rate

A breakdown of faecal culture and bla_{CTX-M} PCR results are covered in chapter 3. Post-travel, bla_{CTX-M} had been acquired by 16/18 (89%) volunteers (Chapter 3, Table 3.4).

After WGS and bioinformatic screening of contigs using ResFinder, none of our post travel isolates contained carbapenemase genes.

4.4.2 CTX-M-EC acquisition is Polyclonal

CTX-M-producing E. coli acquired during travel came from a wide range of sequence types (STs) and phylogenetic groups (Figure 4.1). Among volunteers in our study we found 11/15 (73%) acquired CTX-M-EC belonging to phylogenetic group (PG) A. Other common PGs were PG-D (6/15 volunteers, 40%), PG-B2 (5/15, 33%), and PG-B1 (4/15, 27%). PG-B2 comprised of ST131 in 4/5 volunteers, and ST1193 in 1/5 volunteers (Figure 4.1).

For volunteers acquiring CTX-M-EC, gut colonisation with CTX-M-EC was often polyclonal (8/15 volunteers with sequenced E. coli had >1 MLST producing CTX-M). (Table 4.1 and Table S1, appendix 1). The mean number of acquired E. coli STs per volunteer was 3 (median 3, range 1-9). 7/15 travellers had >3 E. coli ST with bla_{CTX-M}. Taking the number of volunteers with WGS data as the denominator, the commonest ST among CTX-M-producing *E. coli* was ST131 (4/15), with other common STs being ST38 (3/15), ST10 (3/15), and ST43 (3/15).

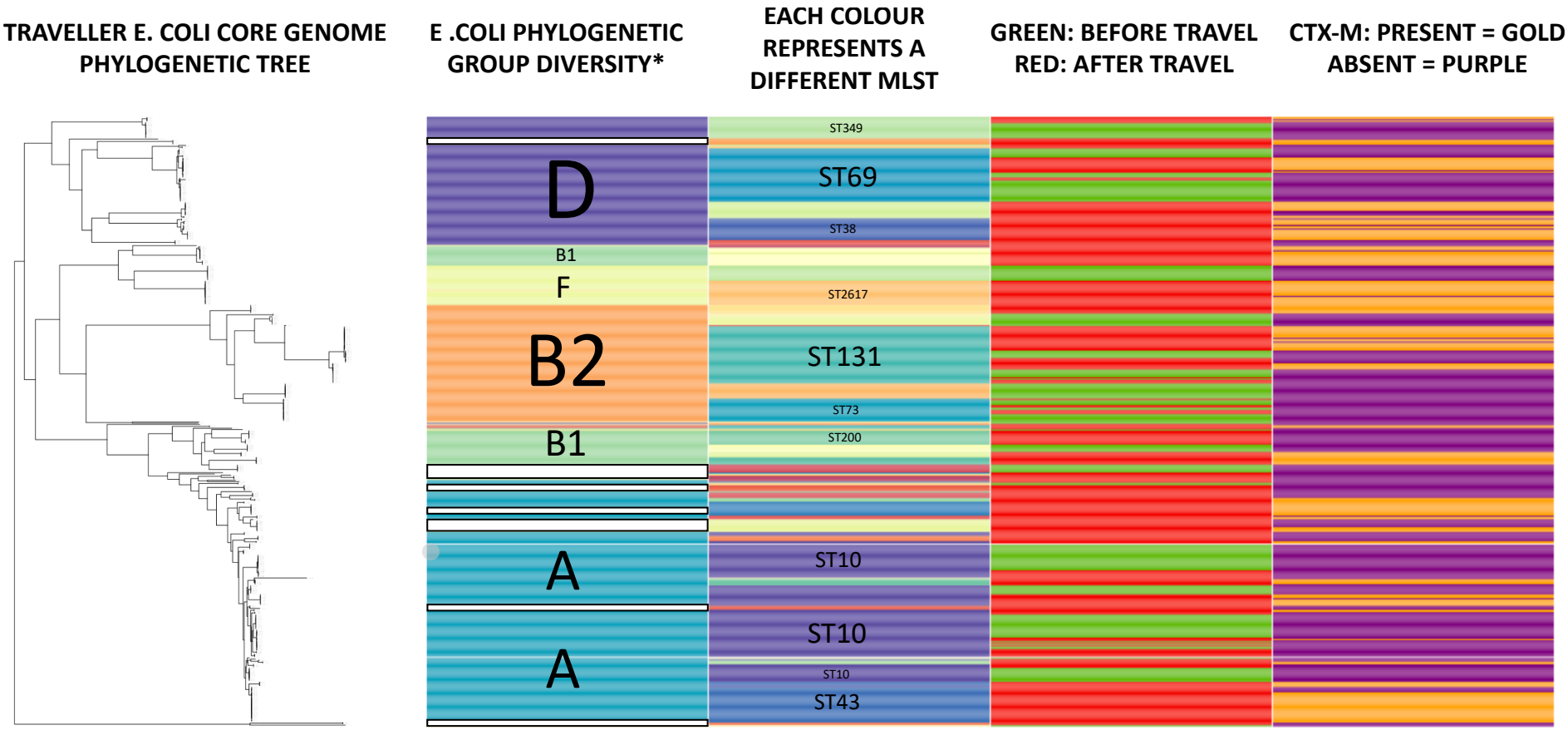


Figure 4.1. Phylogenetic relationships of pre- and post-travel E. coli strains. Displays the phylogenetic groups, majority MLST types, and presence of $bla_{CTX\text{-}M}$ before and after travel top South Asia. Four hundred eighty-three E. coli strains from 15 volunteers. *white bars indicate where the phylogenetic group undetermined.

Table 4.1. Pre- and post-travel *E. coli* from each volunteer indicating CTX-M-EC, non-CTX-M-EC, and tooth-picked strains.

Volunteer	Number of faecal samples		*E. coli* MLST after illumina sequencing		
	Pre-travel	Post-travel	Pre-travel	Post travel	ST of non-CTX-M-EC from tooth-picking experiment
1	2	4	131	**349**, 394, 69, 131	
3	3	6	69, 941, 2008	**43, 38, 1193, 405**	2076, 617, 46 (3 cols sample 3a), 224 (1 col from 3b), 10 (2 cols from 3f)
4	2	5	10	**2732, 69, 648**	
5	1	10	452	**38, 405, 131, 48, 10, 6338, 88, 3478**	
6	3	4	69, 73	**450, 8095, 2221, 73**	
7	3	6	10, 69	**10, 6438, 43, 200**, 3036, 69	
8	2	7	69, 1163, 2712, 10	**448, 10, 648, 131, 38**	*38 (1 col from 8g)
9	3	6	69, 3727, NT	**4, 200, 2617**	*2617 (1 col 9f)
10	3	4	354	642, 200, 34, 10	
12	2	8	1314, 10, 752, 770	**43,** 1276, 189, 5919, 216	349 (1 col from 12e)
15	2	3	210, 10	**167**, 52, 10	
16	2	5	349	**38, 227, 131**	
17	2	3	NT, 131	**450**, 43, 131	206 (1 col from 17a)
19	2	1	10	**43**	7174 (1 col from 19a)
20	2	6	10,452, NT	**6438, 162, 131, 226, 405,** 10	2797 (1 col 20a), *10 (1 col 20b)
22	2	3	2619	**226**, 607, 2967, 7174	

Blue rows indicate where there is data from tooth-picking experiments

*ST2617, non-CTX-M-EC, in faecal sample 9f, is not the same strain as the CTX-M-producing ST2617, also from volunteer 9 faeces (determined by SNP typing).

For STs, black font indicates non-CTX-M; red font indicated **CTX-M-15**; blue font indicates **CTX-M-14**. Text in parentheses indicates the number of tooth-picked colonies which were sequenced. NT, non-typeable.

4.4.3 CTX-M-EC clones are maintained after travel

The mean duration of carriage of CTX-M-EC was 113 days, with a median of 60 days. This carriage duration must be considered to be an underestimate, because 8/16 volunteers remained CTX-M-EC carriers at the last faecal sampling point. 46% (6/13) volunteers were known to CTX-M-EC positive at 6 months after travel. *E. coli* STs with bla_{CTX-M} which were carried the longest in this study were ST131 (PG-B2) (11 months, volunteer 8), ST405 (PG-D) (9 months, volunteer 3), and ST648 (PG-B1) (8 months, volunteer 8). Considering all strains which colonised the faecal microbiome for the longest duration, there was no predominance of any particular ST or PG.

Eight volunteers provided stool samples at two or more time points after travel. Of these, 8/8 volunteers carried CTX-EC of the same ST in stool samples at different time points (Table 4.2). Using SNP typing, in 8/8 volunteers the isolates found in separate faecal samples are <10 SNPs apart, suggesting stable colonisation with the same strain over several months (Table 4.2). Four volunteers had post travel CTX-M-EC isolates which were indistinguishable (0 SNPs) between separate post travel faecal samples collected at different time points: volunteer 3 (ST1193); volunteer 9 (ST2617); volunteer 12 (ST43) and volunteer 20 (ST162) (Table 4.2).

Table 4.2. SNP typing of Post travel CTX-M-EC.

Strain (preceding number is volunteer number)	Sample collection point (days post travel)	MLST
3b3	7	1193*
3b4		1193^{0}
3c3	28	1193^{0}
3d1	56	1193^{63}
3d2		1193^{52}
3d3		1193^{0}
3d4		1193^{63}
4a6	7	69^{*}
4b1	30	69^{5}
4b2		69^{4}
4b3		69^{4}
4b4		69^{4}
4c1	49	69^{4}
4c4		69^{4}
4e1	152	69^{4}
4e2		69^{5}
4e4		69^{4}
5.2a	4 days (post Uzbekistan)	38^{*}
5.2b		38^{2}
5.2c		38^{2}
5.2e		38^{2}
5.2f		38^{2}
5a1	3 days post India, 5 weeks post Uzbekistan	^48
5a2		$^{46}48$
5a3		$^{42}48$
5a4		$^{39}48$
5b1	8 days post India	$^{48}48$
5b3		$^{33}48$
5b4		$^{26}48$
5c4	28 days	$^{46}48$
5e3	91 days	38^{6}
5e4		38^{6}
5f3	183 days	$^{12765}48$
8b2	16	10*
8b3		10^{1}
8b4		10^{1}
8c1	40	10^{2}
8c2		10^{43}
8c3		10^{2}
8c4		10^{2}
8d1	58	10^{1}
8d2		10^{13331}
8d4		10^{13323}
9b1	14	2617^{19}
9b2		2617*
9c1	35	2617^{9}
9c2		2617^{5}
9c3		2617^{7}
9c4		2617^{1}

9d1	91	2617^{2}
9d3		2617^{2}
9d4		2617^{7}
9e1	152	2617^{4}
9e2		2617^{0}
9e3		2617^{6}
9e4		2617^{1}
9f1	210	2617^{3}
9f2		2617^{7}
9f3		2617^{5}
9f4		2617^{4}
12a1	3	43^{5}
12a2		43*
12a3		43^{0}
12a4		43^{0}
12b1	21	43^{0}
12b2		43^{0}
12b3		43^{0}
12b4		43^{1}
12c1	29	43^{0}
12c2		43^{0}
12c3		43^{0}
12c4		43^{0}
12d1	56	43^{1}
12d2		43^{3}
12d3		43^{2}
12d4		43^{2}
12e1*	115	43^{0}
12e2		43^{0}
12e3		43^{1}
12e4		43^{1}
16b2	40	131^{*}
16c1	91	131^{4}
16c2		131^{3}
16c4		131^{2}
16e1	179	131^{3}
16e2		131^{4}
16e3		131^{3}
16e4		131^{6}
20a3	7	162^{5}
20a4		162*
20b1	42	162^{0}
20b2		162^{0}
20b3		162^{0}

*indicates reference strain for SNP comparisons for any given volunteer. SNPs are displayed as super-script after the ST;

^indicates 2nd reference strain where 2 STs are present (volunteer 5). SNPs are displayed as super-script before the ST.

4.4.4 *E. coli* without CTX-M acquired during travel co-colonise the GI tract alongside CTX-M acquired strains after travel

The aim of this experiment was to determine whether faecal samples containing CTX-M-EC co-colonise the faecal microbiome with CTX-M non-producers. Tooth-picking experiments were undertaken for faecal samples from eight volunteers who were known to have acquired CTX-M-EC.

Data from tooth-picking 100 colonies per faecal sample, followed by spotting on to plain LB agar, followed by onto cefotaxime-containing agar, is displayed in Table 4.1 and Figure 4.2. In 3/4 volunteers, for whom we collected tooth-picking data on multiple faecal samples, the proportion of cefotaxime-resistant strains diminishes over time (Figure 4.2). In 7/7 cases where non-CTX-M tooth-picked isolates were sequenced, we found unique non-CTX-M-producing E. coli strains in stool samples that contained CTX-M-producing E. coli (Table 4.1).

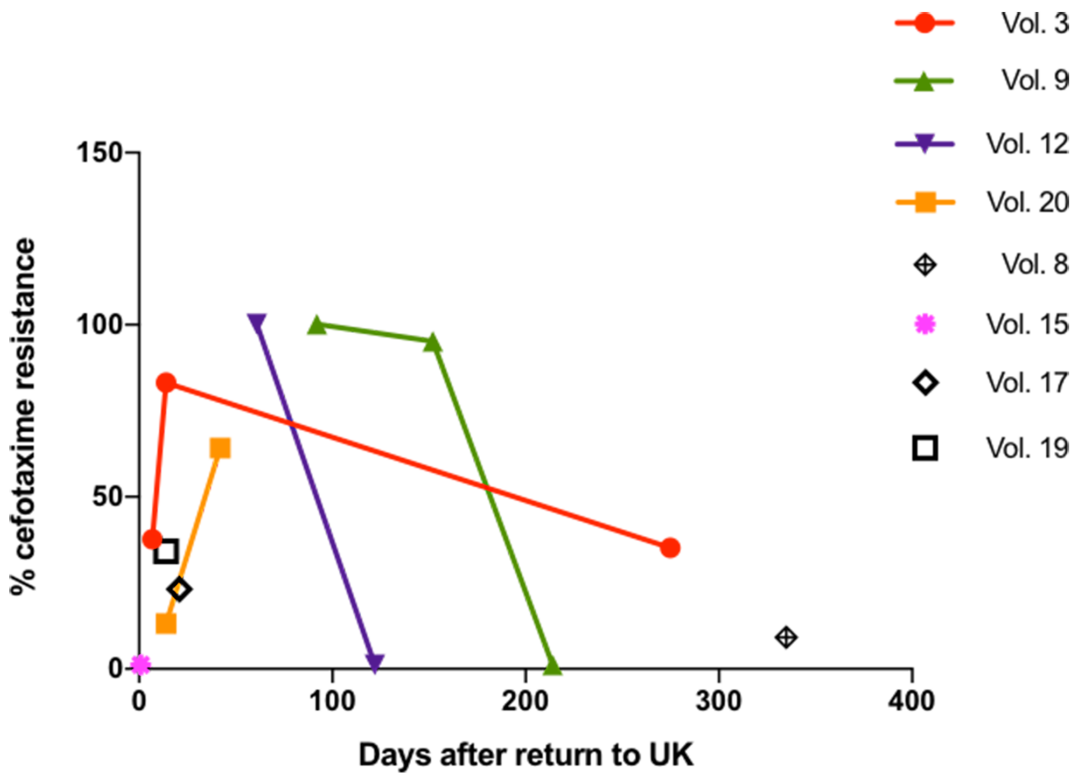


Figure 4.2. Proportion of cefotaxime-resistant strains in post-travel fecal samples containing CTX-M-EC.
Vol., volunteer.

4.4.5 Resident *E. coli* Persist as a minority population in the faecal microbiome

For seventeen volunteers, non-CTX-M-EC populations were sequenced before and after travel. In the case of seven volunteers who had acquired and then lost CTX-M-EC, we detected the same non-CTX-M-EC MLSTs before and after travel. We used SNP typing to clarify whether these strains were the same clonal lineage of non-CTX-M-EC before versus after travel. In 5/7 volunteers we found near-indistinguishable strains (<20 SNPs) before versus after travel (Table 4.3). All 5 volunteers also acquired, and lost co-colonising CTX-M-EC.

Table 4.3. SNP comparisons of pre and post travel non-CTX-M E. coli for volunteers with the same MLSTs before vs. after travel.

	Sample collection point[+] (days pre or post travel)	MLST
1_1a	10 days pre	131*
1_1b		131^{5}
1_1c		131^{1}
1_1d		131^{19}
1_2a	5 days pre	131^{17}
1_2b		131^{18}
1_2c		131^{6}
1_e_1	122 days post	131^{3}
1_e_2		131^{6}
1_e_3		131^{2}
6_1c	42 days pre	73^{17}
6_1e		73^{14}
6_2a	28 days pre	73^{77}
6_2b		73*
6_2c		73^{14}
6_2d		73^{22}
6_3a	1 day pre	73^{23}
6_3b		73^{17}
6_3c		73^{27}
6_3d		73^{17}
6_3e		73^{20}
6_3f		73^{88}
6c2	42 days post	73^{1}
6c4		73^{30}
6d1	180 days post	73^{24}
6d2		73^{24}
6d3		73^{5}
6d4		73^{20}

Green shading: pre-travel; orange: post travel.

*indicates reference strain for SNP comparisons for any given volunteer. SNPs are displayed as super-script after the ST

[+]Sample collection point only includes faecal samples where the E. coli sequence type displayed for that volunteer was detected. For the full list of isolates see Table S1.

Table 4.3. (Continued). SNP comparisons of pre and post travel non-CTX-M E. coli for volunteers with the same MLSTs before vs. after travel.

	Sample collection point[+] (days pre or post travel)	MLST
7_1a	60 days pre	10*
7_1b		10^{2}
7_1c		10^{2}
7_1d		10^{1}
7_1e		10^{0}
7_1f		10^{5}
7_3b	7 days pre	10^{2}
7_3c		10^{3}
7_3d		10^{4}
7_3e		10^{2}
7_3f		10^{2}
7a1	5 days post	10^{18143}
7b1	10 days post	10^{18141}
7b2		10^{18124}
8.2a	1 day pre	10*
8.2b		10^{5}
8.2d		10^{2}
8.2e		10^{17}
8.2f		10^{1}
8b2	16 days post	10^{17093}
8b3		10^{17084}
8b4		10^{17085}
8c1	40 days post	10^{17085}
8c2		10^{17087}
8c3		10^{17084}
8c4		10^{17088}
8d1	58 days post	10^{17084}
8d2		10^{13306}
8d4		10^{13307}
8d5		10^{17084}

Green shading: pre-travel; orange: post travel.
*indicates reference strain for SNP comparisons for any given volunteer. SNPs are displayed as super-script after the ST. [+]Sample collection point only includes faecal samples where the E. coli sequence type displayed for that volunteer was detected. For the full list of isolates see Table S1.

Table 4.3. (Continued). SNP comparisons of pre and post travel non-CTX-M E. coli for volunteers with the same MLSTs before vs. after travel.

	Sample collection point[+] (days pre or post travel)	MLST
15_1c	28 days pre	10*
15_1d		10^{6}
15_2c	14 days pre	10^{10}
15_2d		10^{54}
15c1	150 days post	10^{9}
15c2		10^{7}
15c3		10^{23}
15c4		10^{14}
17_2a	3 days pre	131*
17_2b		131^{18}
17_2c		131^{12}
17_2d		131^{11}
17_2e		131^{27}
17_2f		131^{8}
17c1	84 days post	131^{16}
17c2		131^{29}
17c3		131^{17}
17c4		131^{45}
20_1b	10 days pre	10^{13568}
20_1c		10^{13553}
20_2a	4 days pre	10^{14956}
20bpp	42 days post travel	10*
20f1	120 days post	10^{10}
20g1	183 days post	10^{24}
20g2		10^{9}
20g3		10^{9}
20g4		10^{31}

Green shading: pre-travel; orange: post travel.

*indicates reference strain for SNP comparisons for any given volunteer. SNPs are displayed as super-script after the ST

[+]Sample collection point only includes faecal samples where the *E. coli* sequence type displayed for that volunteer was detected. For the full list of isolates see Table S1.

4.4.6 Co-travelling spouses share non-CTX-M *E. coli* ST200

Volunteers 9 and 10 are spouses who travelled to Sri Lanka and India together. Table 4.4 shows the relatedness of four ST200 strains isolated from faecal samples from both of these volunteers. As can be seen from the SNP matrix these strains are indistinguishable between volunteer 9 and 10, with zero SNPs between isolate 9a2 and 10a1.

4.4.7 Rise in overall number of different AMR genes after travel

Figure 4.3 a-h displays AMR gene presence for fifteen volunteers with WGS data. After travel, *E. coli* in the microbiome carry a greater abundance of antimicrobial resistance genes compared to before travel (11/15 volunteers had increased abundance of AMR genes in post-travel faecal samples compared to pre-travel samples) (Figure 4.3 a-h).

It is notable that volunteer 6 pre-travel *E. coli* contained no detectable AMR genes encoded by the two *E. coli* clones detected in pre-travel faecal samples (ST69 and ST73), compared to post travel *E. coli* (Figure 4.3 d). During the first 6 weeks of post-travel sampling for volunteer 6, antibiotic resistance genes to seven antibiotics were encoded by post travel *E. coli* (clones ST450, ST2221 and a clone with no ST assigned).

Several other volunteers only carried one class of AMR gene pre-travel (tetracycline resistance gene, tet). These were volunteers 4, 5, 9, 19, and 22.

Table 4.4. SNP distance matrix of faecal ST200 *E. coli* from volunteer 9 and 10.

	9a2*	10a1	10b1	10b2
9a2*	0	0	1	7
10a1	0	0	1	7
10b1	1	1	0	8
10b2	7	7	8	0

*experiment reference strain

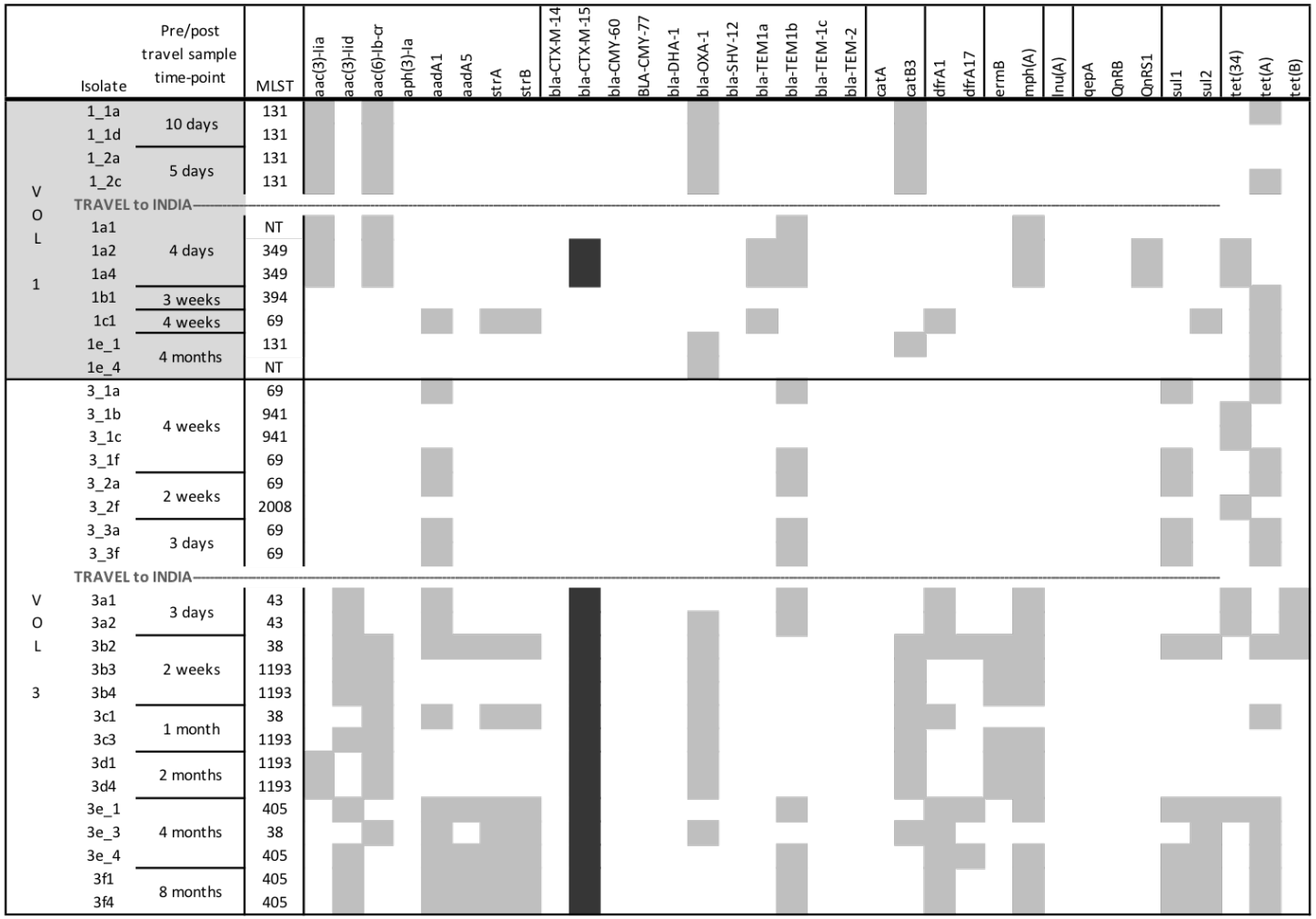


Figure 4.3a AMR gene profile of pre and post-travel *E. coli* for volunteers 1 and 3.

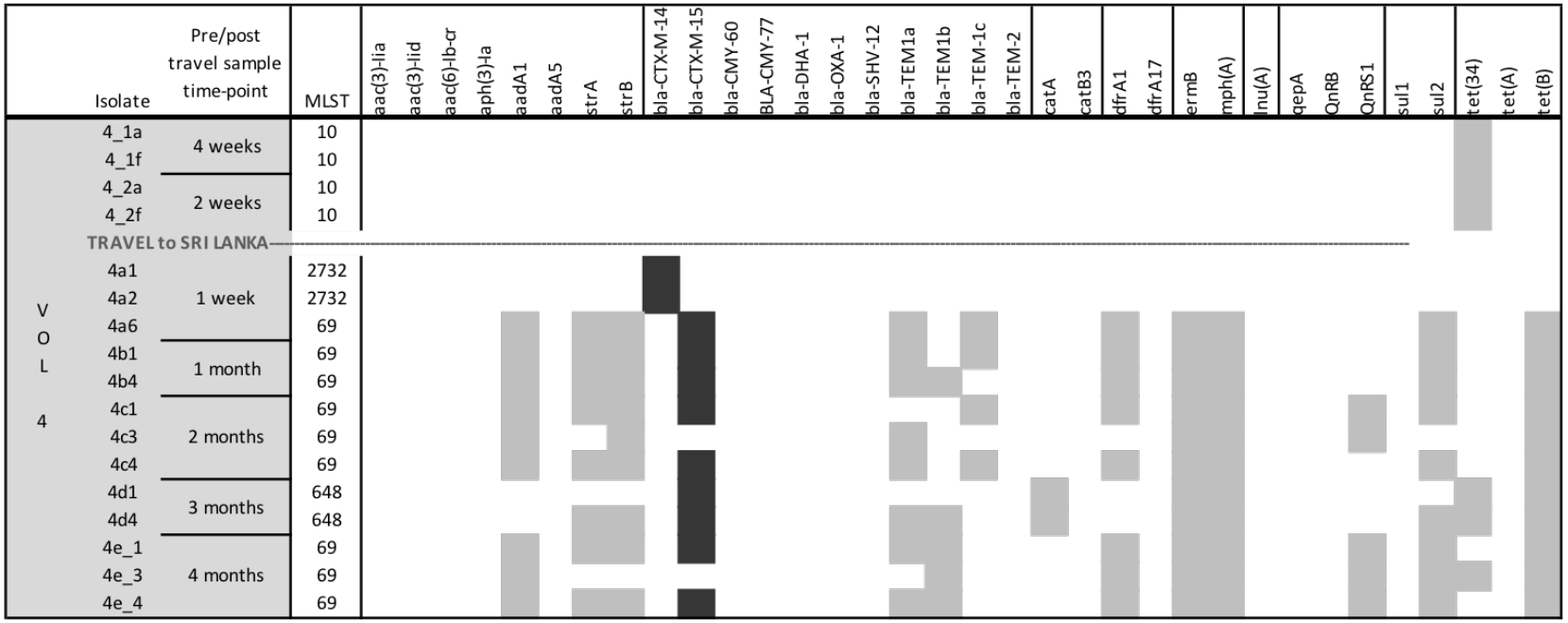


Figure 4.3b AMR gene profile of pre and post-travel *E. coli* for volunteer 4.

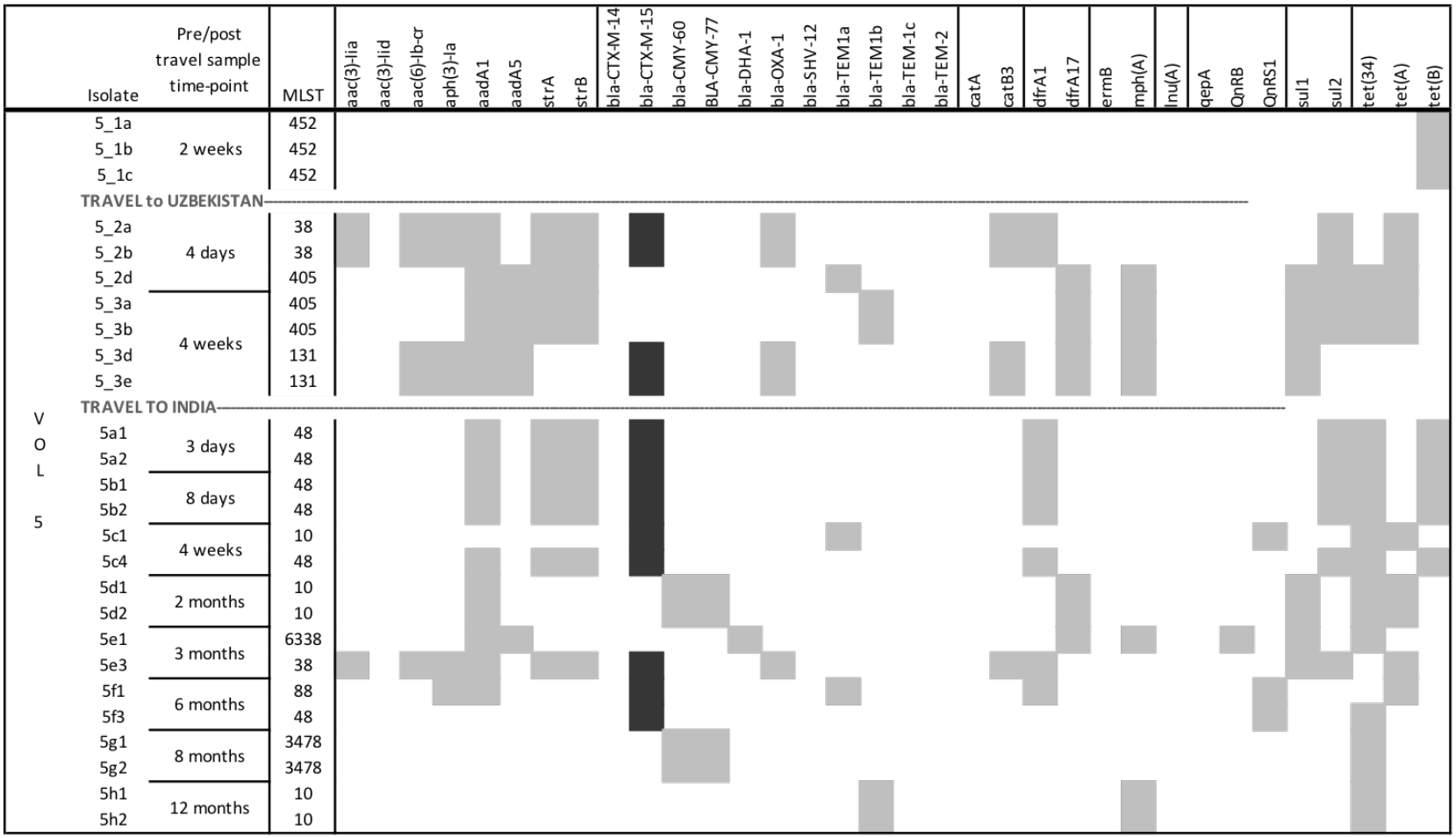


Figure 4.3c. AMR profile of pre and post-travel *E. coli* for volunteer 5.

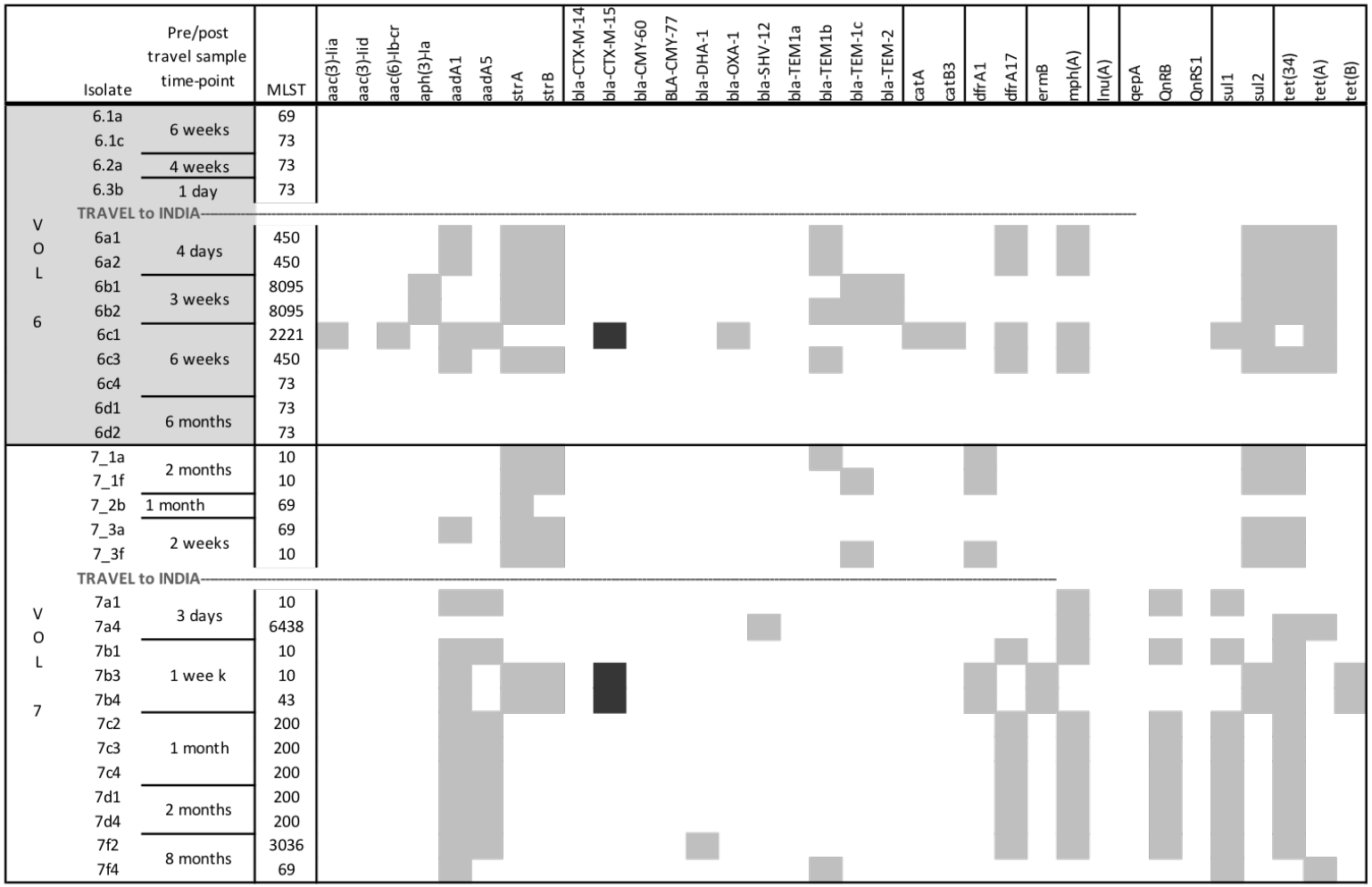


Figure 4.3d. AMR profile of pre and post-travel *E. coli* for volunteer 6 and 7.

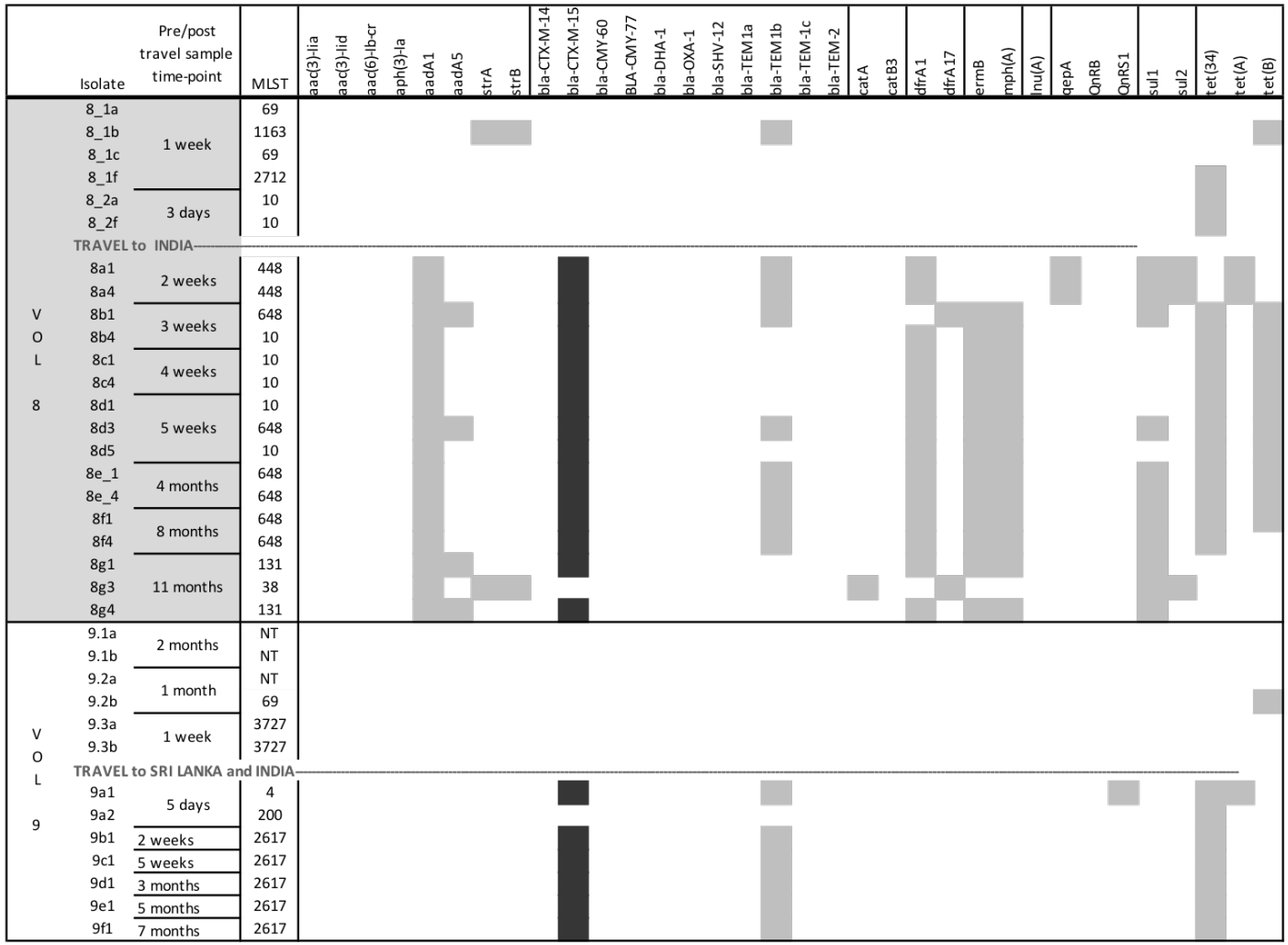


Figure 4.3e. AMR profile of pre and post-travel *E. coli* for volunteer 8 and 9.

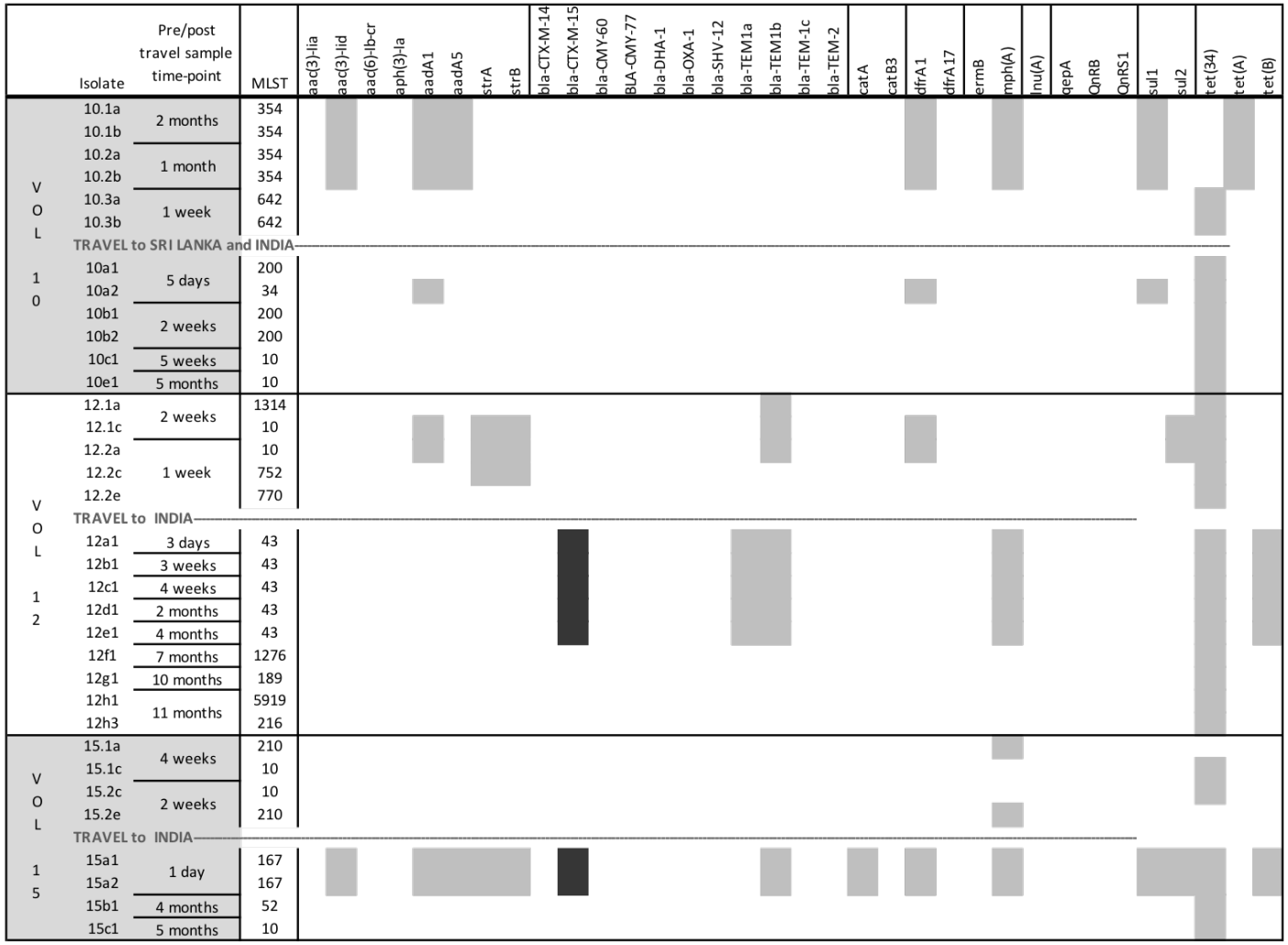


Figure 4.3f. AMR profile of pre and post-travel *E. coli* for volunteers 10, 12 and 15.

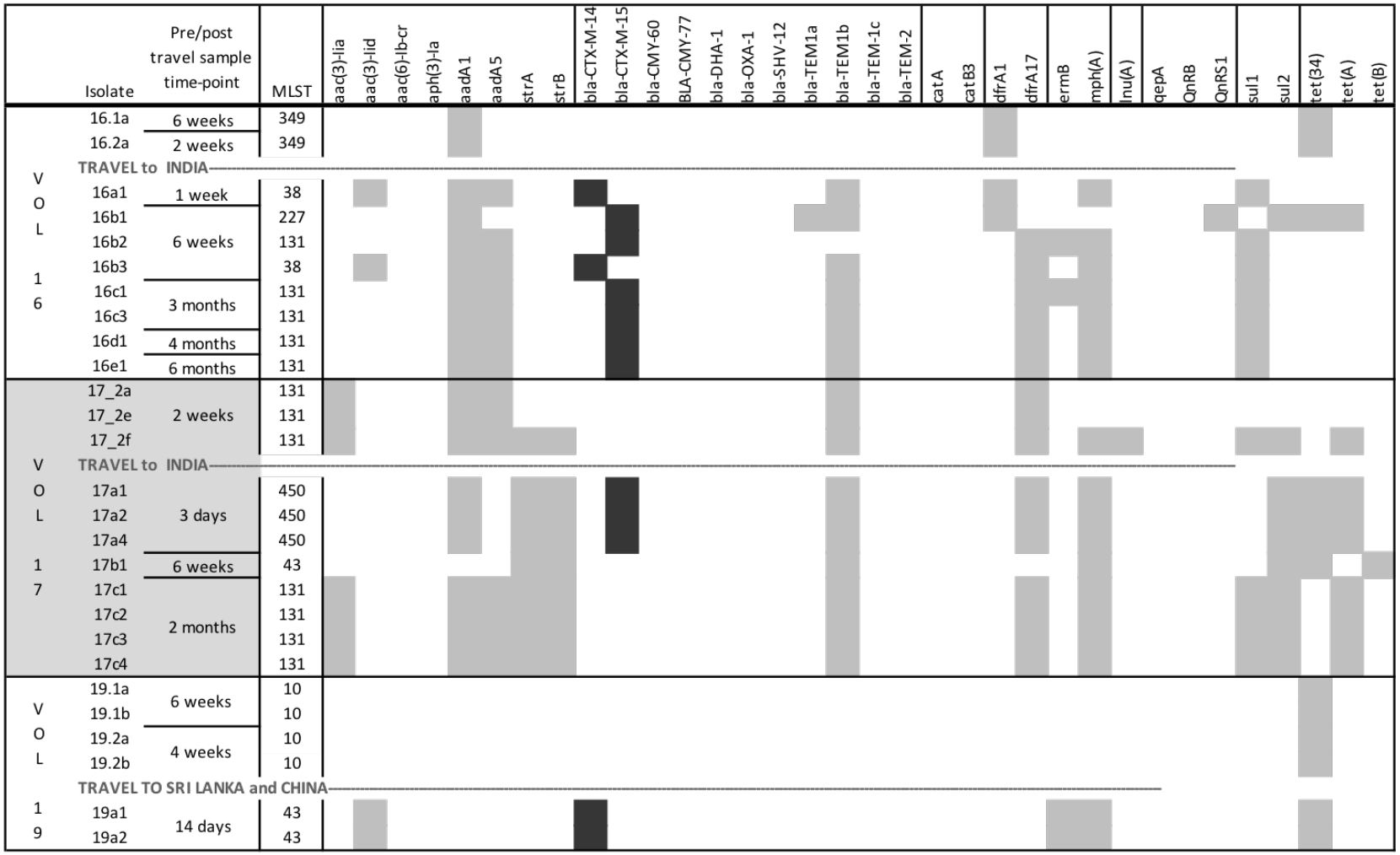


Figure 4.3g. AMR profile of pre and post-travel *E. coli* for volunteers 16, 17 and 19.

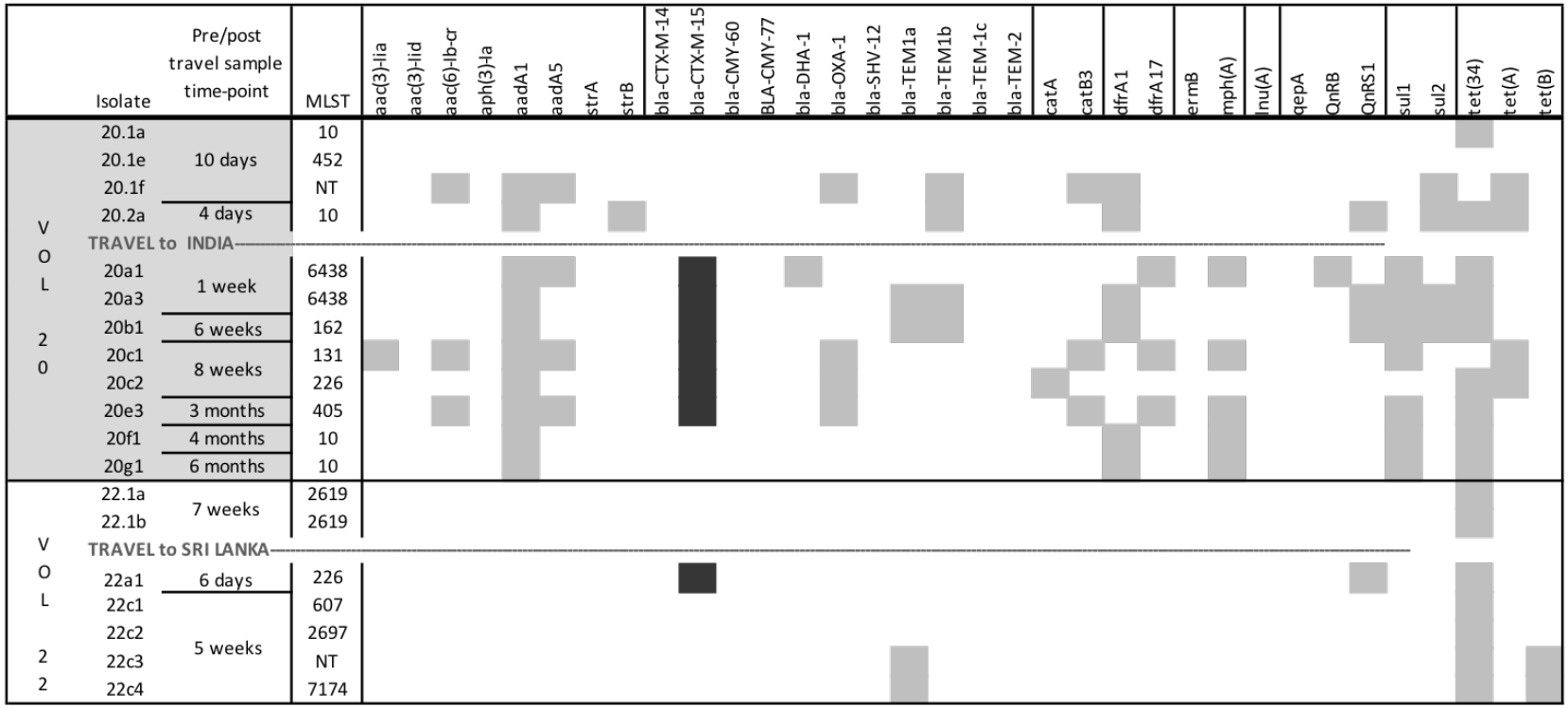


Figure 4.3h. AMR gene profile of pre and post-travel *E. coli* for volunteers 20 and 22.

4.5 Discussion

4.5.1 CTX-M-EC acquisition is polyclonal

CTX-M-producing *E. coli* acquired during travel came from a wide range of sequence types (STs) and phylogenetic groups, in keeping with previous studies (Weisenberg *et al.*, 2012; Paltansing *et al.*, 2013; Kuenzli *et al.*, 2014; Pires *et al.*, 2016; Vading *et al.*, 2016).

The pattern of *E. coli* PG group acquisition in the present study (Figure 4.1) are in keeping with previously published work which found a predominance of PG-A in travel-acquired strains (Valverde *et al.*, 2015; Pires *et al.*, 2016). Moreover, CTX-M-EC belonging to PG-A, PG-D, and PG-B1 are commonly acquired after travel (Pires *et al.*, 2016). In terms of *E. coli* pathogenicity, PG-B2 and PG-D tend to be extra-intestinal pathogenic E. coli (ExPEC), particularly urinary sepsis and bacteraemia, whereas PG-A and PG-B1 usually lack the required pathogenicity genes required for successful extra-intestinal virulence in humans (Boyd and Hartl, 1998).

For each volunteer who acquired CTX-M-EC, the acquisition of >ST was common (8/15 volunteers). The most common STs were ST131, ST38, ST10, and ST43. Limited data suggests that the prevalence of ST131 isolates in Indian CTX-M-EC clinical isolates varies between 9-25% (Hussain *et al.*, 2012; Roy *et al.*, 2015; Ranjan *et al.*, 2017). However, apart from the present study (Bevan *et al.*, 2018), no studies to date have surveyed faecal carriage of *E. coli* in India.

Relatively few studies have assessed the *E. coli* MLSTs from the human microbiome identified after travel, but all found there to be a wide range of ESBL-producing *E. coli* MLSTs acquired through travel, (Weisenberg *et al.*, 2012; Paltansing *et al.*, 2013; Kuenzli *et al.*, 2014; Pires *et al.*, 2016; Vading *et al.*, 2016). Pires and colleagues showed that travel from Switzerland to India results in polyclonal colonisation with CTX-M-producing *E. coli* with a mean of 2 STs and a range of 1-5 (Pires *et al.*, 2016). In addition, Kuenzli et al found that in a representative population of 34 CTX-M-EC acquired after travel to India, there were 24 different STs (Kuenzli *et al.*, 2014).

Pires and colleagues stated that re-colonisation after travel with a pre travel ST648 clone occurred (Pires *et al.*, 2016), but unlike the present study, they did not undertake SNP typing to confirm presence of an indistinguishable strain (Bevan *et al.*, 2018). In the current study, in post travel faecal samples in at least 4 volunteers, we were able to detect the same non-CTX-M *E. coli* clones which had been present before travel, and before CTX-M-EC acquisition. The strains found after travel were almost identical (< 10 SNPs) from the pre-travel strains, therefore the re-acquisition of *E. coli* from the 'home' UK environment can be ruled out. Therefore, the pre-travel *E. coli* are not replaced by acquired CTX-M-EC strains, rather there is co-colonisation with acquired *E. coli* and pre-travel pre-existing *E. coli*. The loss of CTX-M-EC after travel, albeit sometimes up to a year post travel, may be due to the competition from pre-existing non-CTX-M *E. coli*.

Although Arcilla and colleagues conducted the largest prospective traveller study, which included >1000 volunteers, their focus was on risk of acquisition and onward transmission of

CTX-M-EC, and they did not analyse the clonal relationships between Enterobacteriaceae acquired by travellers (Arcilla *et al.*, 2017).

The commonest STs among the volunteer cohort after travel, with CTX-M-producing *E. coli,* were ST131, ST38, ST10, and ST43. These *E. coli* strains were colonisers of the faecal microbiome and were not infecting strains. It is notable that the *E. coli* STs acquired by seven volunteers were either ST131 or ST38 (Figure 4.1). *E. coli* ST131 (part of PG B2) and to a lesser extent, ST38 (part of PG D) are known to be common causes of ESBL-producing-ExPEC infection in humans, shown to cause bacteraemia in recent surveys (Roer *et al.*, 2017; Harris *et al.*, 2018)(see also Chapter 2, section 2.5.2).

The volunteers in this study were healthy, and all participants denied any illness on the days of faecal sampling. Volunteers were not followed up to determine the incidence of ExPEC infection in our cohort, but given that the participants were young, healthy individuals, ExPEC infection is likely to have been low or absent. Further work in this area would involve screening travel acquired CTX-M-EC for pathogenicity genes to give an indication of the clinical significance of these strains, in terms of their potential to cause ExPEC infection.

4.5.2 CTX-M-EC clones are maintained in the faecal microbiome after travel

The median duration of carriage of CTX-M-EC was 60 days, with 46% of volunteers (6/13) known to be CTX-M-EC positive at 6 months after travel. Volunteer 8 carried CTX-M-EC for the longest (11 months). However, a limitation of the carriage-data in this study is that 50%

of volunteers with CTX-M-EC were still colonised at the final sampling point. Therefore, the duration of carriage in 50% of volunteers was not determined, and the overall mean carriage duration is an underestimate.

Arcilla and colleagues found 11.3% were still colonised with ESBL-producing Enterobacteriaceae at 12 months post travel, with a median post travel duration of colonisation of 30 days, but did not report a subgroup analysis on the duration of carriage for 79 travellers who went to India (Arcilla *et al.*, 2017). Correlating with this study, Pires reported a 40% colonisation rate (6/15 travellers), 6 months after returning from India (Pires *et al.*, 2016). In addition, Ruppe et al found that 7.2% (8/111) of ESBL-carriage positive travellers were still carriers at 6 months after travel to Asia (Ruppé *et al.*, 2015). Moreover, Ruppe et al reported that travel to Asia is significantly associated with a longer duration of carriage compared to travel to Africa or Latin America (Ruppé *et al.*, 2015). A more recent Canadian study which found that of 70 travellers to the Indian subcontinent who acquired ESBL-producing *E. coli*, 21% (15/70) were still colonised after return to Canada (Peirano *et al.*, 2017).

Volunteer 5 is particularly notable as having first travelled to Uzbekistan with return to the UK, followed by a second excursion to India, and then return to the UK. Putting the post travel CTX-M-EC-containing faecal samples into perspective, Sample 5.2 was collected 4 days after return from Uzbekistan (sample with isolate ST38), sample 5a1 was collected 5 weeks after the Uzbekistan trip and 3 days after the volunteer returned from India (sample ST48), and sample 5e4 was collected 12 weeks after the travel to India (sample with ST38). The

ST38 isolates in faecal samples 5.2 and 5e4 is only 6 SNPs apart, suggesting that the same CTX-M-EC strain remained as a coloniser within the faecal microbiome in the home environment post Uzbekistan, during the trip to India, and again stably maintained on return to the UK (Table 4.2 and Figure 4.3c).

Given the diversity within *E. coli*, it is not surprising that we also found colonisation with completely different strains of the same MLST group. For volunteer 8 ST10 isolates (Table 4.3), closely related CTX-M-EC are carried for 2-8 weeks after travel, but in addition there is a second ST10 population detected at 8 weeks post travel which is unrelated to the ST10 detected in earlier post travel samples (13323 SNPs apart) (Table 4.3).

4.5.3 *E. coli* without CTX-M acquired during travel co-colonise the GI tract alongside CTX-M-EC after travel

The proportion of cefotaxime-resistant *E. coli* in faecal samples known to contain CTX-M-EC, varied considerably between volunteers, and at different time points for the same volunteer (Figure 4.2). Picking 100 colonies per faecal sample allows the isolation for the major *E. coli* strains in the sample, as previously described (Hedges, Howe and Linton, 1977).

In faecal samples collected from the same volunteer that were analysed using tooth-picking over time, the proportion of cefotaxime-resistant strains did not remain stable (Figure 4.2). In 3/4 volunteers, the proportion of cefotaxime-resistant strains diminishes over time, probably reflecting out competition from non-CTX-M-EC and lack of re-exposure to CTX-M-EC through ingestion in the UK.

A limitation of the tooth-picking approach is that only 15 strains were sequenced from 8 volunteers (in most cases one colony pick per volunteer). Therefore, the true diversity of non-CTX-M *E. coli* in faecal samples containing CTX-M-producers, could not be ascertained. However, the present study is the first to demonstrate co-colonisation of 3-GC- sensitive *E. coli* in the faecal microbiome containing CTX-M-producing strains (Bevan *et al.*, 2018). There are 2 possibilities underlying this phenomenon: (1) Gut colonisation during travel includes CTX-M-producers and CTX-M non-producers; (2) The CTX-M non-producers were acquired after return to the UK and were detected in stool samples co-colonising with travel-acquired CTX-M-producers. It is also possible that both scenarios take place. However, in 7/7 tooth-picking experiments, the 3-GC-sensitive *E. coli* STs detected were new – the STs were different from both the pre-travel CTX-M-free strains – and the post-travel CTX-M-producers. Thus, our data suggests that travel results in acquisition of both CTX-M-producers, and non-producers (Table 4.1). Sampling volunteers while overseas would be required to prove this hypothesis. No such study has been published to-date.

4.5.4 Pre-travel resident *E. coli* persist as a minority population in the faecal microbiome

The present study is the first to show that pre-travel resident *E. coli* persist as an undetectable minority population in the faecal microbiota of volunteers who have acquired CTX-M-EC. This is a novel finding: pre-travel non-CTX-M- *E. coli* are not completely displaced from the faecal microbiome, but are maintained as a minority population throughout travel,

and become detectable again as a majority population after travel. (Table 4.3) (Bevan *et al.*, 2018).

A small number of studies have considered the *E. coli* clonal dynamics of travel-acquired strains over time (Paltansing *et al.*, 2013; Pires *et al.*, 2016; Vading *et al.*, 2016). Pires found 3/15 volunteers who were CTX-M-positive before travel, and in 1/3 cases, the ST648, CTX-M-14-producing strain found before travel - was also detected at 3 months after travel – and was presumably the same pre-travel strain. Similarly, Vading used Rep-PCR to show that 4 volunteers carried the same PG-B2 ESBL-producing Enterobacteriaceae before and after travel (Vading *et al.*, 2016). Moreover, Paltansing showed that 4/7 volunteers who were CTX-M-positive pre travel, had the same CTX-M-strain genotype and ST combination detected in faecal samples after travel (Paltansing *et al.*, 2013). Based on the findings from Pires, Vading and Paltansing, it is reasonable to suggest that pre-travel CTX-M-EC often persist throughout the period of travel and are detectable in post travel faecal samples. However, unlike the present study, none of these studies did SNP-typing of strains, therefore definitive evidence of pre-travel strain persistence was not provided.

4.5.5 Volunteer 9 and volunteer 10 share indistinguishable *E. coli* ST200

Both volunteers 9 and 10 share the same ST200 strain, which is a pre-travel non-CTX-M-producing *E. coli*. (Table 4.4).

When an identical (0 SNPs distance) strain is found from two volunteer samples, the possibility of laboratory cross-contamination must be considered and ruled out. Cross

contamination in the laboratory, but also at the volunteer sample collection stage is a possibility for the samples in question because volunteers 9 and 10 were spouses, and probably used the same bathroom facilities. This is particularly the case for faecal samples 9a and 10a which were submitted on the same day.

However, indistinguishable ST200 strains were found at a later sampling point: 1 SNP between isolate 9a2 (5 days post travel faecal sample, volunteer 9) and 10b1 (30 days post travel faecal sample, volunteer 10). The same *E. coli* ST200 is present in different volunteer faecal samples, on different occasions, separated by in time. Therefore, there has probably been household transmission between volunteers 9 and 10. Such an occurrence has been described in the literature, most recently by Arcilla and colleagues who calculated a 12% risk of transmission of faecal *E. coli* between a returning traveller and a non-travelling household contact (Arcilla *et al.*, 2017).

4.5.6 Mechanism of *bla*$_{\text{CTX-M}}$ acquisition

Typing of CTX-M-EC and non-CTX-M-EC before and after travel using MLST and SNP typing was used to ascertain whether HGT of *bla*$_{\text{CTX-M}}$ had occurred. After MLST of pre- and post-travel *E. coli* for sixteen volunteers, 13/16 had different *E. coli* STs with *bla*$_{\text{CTX-M}}$, to pre-travel non-CTX-M *E. coli* (Table 4.5). For 3/16 volunteers, pre-travel non-CTX-M-EC before travel and CTX-M-EC after travel had the same ST, ST10 (Table 4.5). This suggested that HGT of the *bla*$_{\text{CTX-M}}$ gene onto a pre-existing *E. coli* ST10 had occurred. In order to investigate this initial finding, SNP typing was used to assess the ST10 strain relatedness for each volunteer. Pre-

and post-travel ST10s for each of volunteer 7, 8, and 20 were >13,000 SNPs apart, indicating that these were not the same strain. Therefore, HGT of *bla*$_{CTX-M}$ was not detected (Table 4.5).

Table 4.5. Investigating volunteer isolates for horizontal gene transfer.

Volunteer	Pre- and Post-travel MLST comparison	SNP typing
1	New ST post-travel with *bla*$_{CTX-M}$	N/A
3	New ST post-travel with *bla*$_{CTX-M}$	N/A
4	New ST post-travel with *bla*$_{CTX-M}$	N/A
5	New ST post-travel with *bla*$_{CTX-M}$	N/A
6	New ST post-travel with *bla*$_{CTX-M}$	N/A
7*	ST10 pre (no *bla*$_{CTX-M}$) and post travel (with *bla*$_{CTX-M}$)	Pre vs. post-travelST10s >18,000 SNPs apart
8*	ST10 pre (no *bla*$_{CTX-M}$) and post travel (with *bla*$_{CTX-M}$)	Pre vs. post-travelST10s >13,000-18,000 SNPs apart
9	New ST post-travel with *bla*$_{CTX-M}$	N/A
12	New ST post-travel with *bla*$_{CTX-M}$	N/A
15	New ST post-travel with *bla*$_{CTX-M}$	N/A
16	New ST post-travel with *bla*$_{CTX-M}$	N/A
17	New ST post-travel with *bla*$_{CTX-M}$	N/A
19	New ST post-travel with *bla*$_{CTX-M}$	N/A
20*	ST10 pre (no *bla*$_{CTX-M}$) and post travel (with *bla*$_{CTX-M}$)	Pre vs. post-travel ST10s >13,000-15,000 SNPs apart
21	New ST post-travel with *bla*$_{CTX-M}$	N/A
22	New ST post-travel with *bla*$_{CTX-M}$	N/A

*Three isolates which required SNP typing are boxed.

In eight volunteers, multiple different STs with CTX-M-EC were acquired (Table 4.1), however, no cases were found where a plasmid sequence matched between strains of a different sequence type. Therefore, HGT was not detected occurring between different *E. coli* strains in post travel faecal samples.

4.5.7 Rise in AMR gene frequency after travel

After travel, there was a rise in overall antimicrobial resistant gene abundance, associated with blaCTX-M acquisition, which is usually due to carriage of multiple AMR genes on plasmids (Woodford *et al.*, 2009). Investigation of post travel ESBL-producing (and 3GC-resisitant) *E. coli* in a number of studies has determined antibiotic co-resistance to multiple antibiotics, predominantly, ciprofloxacin (Kennedy and Collignon, 2010; Tängdén *et al.*, 2010; Ostholm-Balkhed *et al.*, 2013; Paltansing *et al.*, 2013; von Wintersdorff *et al.*, 2014; Angelin *et al.*, 2015; Lübbert *et al.*, 2015; Barreto Miranda *et al.*, 2016); gentamicin (Kennedy and Collignon, 2010; Tängdén *et al.*, 2010; Ostholm-Balkhed *et al.*, 2013; Paltansing *et al.*, 2013; Angelin *et al.*, 2015; Barreto Miranda *et al.*, 2016); and co-trimoxazole (Paltansing *et al.*, 2013; Angelin *et al.*, 2015; Lübbert *et al.*, 2015; Barreto Miranda *et al.*, 2016).

Figure 4.3 a-h supports the SNP typing data (Table 4.2 and 4.3), demonstrating that pre-travel non-CTX-M producers are not displaced, but are maintained in the faecal microbiome, and re-emerge as the dominant population after travel. This is shown in Figure 4.3 a-h for five volunteers: 1, 6, 15, 17, and 20. In all 5 cases, the pre-travel strain becomes dominant

again, after CTX-M-EC is lost. CTX-M-EC loss is accompanied by loss of multiple other AMR genes from *E. coli* in the faecal microbiome before the final faecal sampling point.

Dissemination of CTX-M-EC is likely to occur via antibiotic co-selection, particularly in South Asia, where high levels of antibiotics are released into ground water during pharmaceutical production (Fick *et al.*, 2009). Therefore, antibiotic co-selection provides a mechanism for propagation of $bla_{\text{CTX-M}}$ genes and any other genes present on the plasmid in question (Bevan, Jones and Hawkey, 2017). However, the role of antibiotic co-selection is not always involved in $bla_{\text{CTX-M}}$ dissemination, as exemplified by the CTX-M-14-producing ST2732 isolate carried by volunteer 4 after travel. This ST2732 strain only carried one resistance gene, $bla_{\text{CTX-M-14}}$, which is a common phenomenon for this genotype (Cottell *et al.*, 2011; P L Ho *et al.*, 2011). Previous examples of $bla_{\text{CTX-M-14}}$ carriage on a plasmid without other resistance genes includes the globally disseminated, epidemic plasmids: pCT (Cottell *et al.*, 2011); and pHK01 (P L Ho *et al.*, 2011).

Beyond phenotypic studies of AMR in post travel *E. coli* isolates, only two studies were found which used molecular methods to determine the AMR gene content of isolates (von Wintersdorff *et al.*, 2014; Bengtsson-Palme *et al.*, 2015). A study recruiting healthy dutch travellers used a culture-independent PCR, applies directly to faecal samples, finding a statistically significant increased incidence of $bla_{\text{CTX-M}}$, *qnrB*, and *qnrS*, after verses before travel (von Wintersdorff *et al.*, 2014). In keeping with our own data, *tet* genes were present at a high level both before, and after travel, suggesting continued exposure to tet-containing isolates at home, as well as during travel (von Wintersdorff *et al.*, 2014).

A second study used shotgun metagenomic sequencing (illumina MiSeq) to assess the AMR gene content of the faecal microbiota before verses after travel (Bengtsson-Palme *et al.*, 2015). Bengtsson-Palme found a significant increase in abundance of resistance genes to sulphonamides, trimethoprim, and beta-lactams after travel (Bengtsson-Palme *et al.*, 2015), thus providing support to the susceptibility testing undertaken in previous studies (described above) and the genotypic findings in the present study (Figure 4.3, a-h).

Further work in the analysis of the AMR genes in strains would involve selecting representative strains for long read sequencing, to determine sequence of plasmids and/or large insertion sequences with multiple AMR genes. This would provide a picture of whether AMR genes are mobile between strains, as well as an indication as to the origin of any AMR plasmids.

4.6 Conclusion

This chapter has provided evidence that multiple CTX-M-EC clones are acquired by travellers, and that indistinguishable cefotaxime-sensitive strains (non-CTX-M-EC) are present before and after travel to the Indian subcontinent. Furthermore, the same CTX-M-EC strain colonises the human faecal microbiome, which is detectable up to 7 months after travel.

Novel data has been provided on the dynamics of colonisation with CTX-M-EC and non-CTX-M-EC after travel. The post travel faecal microbiome is made up of both cefotaxime-sensitive

and resistant *E. coli* populations (CTX-M-EC), indicating that non-CTX-M-EC are acquired alongside CTX-M-EC during travel. This is the first study to show that a minority population of pre-travel *E. coli* re-emerge as the dominant *E. coli* population after CTX-M-EC is lost. Further studies are needed in order to characterise the pre-travel non-CTX-M-EC.

Travellers usually acquire multiple *E. coli* sequence types carrying $bla_{CTX\text{-}M}$, and that the same *E. coli* clonal lineages are carried in the gut after return to the UK. The polyclonal CTX-M-producing-*E. coli* acquisition suggests multiple acquisition events during travel, which is in keeping with exposure from contaminated food and water supplies with faecal bacteria. Carriage of the same CTX-M-EC clones - often for several months after return to the UK – shows that the acquired strains are not easily displaced and suggests the stable colonisation in the faecal microbiome in the absence of antibiotic selective pressure.

Although the acquired CTX-M-EC are successful colonisers of the faecal microbiome, *E. coli* without CTX-M co-colonise the GI tract alongside CTX-M acquired strains within individual faecal samples collected after travel. The non-CTX-M *E. coli* in this situation are usually novel strains, not found before travel, which implies that travel-acquired strains are both CTX-M-producers and non-producers. This would again be in keeping with multiple acquisition events of faecal bacteria during the period of travel.

This chapter has confirmed that the most common mechanism of CTX-M-EC acquisition in healthy travellers is the acquisition of novel *E. coli* clones. Evidence is provided of the success of both CTX-M-EC in maintaining colonisation for several months after travel, and

also the success of pre-existing non-CTX-M host *E. coli* in remaining in the gut and emerging as the dominant *E. coli* population once the CTX-M-EC is lost.

Chapter 5: Plasmid identification and analysis

5.1 Introduction

5.1.1 Spread of *bla*$_{\text{CTX-M}}$: the role of epidemic plasmids

The transfer of *bla*$_{\text{-CTX-M}}$ genes on conjugative plasmids is fundamental to their evolution and global spread (Carattoli, 2013). Several studies have shown that plasmids of the IncF family are the predominant group which carry *bla*$_{\text{CTX-M-15}}$ (Gonullu *et al.*, 2008; Diestra *et al.*, 2009; Marcadé *et al.*, 2009; Dhanji, Doumith, *et al.*, 2011; Lee *et al.*, 2011) whereas *bla*$_{\text{-CTX-M-14}}$ is carried on a variety of plasmid types including on IncF, especially in the far East, (P. L. Ho *et al.*, 2012) and on IncK, predominantly in Western Europe (Cottell *et al.*, 2011). Horizontal transfer of antimicrobial resistance plasmids by conjugation in Enterobacteriaceae occurs in the human gut, (Smith, 1969; Fernandez *et al.*, 2007; Gona *et al.*, 2014; Knudsen *et al.*, 2018), in animals (Cavaco *et al.*, 2008) and in the environment (Amos *et al.*, 2014; Pehrsson *et al.*, 2016).

The IncF family plasmids are narrow host range, being mainly restricted to Enterobacteriaceae, and contain a number of mechanisms e.g. addiction systems and post segregational killing machinery, favouring plasmid stability (Woodford *et al.*, 2009). As a result, IncF plasmids are adapted to be stably maintained in commensal *E. coli* the gastrointestinal tract of humans and animals, without antimicrobial pressure (Carattoli, 2009).

Identical plasmids which are found across genetically diverse strains have been termed 'epidemic plasmids,'(Carattoli, 2009) and their dissemination helps to explain some of the global trends in *bla*$_{CTX-M}$ epidemiology (see Chapter 2). Examples of epidemic plasmids include pCT (Cottell *et al.*, 2011) and pHK01 (Ho *et al.*, 2012) (which both carry *bla*$_{-CTX-M-14}$), pC151a, (Lavollay *et al.*, 2006) (which carries *bla*$_{CTX-M-15}$) and the closely related plasmids, pEK516 and pEC_B24, (which also encode CTX-M-15) (Woodford *et al.*, 2009; Smet *et al.*, 2010). In South China it has been proposed that a discrete group of *bla*$_{CTX-M-15}$ epidemic plasmids are circulating which share significant homology to pKF3-94 — a Chinese reference plasmid isolated from *K. pneumoniae* — but are quite different from the pC151a-like plasmids described in Europe (Zhuo *et al.*, 2013). An epidemic IncI1 plasmid carrying *bla*$_{-CTX-M-1}$ has also been shown to be highly prevalent across food animal isolates and human isolates (see figure 2) in the Netherlands, suggesting transmission of *bla*$_{-CTX-M-1}$ via the food chain (de Been *et al.*, 2014). Moreover, since the first description of *bla*$_{-CTX-M-1}$ from a clinical isolate in Germany in 1989 (Bauernfeind, Grimm and Schweighart, 1990), *bla*$_{-CTX-M-1}$ has maintained a significant presence in several countries in Western Europe since then (Chapter 2, Figure 2.1 a-c). Globally, CTX-M-1 is less common, and therefore the ongoing persistence of this genotype in Europe could well be due to the spread of epidemic plasmids (de Been *et al.*, 2014).

One of the earliest reports of epidemic plasmid spread was the *bla*$_{-CTX-M-3}$ IncL/M plasmid, pCTX-M-3, which disseminated *bla*$_{-CTX-M-3}$ first in Poland (Gniadkowski *et al.*, 1998), and then throughout Europe. The genotype *bla*$_{CTX-M-15}$ differs from *bla*$_{-CTX-M-3}$ by only one amino acid

substitution, but the different genetic surroundings of these genes in Europe suggests that the evolution and subsequent spread of *bla*$_{\text{CTX-M-3}}$ from Poland occurred independently of the subsequent rise of *bla*$_{\text{CTX-M-15}}$ in the other regions (Livermore *et al.*, 2006). Interestingly, Inc L/M plasmids carrying *bla*$_{\text{CTX-M-3}}$ in China and Australia share significant homology to pCTX-M-3, suggesting a common evolutionary origin (Zhu *et al.*, 2009; Partridge *et al.*, 2012).

5.1.2 Other transferrable elements: insertion sequences, transposons and integrons

Insertion sequences (IS), transposons and integrons have played a key role in the dissemination of *bla*$_{\text{CTX-M}}$ (Cantón, González-Alba and Galán, 2012). A diversity of genetic platforms for the expression of *bla*$_{\text{-CTX-M}}$ have been described (Zhao and Hu, 2012).

Sequence IS*Ecp1* is the most common insertion element associated with *bla*$_{\text{-CTX-M}}$ genes and has been described as being associated with all *bla*$_{\text{-CTX-M}}$ variants and their spread (Zhao and Hu, 2012). IS*Ecp1* has two important characteristics: (1) It encodes a transposase, allowing mobilisation of the *bla*$_{\text{-CTX-M}}$ gene onto the particular plasmid; and (2) it acts as a strong promoter for the expression of *bla*$_{\text{CTX-M}}$ (Poirel, Decousser and Nordmann, 2003).

The transposition element IS26 has also been important in the dissemination of group 1 and group 9 *bla*$_{\text{-CTX-M}}$ variants (Zhao and Hu, 2012). Ensor and colleagues investigated 130 ESBL-producing *E. coli* and *K. pneumoniae* isolates collected 2003-2005 from three Indian hospitals. 73% of these isolates produced CTX-M-15, and in 31% of these, the insertion sequence IS26 was found within the IS*Ecp1* sequence, upstream of *bla*$_{\text{CTX-M-15}}$ (Ensor *et al.*, 2006). However, in a collection of CTX-M-producing Enterobacteriaceae isolated in the late

1990s, IS26 was not found (Walsh, Toleman and Jones, 2007). Therefore, a specific event after 2000 led to capture of *bla*$_{CTX\text{-}M\text{-}15}$ in India by IS26, which has been suggested as causing stable maintenance of *bla*$_{CTX\text{-}M}$ in the genepool thereafter (Ensor *et al.*, 2006). Subsequently, IS26 has been found again in India (Shahid, 2010) and worldwide (Zhao and Hu, 2012). More recently, Johnson and colleagues investigated the role of IncF plasmids and IS26 in the context of the clonal *E. coli* sub-group ST131 *H*30-Rx, and fully assembled a series of plasmids (from isolates of American origin) using long-read sequencing. Their results suggest that plasmids from *H*30-S and *H*30-R sub-groups were highly similar compared to plasmids isolated from the later *H*30-Rx sub-group of ST131, except that only the *H*30-Rx group contained *bla*$_{CTX\text{-}M\text{-}15}$ (Johnson *et al.*, 2016). In contrast to the early studies from India (Karim *et al.*, 2001; Walsh, Toleman and Jones, 2007), it was suggested that IS26, which was found in high copy number across all isolates, was involved in mediating transposition of *bla*$_{CTX\text{-}M\text{-}15}$ onto a common plasmid backbone (Johnson *et al.*, 2016).

It must be stressed that the selective pressure imparted by the use of 3GCs in humans, animals or the environment will increase the chance of the mobilisation of *bla*$_{\text{-}CTX\text{-}M}$. Mobilisation of multi-drug resistant class I integrons, which are often associated with IS*Ecp1* and IS*CR1,* (particularly with group 2 and group 9 CTX-M (Zhao and Hu, 2012), is particularly problematic because they often contain multi-drug resistance cassettes. These include *bla*$_{CTX\text{-}M}$, but also genes encoding resistance to clinically important antibiotics such as fluoroquinolones, chloramphenicol, aminoglycosides and trimethoprim (Cantón and Coque, 2006). Therefore, the overuse of any antibiotic which is encoded within an MDR cassette,

provides evolutionary pressure for the maintenance of all the MDR genes in the gene pool, and will lead to increased spread of the associated plasmid.

5.1.3 Role of bacteriophages in mobilising *bla*$_{CTX-M}$

In addition to the clear roles of plasmid conjugation and transposition, or movement of *bla*$_{CTX-M}$ genes via integration or transposition, the role of bacteriophage mediated transduction is also a key component of *bla*$_{CTX-M}$ transmission between bacteria and their local environment (van Schaik, 2015). Bacteriophages commonly carry antimicrobial resistance genes, and have been found to be associated with *bla*$_{CTX-M}$ variants which include *bla*$_{-CTX-M-10}$, (Oliver *et al.*, 2005) *bla*$_{CTX-M-27}$, (Yang *et al.*, 2017) and *bla*$_{CTX-M-15}$ (Falgenhauer *et al.*, 2014). In addition, AMR genes were found to be more abundant in phage DNA versus bacterial DNA (Subirats *et al.*, 2016). Moreover, in mice treated with ciprofloxacin and amoxicillin, phage DNA was found to be more highly enriched with antimicrobial resistance genes to these antibiotics, compared to untreated mice (Modi SR, Lee H, Spina CS, 2013).

There are clearly a number of different players involved in the transfer of *bla*$_{CTX-M}$: this is likely to be a highly opportunistic process, influenced by the genetic composition of particular ESBL gene reservoirs and differential antibiotic selective pressure, thus leading to convergent evolution in different parts of the world. These differences at the level of conjugation, transposition and transduction, help explain the geographical diversity in *bla*$_{CTX-M}$ genotypes.

5.2 Hypothesis

1. The *bla*$_{CTX-M}$ genes from the CTX-M-EC colonising the faecal microbiome in returning travellers are primarily plasmid mediated, rather than chromosomal in origin;
2. Travel acquired *bla*$_{CTX-M}$-containing plasmids are more closely related to plasmids previously identified from South Asia, than to plasmids of UK-origin;
3. The genetic context of *bla*$_{CTX-M}$ will be reflective of non-UK-type genetic contexts;
4. The ISECp1-CTX-M spacer sequence will vary according to region of origin of the CTX-M-EC isolate;
5. The CTX-M-bearing plasmids in post travel *E. coli* strains will be transferrable by conjugation.

5.3 Methods

Short read Illumina sequencing (see section 4.3.2) was used in combination with long read MinION sequencing to maximise contig size prior to further bioinformatic analysis of plasmid sequences.

5.3.1 MinION long read sequencing

5.3.1.1 DNA extraction

The cationic detergent, cetyl trimethylammonium bromide (cTAB) was used in conjunction with phenol-chloroform for isolation of genomic DNA (Ausubel *et al.*, 2003).

A 5ml BHI broth was grown overnight at 37 degrees for each *E. coli* strain in question. Bacterial pellet was then resuspended in 567µl TE buffer. To achieve bacterial cell wall lysis, SDS 10% (30µl) and proteinase K 0.5% (100µg/ml) were then added before incubation for 1 hour at 37 degrees. Then, NaCl (5M 100µl) was added to allow precipitation of cell wall material, denatured protein and polysaccharides. Next, cTAB/NaCl solution was added, followed by further incubation for 10 minutes at 65 degrees. Then, an equal volume of chloroform/isoamyl alcohol was added, mixed, and micro-centrifuged for 5 minutes.

The aqueous supernatant, containing DNA, was then removed and added to 0.6 volume isopropanol, allowing precipitation of nucleic acid. The string-like DNA pellet can then be transferred to a clean universal with 70% EtOH using a sterile plastic loop. After washing in 70% EtOH, the DNA pellet was re-suspended in 100µl TE buffer.

5.3.1.2 MinION setup

Following DNA extraction, library preparation was carried out using the Oxford Nanopore kit supplied with the MinION, and used the following reagents: FRM fragmentation mix, RBF running buffer, rapid ID adaptor mix, blunt TA ligase. The protocol was followed as per the MinION online guidance https://store.nanoporetech.com/kits-245/pcr-sequencing-kit.html. The 2016 Oxford Nanopore MinION was used which had 1D read capability. After library preparation, 16 µl of pooled DNA was added to the flow cell before starting an overnight MinION run. The DNA from thirty-four isolates were pooled (Isolates in Section 5.4.2.3, Table 5.3).

5.3.2 Plasmid Characterisation

5.3.2.1 Unicycler assembly

Prior to performing a hybrid assembly using short read and long read data, fast5 files produced by the MinION were converted to fastq using Poretools, followed by Porechop to trim the raw reads.

Unicycler was then used for the hybrid assembly of MinION reads combined with illumina reads (https://github.com/rrwick/Unicycler). Illumina reads for each isolate are mapped to a single MinION file consisting of all MinION reads concatenated. Unicycler is a pipeline which uses the SPAdes and Racon assemblers combined with Bowtie2 and Samtools for mapping and polishing.

5.3.2.2 Transconjugant analysis and plasmid identification

For the five transconjugants (4a2T, 12a3T, 12c1T, 12d2T, 12e1T), the putative plasmid contig was identified using BWA-MEM and Samtools. Each transconjugant (fasta file) was used as a reference on which to map the recipient-only (TG1) illumina reads. As the majority of the transconjugant assembly is made up of recipient genomic DNA, the plasmid DNA (the plasmid contig) from the donor was easily identified from regions of the reference which had zero reads mapped from TG1.

5.3.2.3 Confirmation of circular plasmid sequences

A feature of the unicycler hybrid assembly tool is that the assembly output is recognised as a circular DNA sequence, if such a sequence is produced from the assembly. In four cases

where unicycler confirmed that a circular sequence had been assembled, a confirmatory method was used to confirm this using the cloud-based galaxy platform (Afgan *et al.*, 2015). The fasta file opened in a text editor, and 2.5kbp from each end of the sequence were concatenated, with the join highlighted. The concatenated sequence was then saved as a new fasta file, and annotated using prokka (Seemann, 2014). The illumina sequence reads for the isolate in question were mapped to the concatenated file using the BWA-MEM tool (Li and Durbin, 2010) and visualised using artemis (Rutherford *et al.*, 2000). If the illumina reads were seen to cross the concatenation point, then the original sequence was confirmed to be circular. In the four cases where unicycler suggested that an assembly was circular, this was confirmed using this manual approach.

5.3.2.4 Investigation of remaining *bla*$_{\text{CTX-M}}$-containing contigs

Contigs which were not circularised using the above approach but nevertheless carried *bla*CTX-M, were assessed as to whether they were of plasmid or of chromosomal origin.

Several approaches were used to identify plasmid contigs. Initially, the *bla*$_{\text{CTX-M}}$ contigs were screened for 'plasmid features.' Plasmid features included: (1) presence of a plasmid replicon; (2) presence of plasmid transfer genes; or (3) presence of other AMR or metal-resistance genes.

Further interrogation of *bla*CTX-M contigs was carried out using the basic local alignment search tool (*BLA*ST), provided by the National Center for Biotechnology Information (NCBI). If

the *BLA*ST results matched to plasmid sequences, then the contig was confirmed as a plasmid fragment, however, if the *BLA*ST results only matched to bacterial genomic DNA, then the contig in question was confirmed to be of chromosomal origin. The size of the contig was also used as an indication of plasmid or chromosomal origin: contigs >500kbp are more likely to be chromosomal, and this was confirmed using *BLA*ST. Further confirmation that a contig was chromosomal was carried out by determining the genes present by annotation using RAST (Aziz *et al.*, 2008; Overbeek *et al.*, 2014; Brettin *et al.*, 2015).

5.3.2.5 Sequence comparisons

*BLA*ST was used to investigate which previously sequenced plasmids were homologous to the plasmids identified in the present study. The *BLA*ST expect value (e-value), which is the number of *BLA*ST hits expected to occur by chance, has a default value of 10. The e value used in this study was lowered to $1x10^{-6}$, increasing the likelihood that *BLA*ST matches are due to a biological sequence alignment rather than a random chance occurrence. *BLA*ST filters out low complexity regions by default, which includes highly repetitive regions. When searching for plasmid sequence matches using *BLA*ST, the low complexity filter was disabled, because plasmid sequences are often made up of highly repetitive regions.

Between 5-10 sequences were selected from *BLA*ST results for presentation using *BLA*ST ring image generator (BRIG) (Alikhan *et al.*, 2011). Sequences were selected for comparison if (1) there was >50% coverage of the query sequence, and (2) the *BLA*ST result contained metadata or annotation including the species and/or host and location of origin. BRIG

annotations for each query sequence were based on a tab-delimited file created using RAST annotation data (Aziz *et al.*, 2008; Overbeek *et al.*, 2014; Brettin *et al.*, 2015).

5.3.3 Investigating the genetic context of *bla*$_{CTX-M}$

The genetic surroundings of *bla*$_{CTX-M}$ were investigated using *BLA*ST searches, Artemis (Rutherford *et al.*, 2000) and Mauve (Darling, Mau and Perna, 2010). Thirty-five representative *bla*$_{CTX-M}$ contigs were selected for analysis of the *bla*$_{CTX-M}$ genetic context. Genbank files (gbk) were viewed in Mauve, where all open reading frames can be seen. If the gbk annotation was absent or non-specific, this was checked using RAST or *BLA*ST data. Common genetic contexts were displayed using Mauve and additional coloured arrows.

5.3.3.1 Investigating the ISECp1—*bla*$_{CTX-M-15}$ spacer sequence

Using the data generated in the aforementioned genetic context analysis, the non-coding spacer sequence between ISECp1 and *bla*$_{CTX-M}$-15 was copied and saved as a new fasta file using Artemis (Rutherford *et al.*, 2000). This was repeated for the 23 occurrences of 'ISECp1—*bla*$_{CTX-M}$-15' identified in the genetic context analysis. In addition, sequences containing *bla*$_{CTX-M}$-15 in the NCBI Genbank database were also used, including isolates of UK and Indian origin, as well as those spacer sequences described in a previous traveller study (Dhanji, Patel, *et al.*, 2011) (Section 5.4.4). In order to compare the spacer sequences, Mauve was used to line up the above sequences (Notredame, Higgins and Heringa, 2000).

5.3.4 Conjugation Experiments

The ability of post travel *E. coli* strains to transfer *bla*$_{CTX-M}$ containing plasmids in vitro was determined in a series of experiments. Firstly, post travel CTX-M-producing *E. coli* (the potential donor) were subjected to overnight conjugation on filters with J53 *E. coli* (the potential recipient). Where a successful conjugation had occurred for a donor/recipient with overnight incubation, the conjugation frequency was determined with the same donor/recipient combination after incubation for 3 hours.

5.3.4.1 Overnight conjugation

The recipient strain used was TG1: a J53 *E. coli* derivative which was rifampicin resistant and non-lactose fermenting. All potential donor *E. coli* were first checked for rifampicin MIC, lactose fermenting status on MaConkey agar, and PCR for *bla*$_{CTX-M}$. Only strains which were rifampicin susceptible (at 100µg/ml), lactose fermenting, and PCR positive for *bla*$_{CTX-M}$, were put forward as candidate donor organisms for conjugation experiments.

One post travel CTX-M-producing *E.coli* colony (donor strain) was inoculated into 10mls Luria-Bertani broth (LBB) supplemented with 4µg/ml cefotaxime and incubated overnight at 37^{o}C at 200rpm. A colony of TG1 was also cultured overnight in the same way using LBB supplemented with 100µg/ml rifampicin. 1ml was removed from each overnight culture and centrifuged at 13,000rpm for 3 minutes. The supernatant was then removed, and the pellet washed in fresh LBB. The bacterial pellets from donor and recipient strains were then suspended in 125µl and 1000µl of LBB, respectively. Next, to achieve a donor: recipient ratio of 8:1, 50µl of each of donor and recipient suspensions were mixed in an Eppendorf. The

donor/recipient mixture was then transferred to a sterile nylon filter which had been placed in the centre of an antibiotic-free LB agar plate. These conjugation plates were incubated overnight at 37^{o}C.

After overnight incubation, sterile filters were vortexed in 1ml of fresh LBB, so that all bacterial culture was re-suspended. Serial 10-fold dilutions of this re-suspension were made in Eppendorfs up to x1000. 100µl of each dilution was then spread to LB agar plates containing rifampicin (100µg/ml and cefotaxime 4 µg/ml) and incubated overnight at 37^{o}C. Negative control plates were also made up using agar containing rifampicin (100µg/ml) and cefotaxime (4 µg/ml). The negative controls were made from the original donor and recipient LB broths after serial dilution up to x1000. If a potential donor isolate failed to lead to transconjugants growth, the experiment was repeated a second time for that particular donor isolate.

5.3.4.2 Experiments to determine conjugation frequency

For the determination of conjugation frequency, a colony of fresh overnight culture of the donor and recipient *E. coli* were incubated in LBB at at 37^{o}C at 200rpm until each broth had reached OD 0.6, measured using a spectrophotometer. Each donor/recipient mixture was made using an 8:1 ratio and transferred to a sterile filter on antibiotic-free LB agar. These conjugation plates were incubated for 3 hours at 37^{o}C. Next, growth from filters was re-suspended, and 10-fold dilutions were made and spread to LB agar containing rifampicin (100µg/ml) and cefotaxime (4 µg/ml) as in Section 5.3.4.1. In order to determine

conjugation frequency, viable count plates of the donor isolate were determined by spreading serial dilutions of the original broths up to x10,000 and colony counting.

$$\text{Conjugation Frequency} = \frac{\text{Median Number of Transconjugants}}{\text{Median Number of Recipients} \times \text{Donor Viable Count/Recipient viable count Recipient Viable Count}}$$

Each donor which successfully conjugated overnight, was subjected to 3 separate replicate conjugation frequency experiments, with a mean value used to calculate standard deviation and 95% confidence intervals.

5.3.5 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was used as a tool to confirm plasmid transfer to recipient *E. coli* after conjugation experiments.

Isolates were sub-cultured from -80 bead stock onto isosensitest agar plates and incubated aerobically overnight at 37°C. Whole plates were scraped off with a sterile cotton swab and the culture was thoroughly suspended in 2mls tris-EDTA (TE) buffer (100mM tris, 100mM EDTA). Molten 1% low melting point agarose (LMPA) was made up by dissolving 1g LMPA in 100mls 0.5M TE buffer and heating in a microwave until fully dissolved. Molten LMPA was then immediately transferred to a water bath pre-heated to 55°C to prevent premature setting. PFGE plug moulds were inverted and placed on the adhesive tape, forming a temporary seal. 400µl of cell suspension was transferred to a sterile 1.5ml Eppendorf for

each isolate, within the 55°C water bath. 20 µl of Proteinase K (20mg/ml stock) was added to each Eppendorf and mixed carefully by gentle pipetting. 400µl of melted LMP was then added to the cell suspension/proteinase K mixture, (final concentration proteinase K, 0.5mg/ml). Plug moulds were then filled with the molten mixture containing cell suspension, proteinase K, and LMPA. Five plugs were made for each isolate. Plugs were left to set for 20 minutes at room temperature.

Cell lysis buffer (250mls) was prepared using 125mls of TE buffer (100mM tris, 100mM EDTA), 100mls SDW, and 25mls 10% sarcosyl, and adjusted to pH 8.0. 400µl proteinase K stock solution (20mg/ml) was added to 80mls of cell lysis buffer (final concentration proteinase K, 0.1mg/ml). 5mls of cell lysis buffer was transferred to a sterile universal for each isolate. Excess agarose from the top of plugs was trimmed off with a scalpel. A sterile plastic loop was used to push five plugs (per isolate) into each universal containing. Plugs were incubated with cell lysis buffer at 50 rpm in a 55^{o}C water bath for 90 minutes. Lysis buffer was drained from universals using a sterile fine-tipped pastette, without damaging agarose plugs. 10mls of pre-warmed SDW (55^{o}C) was added to each universal which was incubated at 50 rpm in a 55^{o}C water bath for 15 minutes. The SDW was removed and the plug washing step was repeated again with fresh SDW. The 15-minute washing steps were repeated for four times more using TE buffer (10 mM Tris:1 mM EDTA, pH 8.0) which had been pre-heated to 55^{o}C.

S1 nuclease buffer (100mls) was made up using 300mM sodium acetate, 10mM zinc acetate, and 50% glycerol w/v). 1ml S1 nuclease buffer was added to a sterile universal for each

isolate (5 plugs per isolate). 2.5µl of S1 nuclease 100 U/µl was added to each universal containing 1ml of S1 nuclease buffer, (final concentration S1 nuclease, 0.25 U/µl). Isolate plugs were added to each universal and incubated at 4^{o}C for five days.

1g PFGE-grade agarose was dissolved by boiling in a microwave in 110mls 0.5 M TBE buffer (approx. 1% gel). This was allowed to set at room temperature in a gel tray. Agarose plugs were removed from S1 nuclease universal containers and were cut to an appropriate size using a scalpel. Plugs were then carefully loaded individually into the wells of the gel. The final lane of the gel was loaded with a slice of lambda DNA size marker. Molten PFGE-grade agarose was used to seal wells. A PFGE tank was then filled with 2.5L of pre-chilled 0.5M TBE. The following PFGE conditions were then set: 19 hours, switch time initial 5 sec, final 45 sec, 6V/cm, 120^{o} angle, 14^{o}C.

After running, the gel was removed from the tank and placed in 250mls TBE buffer/ 80mcl gel red dye. This was incubated at room temperature for 30 mins at 60rpm, followed by washing in SDW for 5 minutes at 60rpm. The gel was imaged using a UV trans-illuminator and photographed. The gel was then dried in an oven at 50 degrees overnight between thick blotting pads.

5.4 Results

5.4.1 MinION outputs

A single MinION run was undertaken. After setting up, the MinION was left to run overnight. Unfortunately, due to an unexpected hospital generator test, the MinION did not run in full, but did lead to the generation of nine folders of fast5 files.

Calculation of read coverage for a draft genome was not possible as this was a mixed minion run containing DNA from 34 isolates. However, an estimate of coverage per isolate can be made. The overall read coverage for the pooled MinION run was 28.71. This coverage figure was obtained after fast5 to fastq conversion using canu. The average coverage per isolate (n=34): 28.71/34 = 0.84.

Table 5.1: Poretools output for MinION reads from one sequencing run

Total reads	24053
Total base-pairs	145,463,948
Mean read length (bp)	6047.64
Median read length (bp)	2516
Min read length (bp)	16
Max read length (bp)	1,125,149

5.4.2 Identification of plasmid DNA

The isolation and characterisation of CTX-M-bearing plasmids was undertaken using several approaches which included: screening illumina data for putative plasmid contigs, transfer of the CTX-M-plasmid to a plasmid free laboratory strain followed by transconjugant sequencing, and hybrid assembly using both short and long read sequencing.

5.4.2.1 Identification of plasmid contigs

After WGS and analysis of draft assemblies using abricate software (Seemann, 2017a), 198 isolates of *E. coli* from travellers were identified which carried a $bla_{CTX\text{-}M}$ gene (See Chapter 3). Artemis software was used to locate the CTX-M contig for each isolate, to determine whether any plasmid genes were co-located on the same contig, and to establish the genetic context of $bla_{CTX\text{-}M}$. At this stage, plasmid genes included plasmid replicon and plasmid transfer operon (tra) genes. Where a volunteer faecal sample yielded >1 *E. coli* MLST type, or if clonally disparate isolates within the same ST were found by SNP-typing, an isolate from each clone was subjected to an Artemis search.

Thirty-five CTX-M-15-producing isolates, and four CTX-M-14 producing isolates were identified for further investigation using this screening approach (Table 5.2). CTX-M-contigs were found to be of plasmid origin in 22/39 (56%); chromosomal in 15/39 (38%), and not known in 2/39 cases (Table 5.2). Contigs assembled using the hybrid assembly are indicated (*) in Table 5.2.

Table 5.2. CTX-M-bearing contigs with origin designated as plasmid, chromosomal or NK (not known). N=39.

Isolate	MLST	CTX-M genotype	Contig size, bp	Origin
1a4	349	15	50865	plasmid
3a1	43	15	6868	NK
3b2	38	15	495603*	chromosome
3b4	1193	15	642401	chromosome
3c1	38	15	107577	chromosome
3c3	1193	15	642401	chromosome
3d1	1193	15	318516	chromosome
3d3	1193	15	80715	chromosome
3e1	405	15	110977	plasmid
3e2	405	15	110977	plasmid
3f3	405	15	110850	plasmid
4a2T	donor 2732	14	68680	plasmid
4a6	69	15	461991	chromosome
4d1	648	15	709547*	chromosome
5.3e	131	15	128593	plasmid
5a1	48	15	235946*	chromosome
5b4	48	15	86675	chromosome
5c1	10	15	26815	plasmid
5e4	38	15	10044	NK
5f1	88	15	86170	plasmid
6c1	2221	15	108112	chromosome
8a1	448	15	101796	plasmid
8b1	648	15	4992	plasmid
8g1	131	15	358309*	chromosome
9a1	4	15	80715	plasmid
9c3	2617	15	6451	plasmid
12a2	43	15	95470	plasmid
12a3T	donor 43	15	71111*	plasmid
15a3	167	15	33988	plasmid
16a1	38	14	1112871	chromosome
16b1	227	15	35059	plasmid
16b2	131	15	290931	chromosome
17a1	450	15	5669	plasmid
19a1	43	14	4153	plasmid
19a2	43	14	13142	plasmid
20a3	162	15	99441	plasmid
20c4	226	15	166927	plasmid
20e4	405	15	872498*	chromosome
22a1	226	15	271110	chromosome

Nb. Where the isolate is a transconjugant, the donor MLST is indicated. Contigs originated from the illumina assembly, unless marked with*, designating a hybrid assembly.

5.4.2.2 CTX-M plasmid identification after conjugation into a sensitive recipient

In vitro conjugation was used to transfer the *bla*$_{CTX\text{-}M\text{-}14}$ plasmid from strain 4a2 to a laboratory *E. coli* K12 strain. The resulting transconjugant, 4a2T, and plasmid-free recipient strain were then sequenced, allowing identification of the transferred plasmid. The same procedure was carried out for CTX-M-15-producing *E. coli* strain 12a3, leading to the creation of the transconjugant 12a3T.

Figure 5.1 shows a line-up of the transconjugant 4a2T, and the recipient strain, TG1. The transferred plasmid can be identified as the sequence containing *bla*$_{CTX\text{-}M}$ which is only present in 4a2T, and not present in TG1. Figure 5.2 shows a different representation of the same experiment carried out for 12a3, displaying a mauve output for TG1 which lacks the CTX-M-contig (p12a3T) which is only found in the transconjugant 12a3T strain.

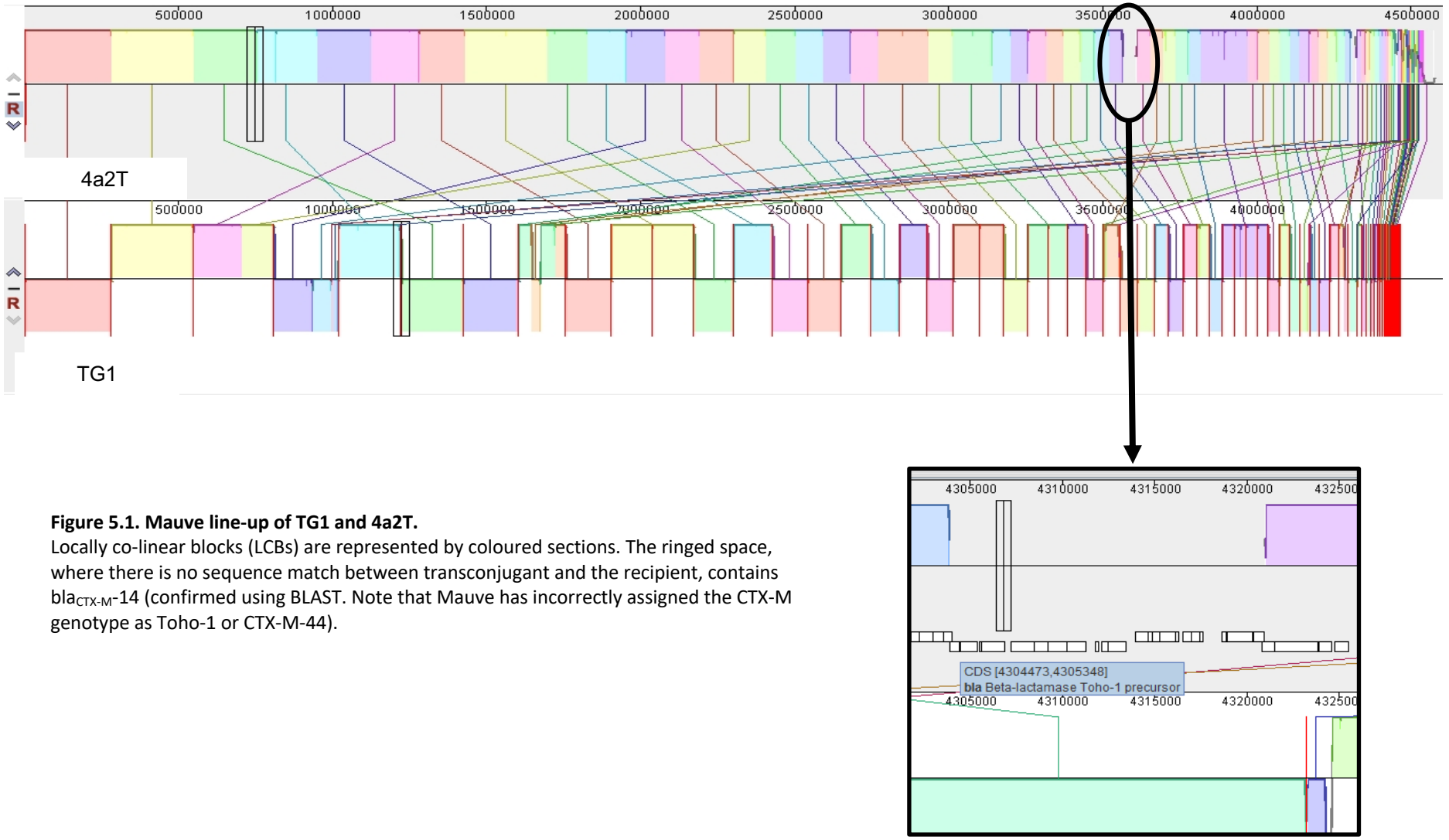


Figure 5.1. Mauve line-up of TG1 and 4a2T.
Locally co-linear blocks (LCBs) are represented by coloured sections. The ringed space, where there is no sequence match between transconjugant and the recipient, contains $bla_{CTX\text{-}M}$-14 (confirmed using BLAST. Note that Mauve has incorrectly assigned the CTX-M genotype as Toho-1 or CTX-M-44).

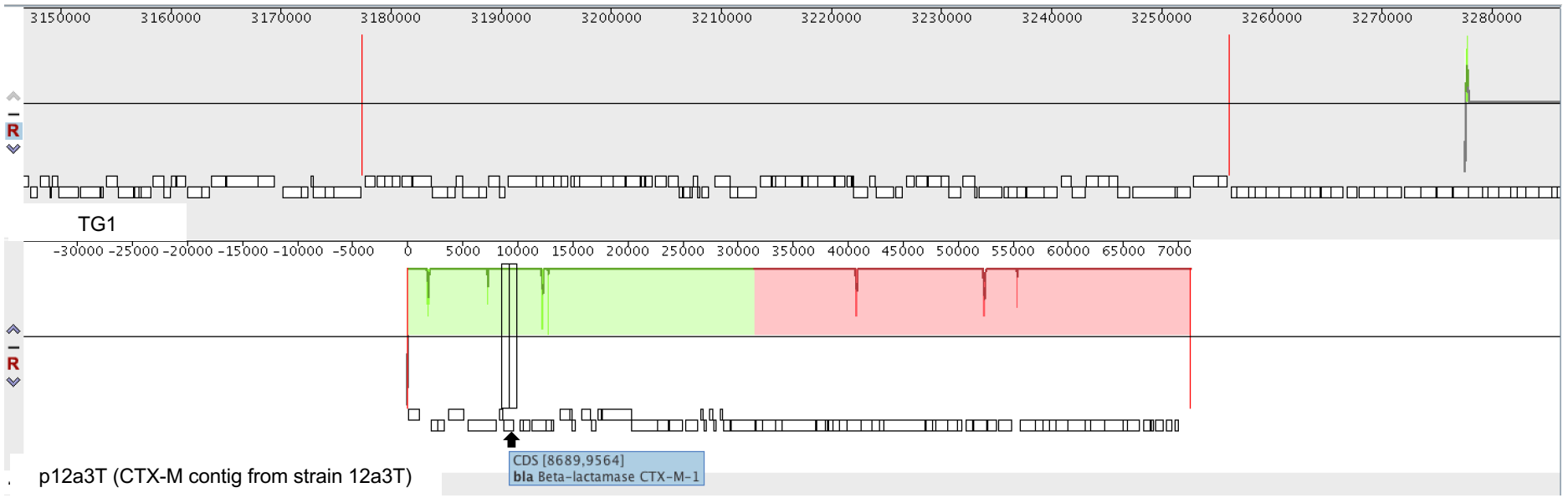


Figure 5.2 Mauve line-up of TG1 and p12a3T.
Locally co-linear blocks (LCBs) are represented by coloured sections. Mauve assigns blaCTX-M genes according to their group: group 1 CTX-M in this case, which was confirmed to be blaCTX-M-15 after BLAST analysis.

5.4.2.3 Hybrid assembly after long and short read sequencing

A hybrid assembly approach using long and short read sequencing, with both illumina and Oxford Nanopore sequencers, (Wick *et al.*, 2017), was a further approach used to determine $bla_{CTX\text{-}M}$ plasmid sequences. Thirty-four isolates were subjected to the hybrid assembly (Table 5.3). For 59% (20/34) of isolates, showed an increase in the assembled contig size after unicycler assembly compered to illumina short read assembly alone. In the remaining 14 isolates there was a decrease in the assembled contig size (35%; 12/34) or no difference after unicycler assembly, compared to the initial illumina assembly (6%; 2/34). However, it must be noted that the illumina assembly method when used in isolation, gave contig sizes >1000 bp higher than the unicycler assembly in only 5/34 cases (15%).

Four plasmid sequences were circularised using the unicycler hybrid assembly (Table 5.3). Unicycler confirms that a sequence is circular, but an extra check for circularisation was performed. Contigs were confirmed to be circular sequences by mapping illumina sequence reads to the concatenated ends of the contig under investigation. The circularised plasmids are from donor isolates 3f3 from volunteer 3 (plasmid pERB3f3); 9a1 from volunteer 9 (pERB9a1); 4a2T from volunteer 4 (pERB4a2); and isolate 12a3T from volunteer 12 (pERB12a3). An example of the circularistion check is shown for pERB3f3 (Figure 5.3).

Table 5.3. CTX-M-EC strains subjected to unicycler hybrid assembly.
Boxed isolates indicate circularised plasmids. Rows are shown in light grey where the unicycler assembly produced the largest contig; and in dark grey where the illumina assembly produced the largest contig.

Isolate	CTX-M genotype	MLST	Unicycler CTX-M contig, bp	Illumina CTX-M contig, bp
1a2	15	349	127505	50865
3a1	15	43	6614	6868
3b2	15	38	495603	107492
3b4	15	1193	1193064	642041
3c1	15	38	496320	107577
3f3	15	405	110850	110977
4a2T	14	donor 2732	68680	17093
4d1	15	648	709547	487721
4e1	15	69	5181	461991
4e2	15	69	5224	461029
5a1	15	48	235946	86674
5e4	15	38	10044	2692
8a1	15	448	101542	101796
8b1	15	648	4736	4992
8e1	15	648	73044	4992
8g1	15	131	358309	288622
9a1	15	4	80715	5621
9c2	15	2617	70913	6451
9c3	15	2617	70900	6451
12a3T	15	donor 43	71111	71979
12c1T	15	donor 43	71079	72822
12d2T	15	donor 43	72068	72068
12e1T	15	donor 43	72765	72765
12a2	15	43	95470	71048
12c2	15	43	95462	61093
12d4	15	43	96294	71879
15a1	15	167	33796	28790
16a1	14	38	1112871	677002
17a2	15	450	97215	5734
19a1	14	43	3732	4153
19a2	14	43	3732	13147
20a3	15	162	27983	99441
20c1	15	131	116824	3419
20e4	15	405	7744	30655

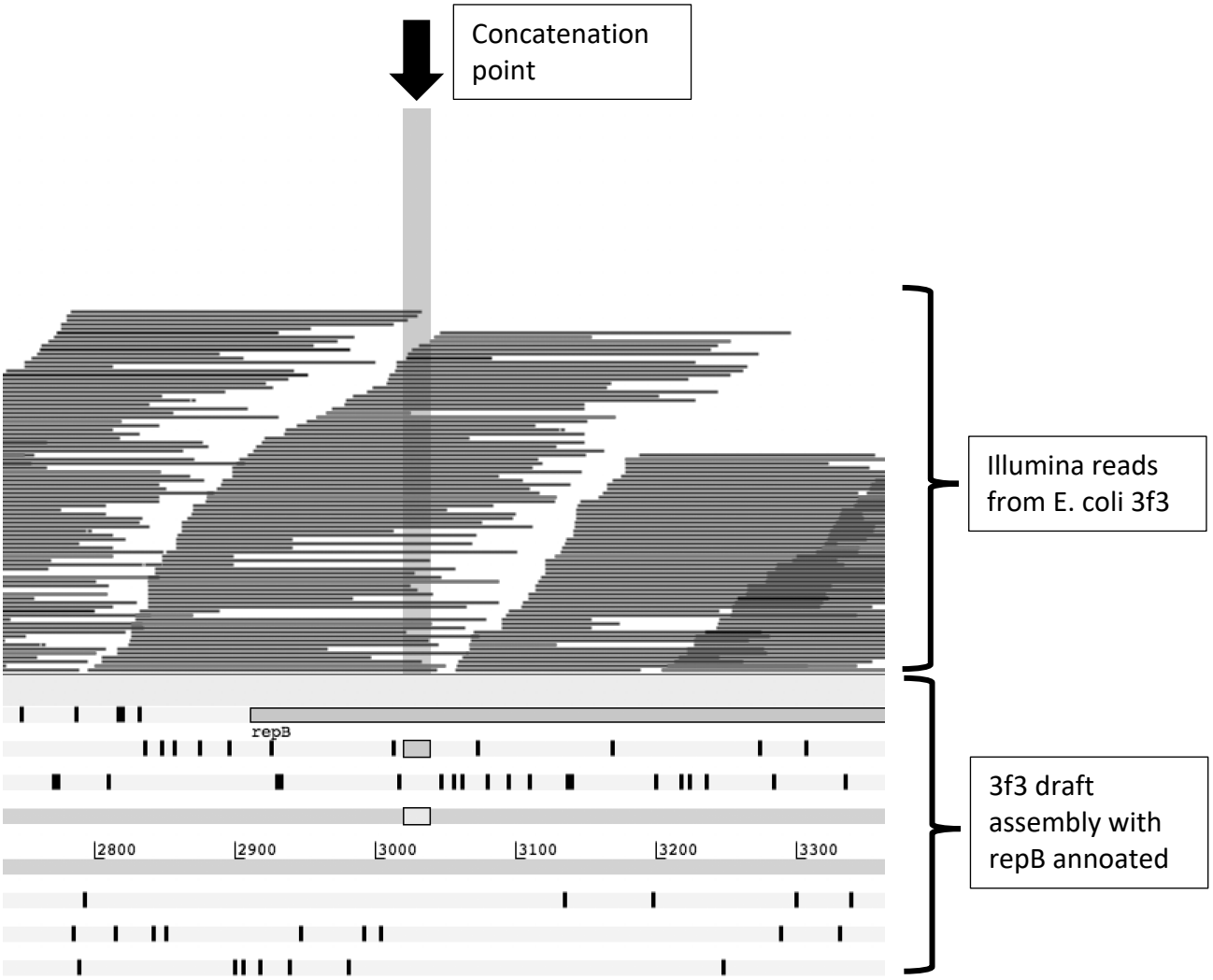


Figure 5.3 Artemis image confirming circularisation of pERB3f3.
Vertical band is a 20 bp selection which covers the concatenation point, occurring within ORF of repB.

5.4.3 Genetic contexts of *bla*$_{CTX-M}$

Six genetic context types designated (i)-(vi) are displayed in Table 5.4. These were distributed among 33 CTX-M-EC strains (Figure 5.4).

5.4.3.1 Context (i)

The most common genetic context was ISECp1-*bla*$_{CTX-M}$-15-orf477-Tn3, context (i), which was found in 11/35 cases among eight volunteers. For isolate 4d1, the Tn3 element was truncated by a hypothetical protein, and for 9c3 the ISECp1 was truncated by a spacer sequence between ISECp1 and catA2 (Figure 5.4; and Figure 5.6e).

5.4.3.2 Context (ii)

There were nine occurrences of CTX-M-EC with context (ii), ISECp1-*bla*CTX-M-15-orf477, across seven volunteers, although it must be noted that in two cases the genetic environment could not be fully revealed due to being present close to the end of a contig. In one case (isolate 4a6), the orf477 was found to be truncated by non-coding DNA (Table 5.4 and Figure 5.4).

5.4.3.3 Context (iii)

The CTX-M Context ParA-*bla*$_{CTX-M}$-15-orf477-Tn3 (iii), was found on six occasions in *E. coli* from five volunteers (Table 5.4 and Figure 5.4). In 5/6 cases for context (iii), the Tn3 was followed by an IS3 element (Table 5.4).

5.4.3.4 Context (iv)

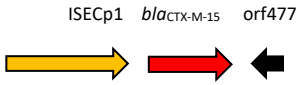
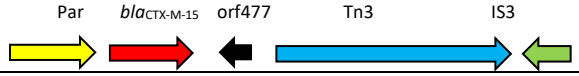
Context (iv), which was defined as IS26-ISECp1-*bla*$_{CTX-M-15}$-orf477-Tn3-IS26, (or minor variations thereof) was found in three *E. coli* clones. These included the ST648 strain 8e1, (Section 5.4.6.3, Figure 5.6g) and presumably the 8b1 strain which was truncated by being present on a short contig (Figure 5.4). For 8b1, the presumed position of IS26 is indicated in Figure 5.4, according to the position of IS26 on the clonal isolate 8e1 (Figure 5.6g). Context (iv) was also found in strain 9a1, with IS26 flanking both ends of IS26-ISECp1-*bla*$_{CTX-M-15}$-orf477-Tn3-IS26 (Figure 5.4 and Section 5.4.5.3, Figure 5.6c). IS26 was also present in strain 20c4 in the formation -*bla*$_{CTX-M-15}$-orf477-Tn3-IS26 with unknown genes upstream of *bla*$_{CTX-M-15}$ due to being present at a contig boundary (Figure 5.4). More detailed analysis from strain 9a1 allowed identification of the plasmid pERB9a1, with the following genes surrounding *bla*$_{CTX-M}$: qnrS1-IS2-IS26-ISECp1-*bla*$_{CTX-M-15}$-orf477-Tn21-IS26 (Figure 5.6c).

5.4.3.5 Contexts (v) and (vi)

Context (v), -*bla*$_{CTX-M-15}$-orf477-Tn3-IS26, was found in two isolates, 5.3e and 20e4. The context IntI1-ΔTn3 -*bla*$_{CTX-M-15}$-orf477-Tn3-IS26, was found in one isolate, 5.3e, where Tn3 has been truncated by xerD, a tyrosine recombinase (5.4). 20e4 had a slightly different *bla*$_{CTX-M}$ genetic context, ISECp1-*bla*$_{CTX-M-15}$-orf477-Tn3-IS26 (Table 5.4 and Figure 5.4). However, the contig boundary for 20e4 just preceded ISECp1 and so additional upstream genes could not be determined.

Context (vi), ISECp1-*bla*$_{CTX-M-15}$-orf477-Tn3-IS3, was found in a single isolate, 22a1 (Table 5.4 and Figure 5.4).

Table 5.4. *bla*$_{CTX-M-15}$ genetic context types for post travel *E. coli*

Context	Context and schematic representation	Isolates [frequency]	Previous descriptions
i		[11]: 3a1, 3b2, 3b3, 3d1, 6c1, 7b3, 20c3, 16b4, 9c3(ΔISECp1), 8c2, 4d1(ΔTn3)	(Lartigue, Poirel and Nordmann, 2004; Smet *et al.*, 2010)
ii *Group A**		[9]: 3f3, 4a6 (Δorf477), 5a1, 5f2, 5f3, 7b4, 8a1, 15a3, 17a1	(Eckert, Gautier and Arlet, 2006)
iii		[6]: 1a4, 5c1, 5f1, 20a3, 16b1, 12a3 (IS3 absent)	(Akiba *et al.*, 2016)
iv *Group B & N**		[3]: 8b1(t), (see plasmid pERB8e1_f) 9a1, 20c4(t)	(Fortini *et al.*, 2015; Akiba *et al.*, 2016; Jousset *et al.*, 2018)
v		[2]: 5.3e, 20e4 (t)	(Woodford *et al.*, 2009; Forde *et al.*, 2014; Stoesser *et al.*, 2016)
vi		[1]: 22a1	(Johnson *et al.*, 2016)
Other	Contig truncation-*bla*CTX-M-15-orf477 (8g1, 16d1); IS26-*bla*CTX-M-15-orf477-Contig truncation (9d2)		

(t) = sequence truncated by contig boundary. +The second IS26 downstream of Tn3 was not present in the ST349 strain 8b1 or plasmid pERB8e1_f. Plasmid pERB8e1_f (BRIG figure X) shows the full non-truncated sequence.
*Detail in italics describes where the groups match to genetic environments using the A-R designation, as previously described (Dhanji, Murphy, *et al.*, 2011; Amos *et al.*, 2014)

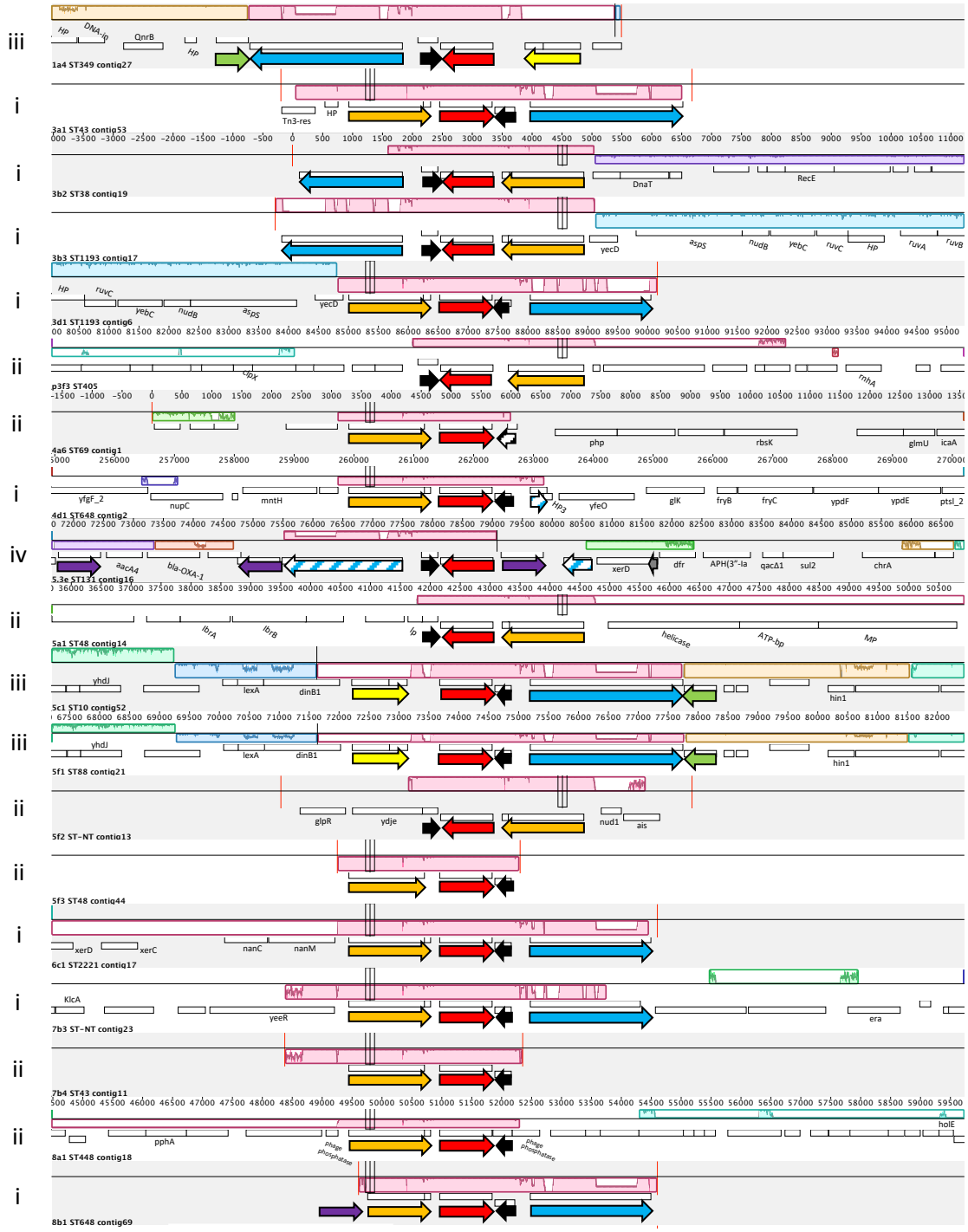


Figure 5.4 Genetic contexts of CTX-M-15-producing post travel *E. coli.*
Coloured arrows represent genetic context elements as in table 5.4. Pastel shaded coloured blocks are Mauve local co-linear blocks. White rectangles are ORFs. White rectangles without annotation are hypothetical proteins according to RAST.

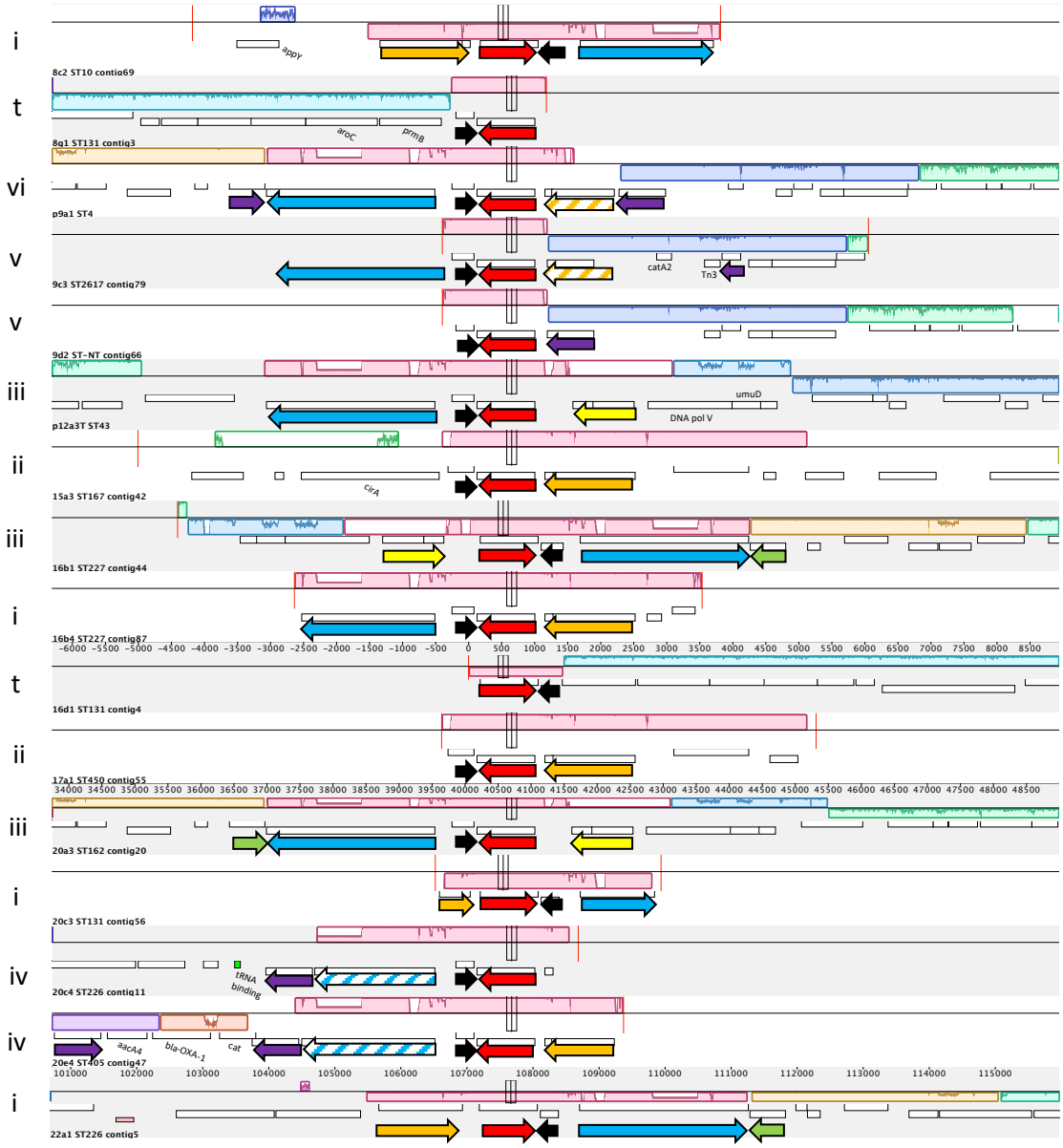


Figure 5.4 (Continued) Genetic contexts of CTX-M-15-producing post travel *E. coli*
Coloured arrows represent genetic context elements as in table 5.4. Pastel shaded coloured blocks are Mauve local co-linear blocks. White rectangles are ORFs. White rectangles without annotation are hypothetical proteins according to RAST.

5.4.4 ISECp1-*bla*$_{CTX-M}$-15 spacer sequence

Twenty-three contigs containing *bla*$_{CTX-M-15}$ contained the ISECp1-*bla*$_{CTX-M-15}$ configuration. In 23/23 cases, the spacer sequence between ISECp1 and *bla*$_{CTX-M-15}$ were indistinguishable. The spacer comprised of 49bp from the end of ISECp1 to the start codon of *bla*$_{CTX-M-15}$ (Figure 5.5). Comparisons were also made between the 23 isolates from the present study, and *bla*$_{CTX-M-15}$-ISECp1 spacer sequences previously deposited in the NCBI genbank database, finding no variation between the spacer sequences already deposited. These included the ISECp1-*bla*$_{CTX-M-15}$ spacer sequences described by Dhanji and colleagues (Dhanji, Patel, *et al.*, 2011), as well as the *bla*$_{CTX-M-15}$-contianing plasmids p2189 (no publication, origin: *E. coli* isolated from human urine in Ghana), pKJNM8C2.1, (no publication: origin: K. pneumoniae isolated from a nasal swab of a preterm human infant, West Bengal, India), and pV234a (Akiba *et al.*, 2016).

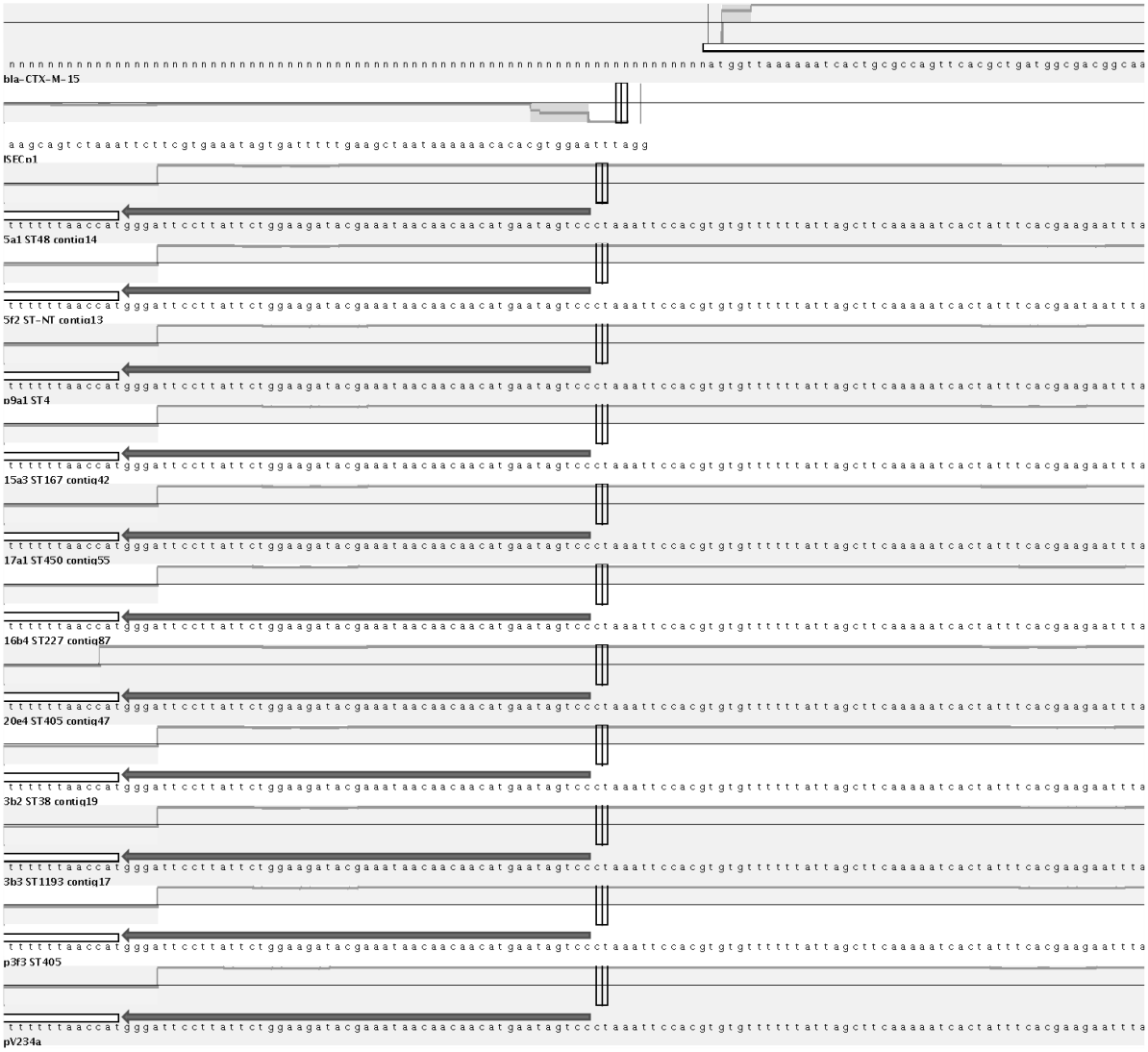


Figure 5.5 Nucleotide sequences of spacer between ISECp1 and *bla*CTX-M-15. (Mauve).
Includes representative isolates from the present study and previously published sequence data deposited in NCBI genbank. This panel is the reverse strand. Isolate or plasmid names are located below each sequence. The arrow represents the spacer sequence. The ISECp1 stop-codon is indicated by the *bla*ck cursor. ISECp1 and *bla*CTX-M-15 sequences are displayed on the first two rows.

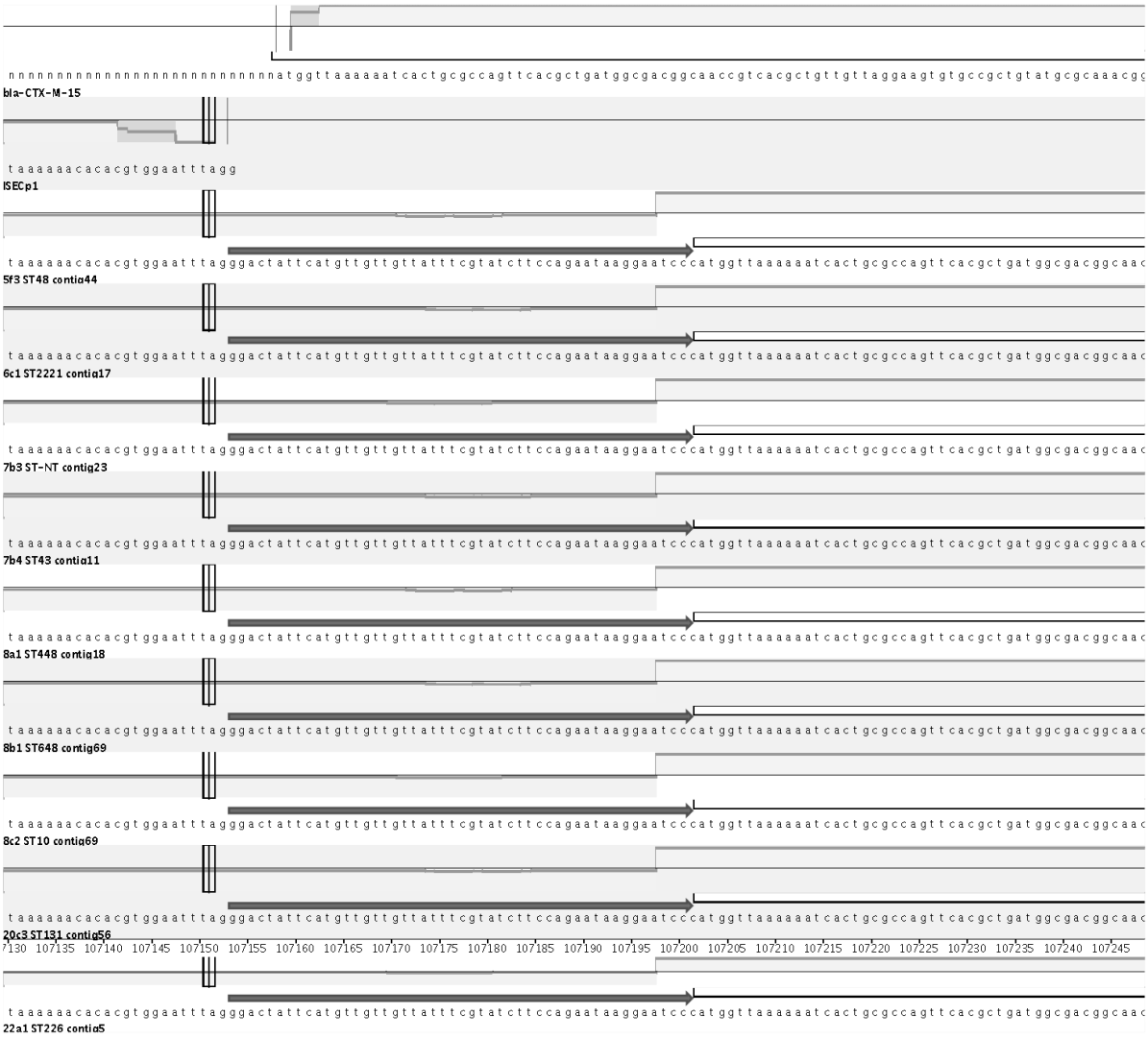


Figure 5.5 (Continued). Nucleotide sequences of spacer between ISECp1 and *bla*$_{CTX-M-15}$. (Mauve).
Includes representative isolates from the present study and previously published sequence data deposited in NCBI genbank. This panel shows the forward strand. Isolate or plasmid names are located below each sequence. The arrow represents the spacer sequence. The ISECp1 stop-codon is indicated by the *bla*ck cursor. ISECp1 and *bla*$_{CTX-M-15}$ sequences are displayed on the first two rows.

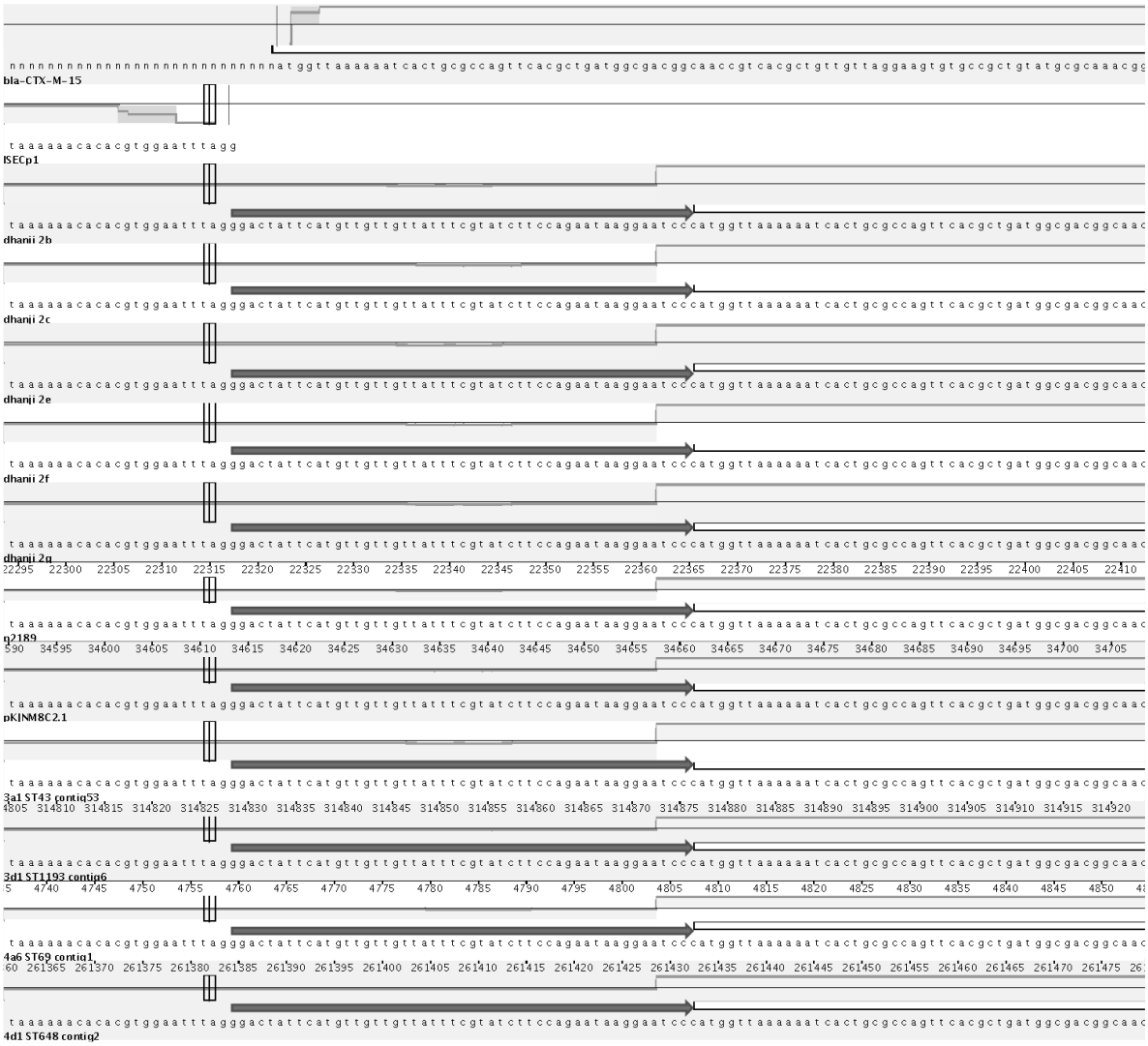


Figure 5.5 (Continued). Nucleotide sequences of spacer between ISECp1 and *bla*$_{CTX-M-15}$. (Mauve).
Includes representative isolates from the present study and previously published sequence data deposited in NCBI genbank. This panel shows the forward strand. Isolate or plasmid names are located below each sequence. The arrow represents the spacer sequence. The ISECp1 stop-codon is indicated by the *bla*ck cursor. ISECp1 and *bla*$_{CTX-M-15}$ sequences are displayed on the first two rows.

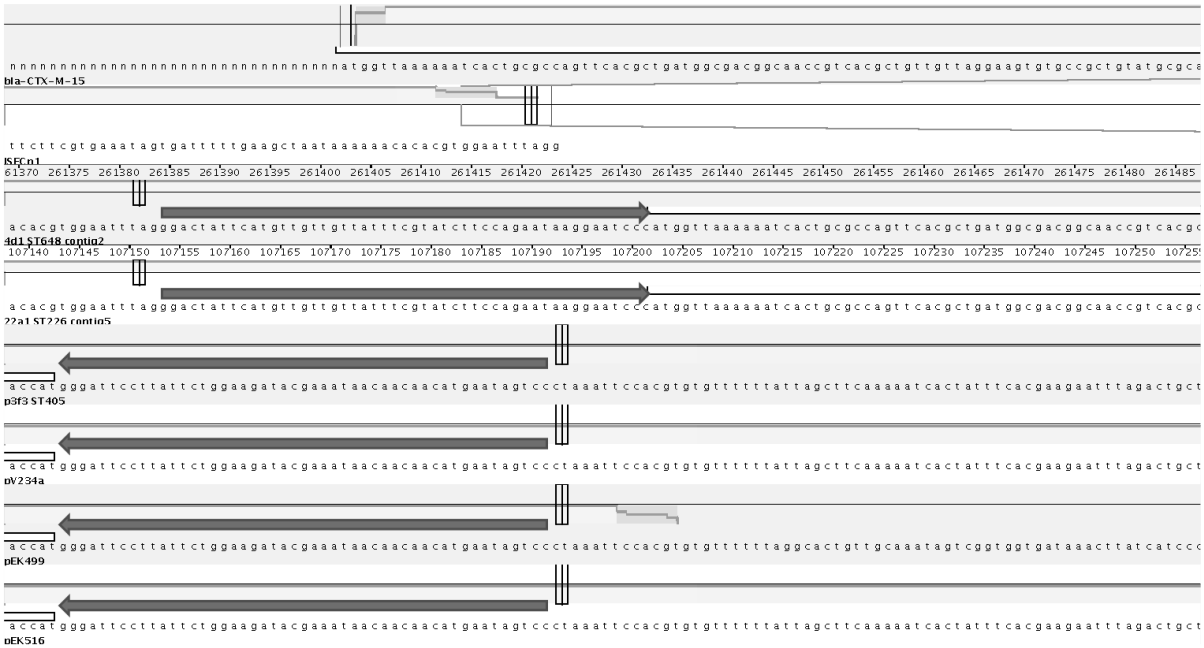


Figure 5.5 (Continued). Nucleotide sequences of spacer between ISECp1 and *bla*$_{CTX\text{-}M\text{-}15}$. (Mauve).
Includes representative isolates from the present study and previously published sequence data deposited in NCBI genbank. This panel shows reverse and forward strands. Isolate or plasmid names are located below each sequence. The arrow represents the spacer sequence. The ISECp1 stop-codon is indicated by the *bla*ck cursor. ISECp1 and *bla*$_{CTX\text{-}M\text{-}15}$ sequences are displayed on the first two rows.

5.4.5 Sequence comparisons of circularised plasmids

Each of the four circularised plasmids (pERB4a2, pERB3f3, pERB9a1, and pERB12a3) were subjected to a *BLA*ST search with results then run through *BLA*ST Ring Image Generator (BRIG) and presented visually (Tables 5.5 a-d, and Figures 5.6 a-d). In addition to the circularised plasmids, CTX-M-containing contigs which were not found to be circular, but were nevertheless confirmed to be plasmid fragments, are displayed in Figures 5.6 e-j with three *BLA*ST comparisons per contig.

The nomenclature for plasmids arising from this study were devised based on the following rules, using pERB3e1(f) as an example. (1) 'pERB' at the start of each plasmid name denoting 'plasmid' (p) followed by the author's initials (ERB); (2) The following numbers and letters correspond to the isolate from which the plasmid originated, therefore for pERB3e1 with '3' representing volunteer 3, 'e' representing the fifth post-travel faecal sample, with the following '2' denoting the second *E. coli* colony pick from the stool culture plate; (3) where followed by '(f)' the plasmid is incomplete and is a non-circularised fragment (CTX-M contig, confirmed to be plasmid in origin).

Table 5.5a. *BLA*ST matches to pERB4a2 (68680bp). Note: volunteer 4 travelled to Sri Lanka.

Plasmid	bp	Organism	Source	Location	CTX-M genotype	Max score	% Query Cover,	% identity	Reference
pHK01	70262	*E. coli*	Human/animal isolates	China	14	72670	100	99	(Ho *et al.*, 2012)
pEG356	70275	*S. sonnei*	Human faeces	Vietnam	24	72670	100	99	(Nhu *et al.*, 2010)
pSJ_82	82288	*E. coli*	Pheasant	China	14	54580	100	99	none
pEC545	70152	*E. coli*	Human	Vietnam	27	46119	97	99	none
pCA08	154789	*E. coli*	Human infection	USA	14	51014	85	99	(J.-J. Li *et al.*, 2015)
pBH100	105801	*E. coli*	Human urine	Brazil	None	73876	83	99	none
pHK23a	73607	*E. coli*	Porcine	China	3	53518	83	99	(Ho *et al.*, 2013)
pTC1	91019	*E. coli*	Porcine ETEC	Hungary	None	73928	81	99	(Fekete *et al.*, 2012)
pVR50	70533	*E. coli*	Human urine	Australia	None	54307	81	99	(Beatson *et al.*, 2015)
pV035b	77687	metagenome	WWTP	India	15*	50639	63	99	(Akiba *et al.*, 2016)

*unpublished data determined with individual BLAST search of NCBI sequence file. NK: not known. WWTP: wastewater treatment plant.

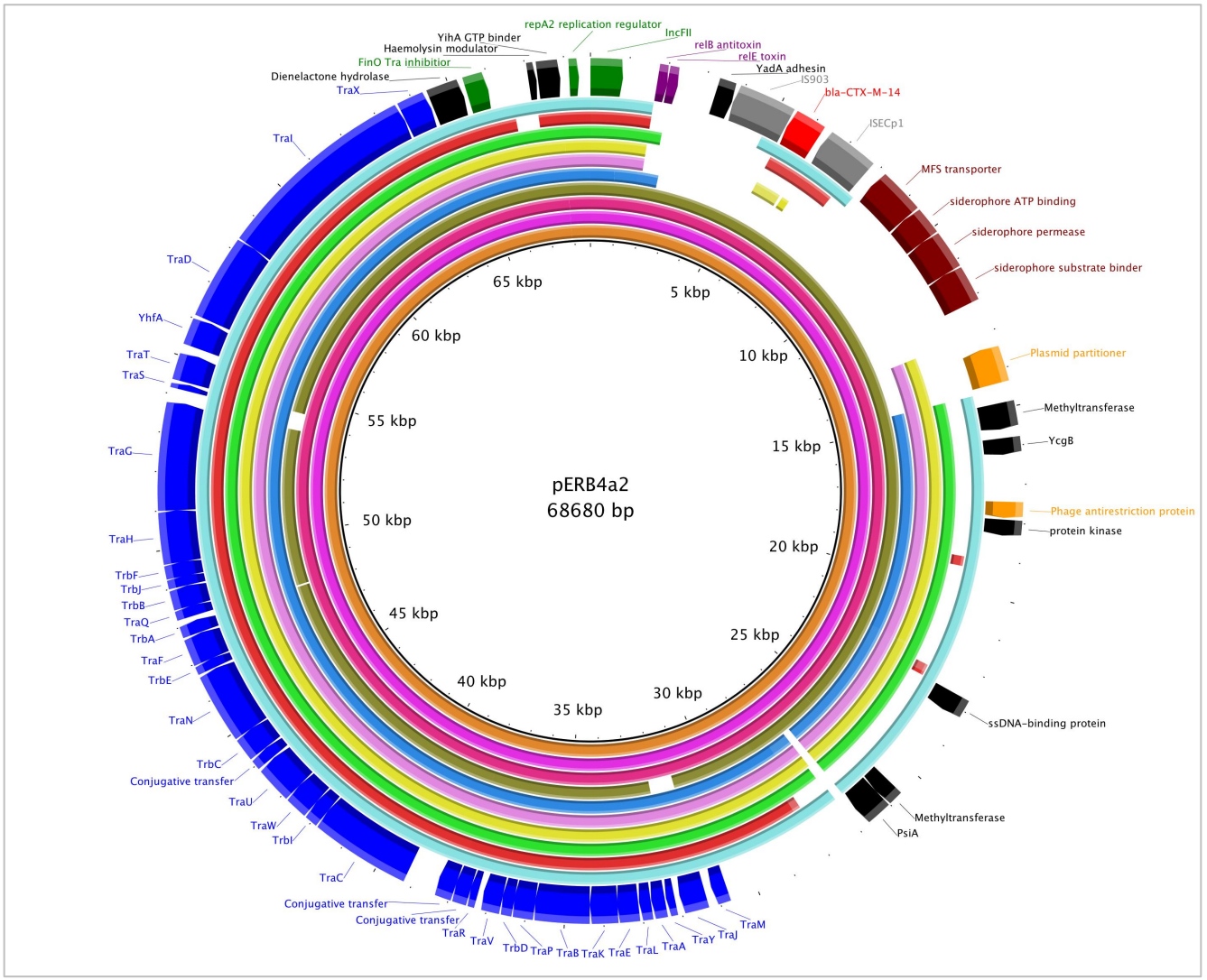


Figure 5.6a. BRIG visualisation of pERB4a2 with *BLAST* matches

Table 5.5b. ***BLA*ST matches to pERB3f3.** (110850bp). Volunteer 3 travelled to India.

Plasmid	bp	Organism	Source	Location	CTX-M genotype	Max Score	% Query Cover	% Identity	Reference
pECAZ162	109057	*E. coli*	Human	USA	None	$1.2x10^5$	95	99	N/A
pECOH89	111741	*E. coli*	Human WS	Germany	15	45740	92	98	(Falgenhauer *et al.*, 2014)
pV234a	112009	metagenome	WWTP	India	15*	68530	91	99	(Akiba *et al.*, 2016)
pAnCo1	112210	*E. coli*	Bov. feedlot	USA	15	68511	91	99	(Colavecchio *et al.*, 2017)
pPSUO78_2	109613	*E. coli*	Chicken Per.	USA	None	51273	89	97	N/A
pM160133_p3	113428	*E. coli*	Human U	USA	None	39449	86	99	(Gilrane *et al.*, 2017)
pMRY16-002_2	108986	*E. coli*	Swine	Japan	None	39449	88	97	(Sekizuka *et al.*, 2017)
pTIC	113109	metagenome	WWTP	Canada	None	45794	83	97	N/A
pSLy3	114472	*E. coli*	Swine	USA	None	38797	83	99	(Meinersmann *et al.*, 2016)
p09EL50	109274	*E. coli*	VTEC stool	Georgia	None	34321	82	98	(Ahmed *et al.*, 2012)

*unpublished data determined with individual *BLA*ST search of NCBI sequence file. WS, wound swab; WWTP, wastewater treatment plant; Bov., bovine; Per., peritonitis; U, urine; VTEC, verotoxigenic *E. coli*.

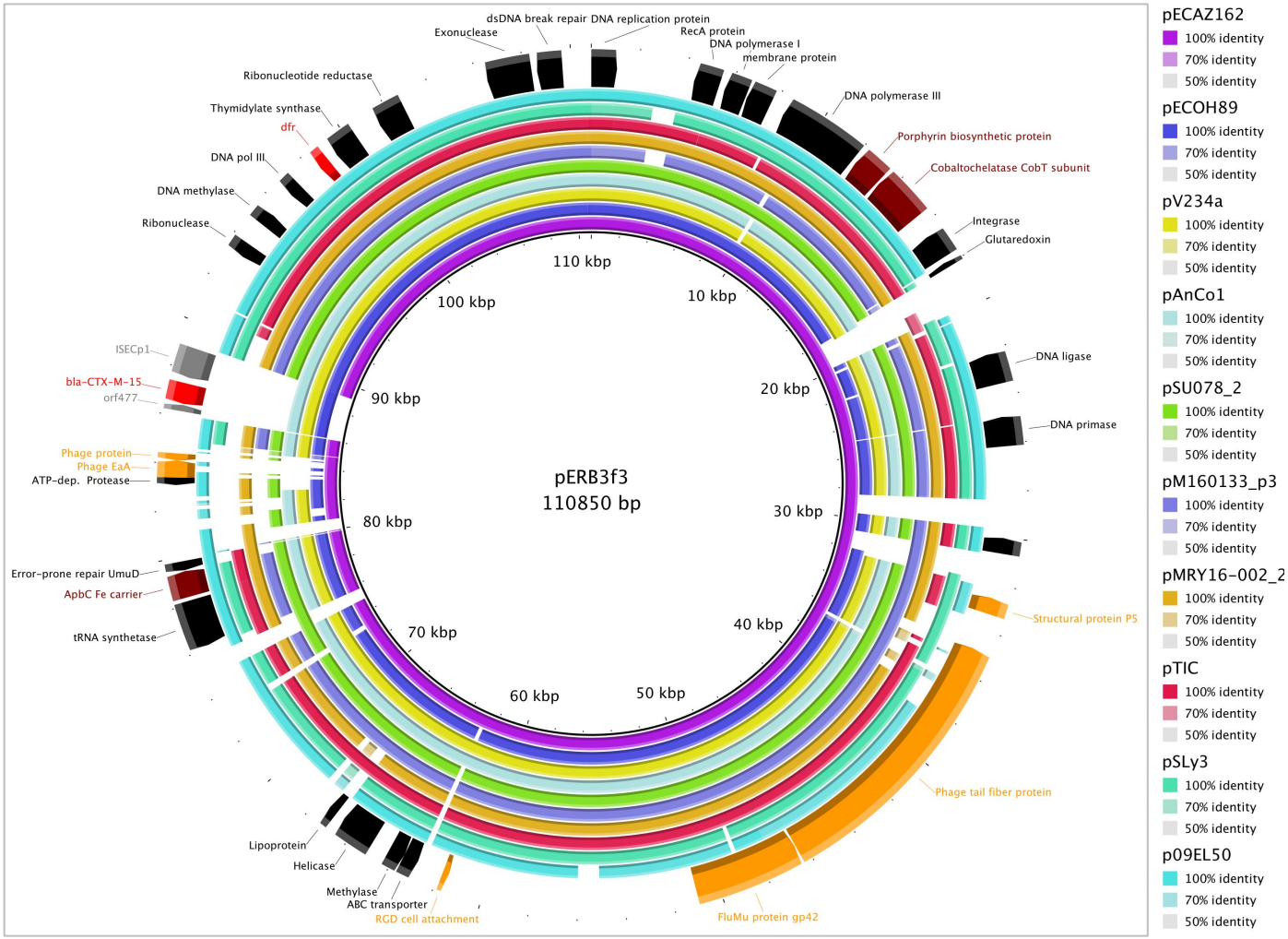


Figure 5.6b. BRIG visualisation of pERB3f3 with *BLAST* matches.

Table 5.5c. *BLA*ST matches to pERB9a1. (80715bp). Volunteer 9 travelled to Sri Lanka.

Plasmid	bp	Species	Source	Location	CTX-M genotype	Max. Score	% Query cover	% Identity	Reference
pC15-1a	92353	*E. coli*	Human	Canada	15	20111	76	99	(Boyd *et al.*, 2004)
pV035-b	77687	*metagenome*	WWTP	India	15*	38978	73	100	(Akiba *et al.*, 2016)
pU25P002	172679	*K. pneumoniae*	Human U	India	15	20094	71	99	(Rafiq, Sam and Vaidyanathan, 2016)
p1002-1	183508	*E. coli*	Human BSI	China	14^{+}	20094	69	99	N/A
pFAM22321	78962	*E. coli*	Dairy cow	Switz.	14	20094	69	99	unpublished
p48896_1	131243	*K. pneumoniae*	Human RS	Pakistan	15	18733	69	98	(Nahid, Zahra and Sandegren, 2017)
pEC743_3	65196	*E. coli*	Human	UAE	none	18733	66	98	unpublished
pTC_N37410PS	66289	*E. coli*	Cattle farm	USA	27	19344	65	98	unpublished
$pEco_{CTX\text{-}M\text{-}15}$	68362	*Shewanella* spp.	Human bile	France	15	37768	26	100	(Jousset *et al.*, 2018)
pPGRT46	83155	*E. coli*	Human FC	Nigeria	15	36766	26	100	(Fortini *et al.*, 2015)

*unpublished data determined with individual *BLA*ST search of NCBI sequence file. WWTP, waste-water treatment plant; U, urine; BSI, bloodstream infection; RS, respiratory secretion; FC, faecal colonisation, Switz., Switzerland.

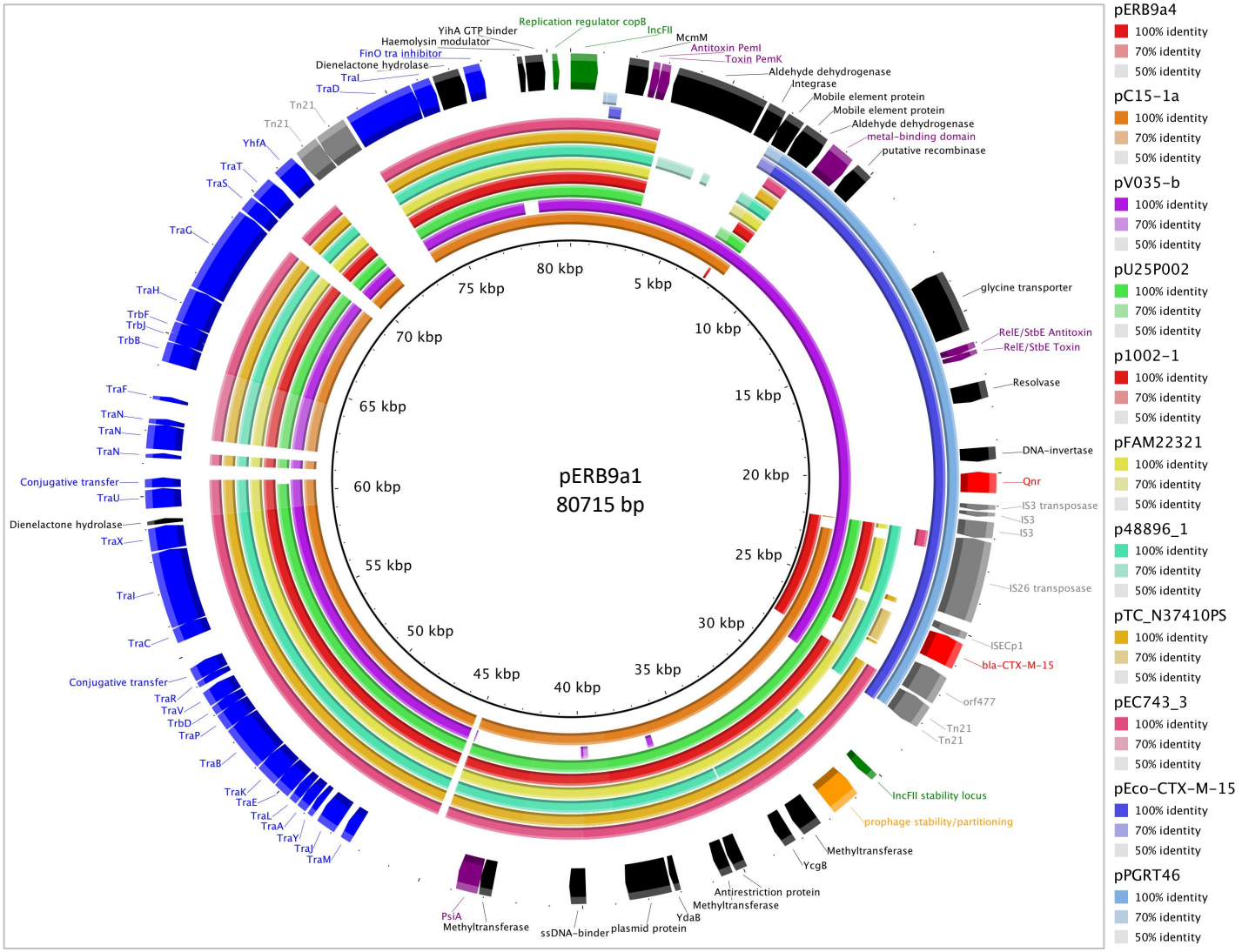


Figure 5.6c. BRIG visualisation of pERB9a1 with *BLAST* matches.

Table 5.5d. *BLAST* matches to pERB12a3 (71111bp). Volunteer 12 travelled to India.

Plasmid	bp	Organism	Source	Location	CTX-M genotype	Max score	% Query cover	% Identity	Reference
pSH4469	91109	S. sonnei	Human faeces	S. Korea	15	72448	99	99	(Kim *et al.*, 2014)
pEK204	93732	*E. coli*	Human clinical	UK	3	72323	99	99	(Woodford *et al.*, 2009)
pEC545_1	96926	*E. coli*	Human	Vietnam	27	70210	99	99	N/A
pKHSB1	94089	S. sonnei	Human faeces	Vietnam	15	67839	99	99	(Holt *et al.*, 2013)
pH1519-88	88678	*E. coli*	Human faeces	Switz.	1	70288	97	98	(Wang *et al.*, 2014)
p2411	91353	*E. coli*	Canine	Switz.	1	68781	97	99	N/A
pKPC-LKEc	145401	*E. coli*	Clinical isolate	Taiwan	3[+]	52259	96	99	(Chen *et al.*, 2014)
pSKLX3330	89672	*E. coli*	Human urine	China	55	72535	96	99	N/A
p2474-3	86725	*E. coli*	Human BSI	China	15[^]	54373	95	99	(Zheng *et al.*, 2016)
pV233_b	97055	metagenome	WWTP	India	none	52381	93	99	(Akiba *et al.*, 2016)

BSI, bloodstream infection; Switz., Switzerland. [+]Also included *bla*-KPC-2. ^Also included MCR-1 and *bla*-NDM-1

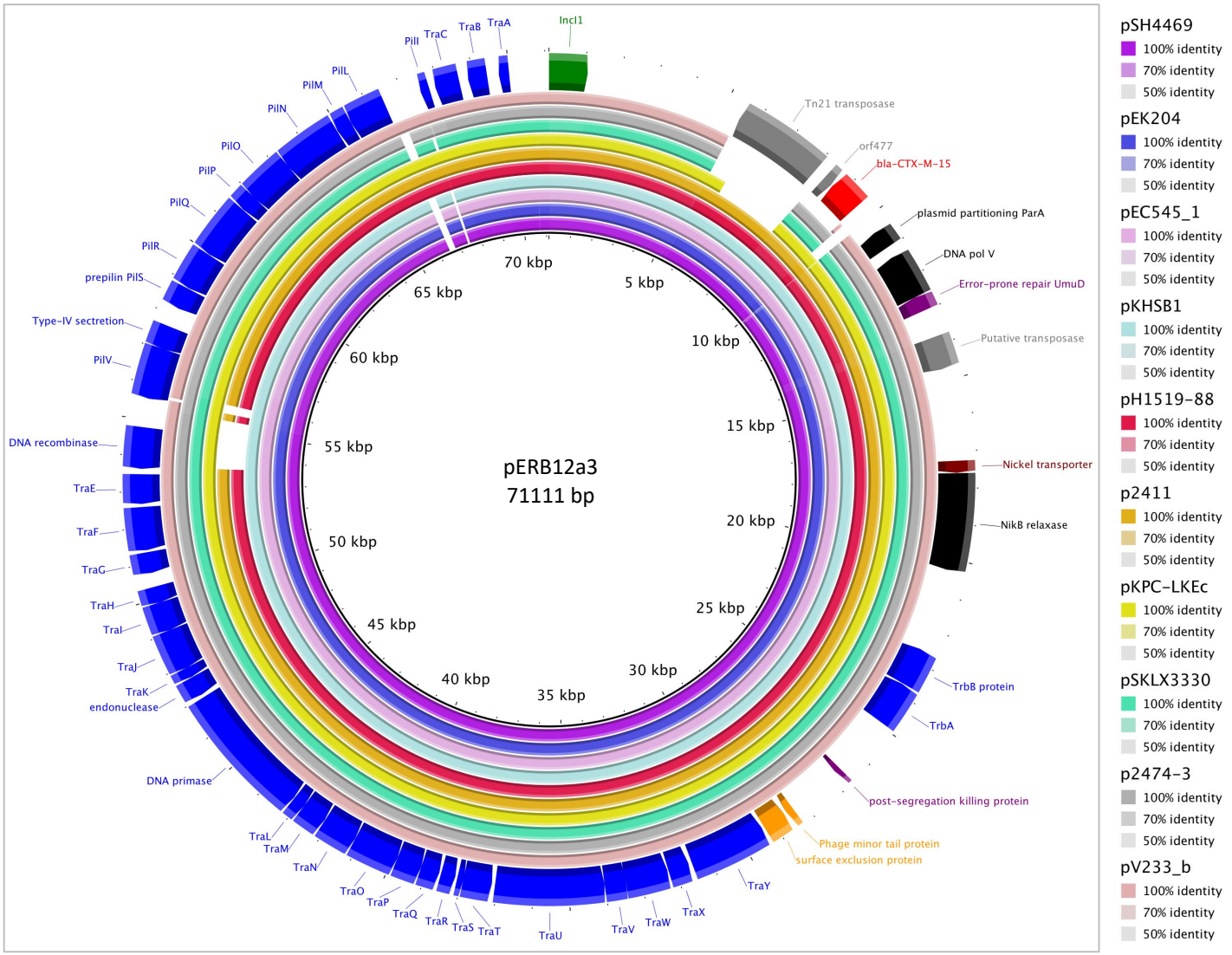


Figure 5.6d. BRIG visualisation of pERB12a3 with *BLAST* matches.

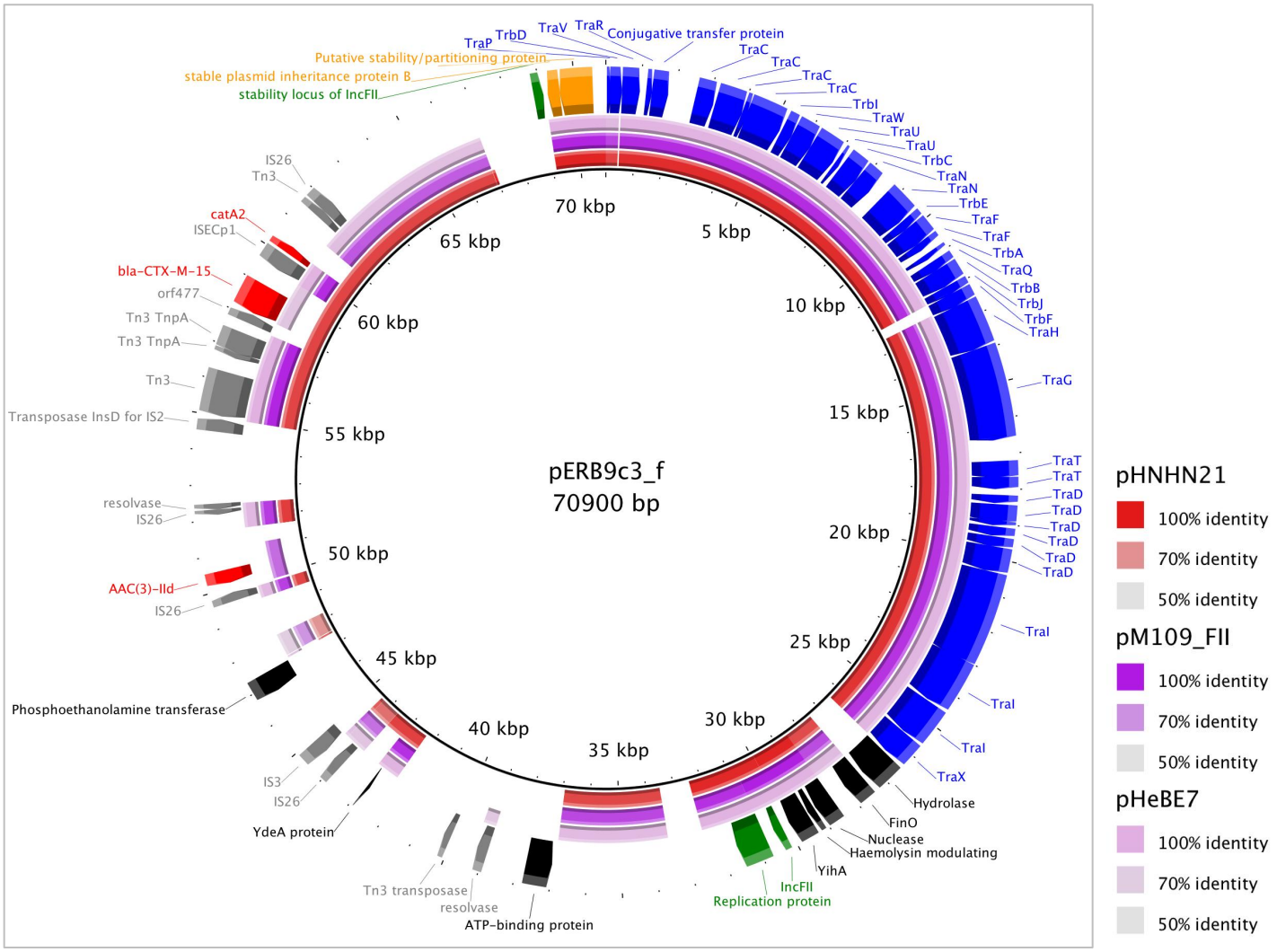


Figure 5.6e. pERB9c3_f with *BLAST* matches.

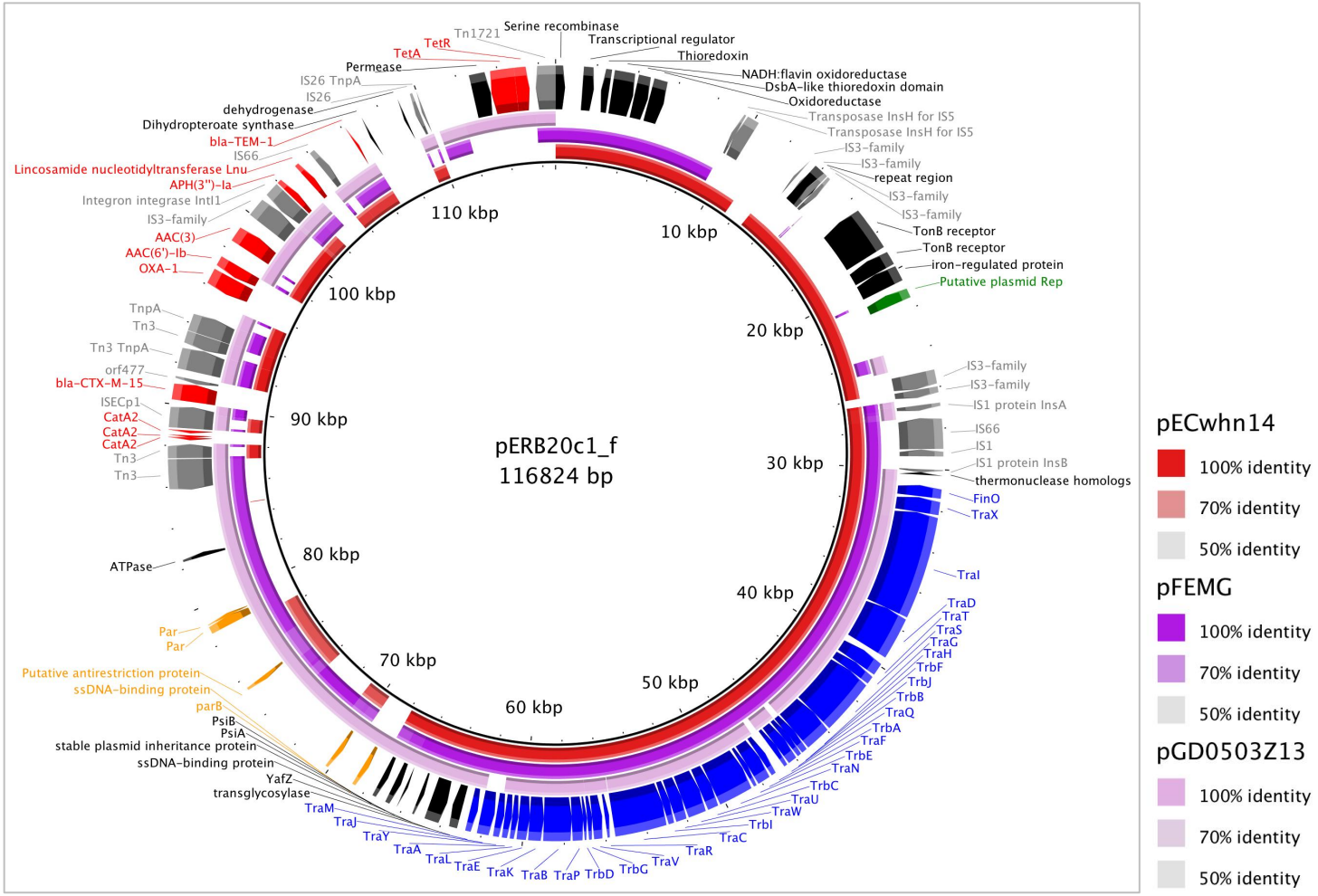


Figure 5.6f. pERB20c1_f with *BLAST* matches.

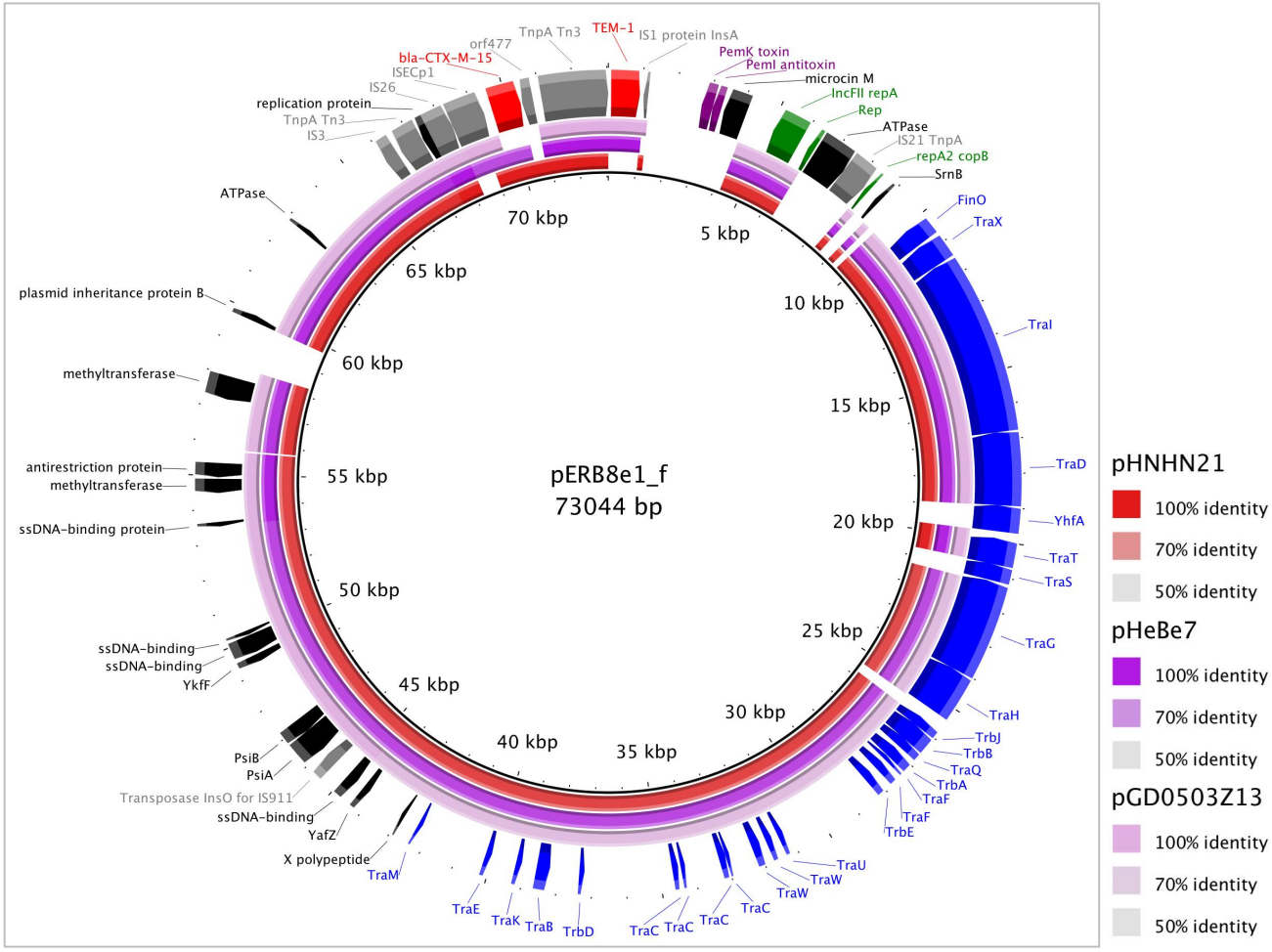


Figure 5.6g pERB8e1_f with *BLAST* matches.

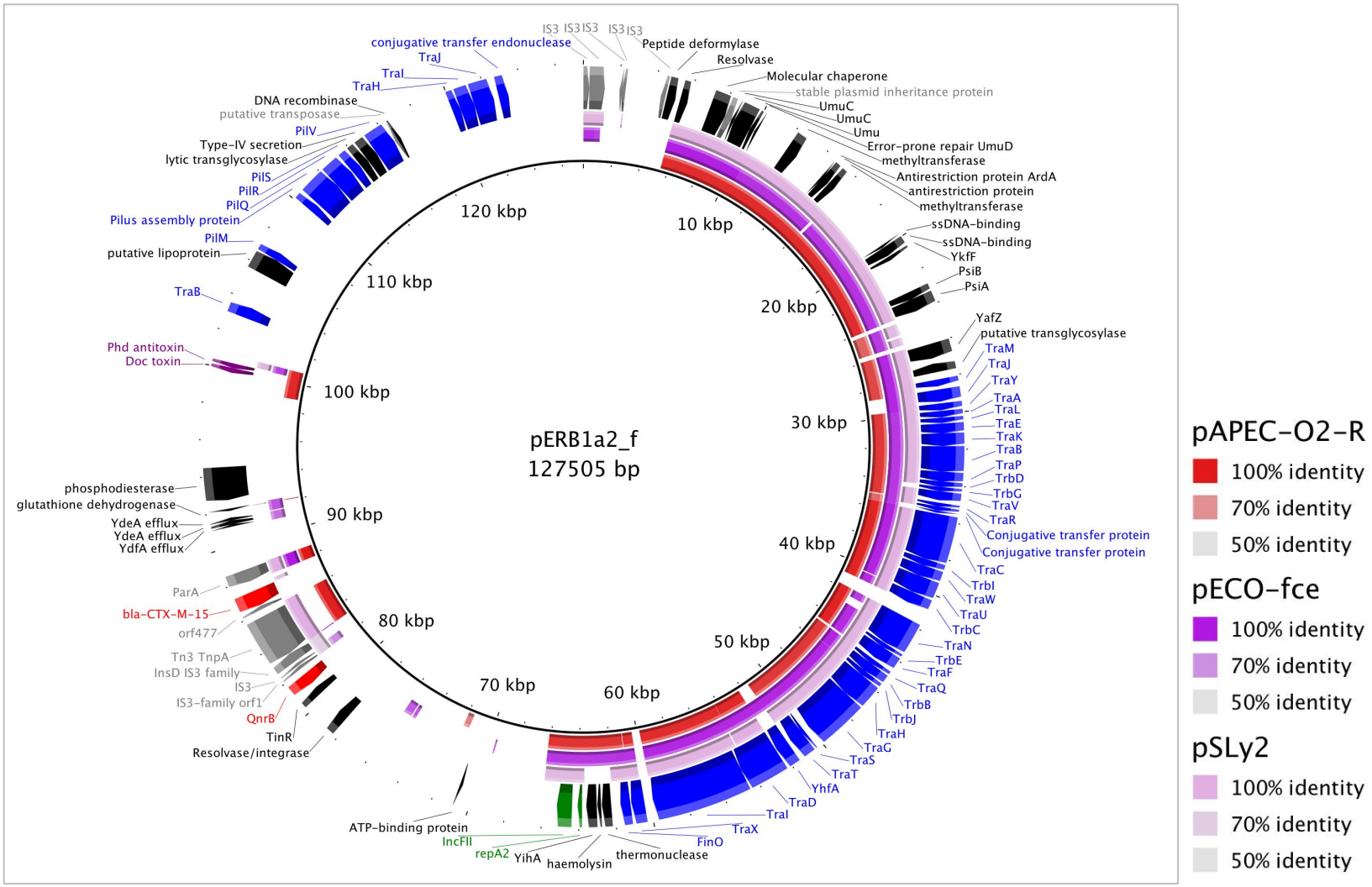


Figure 5.6h. pERB1a2_f with *BLAST* matches.

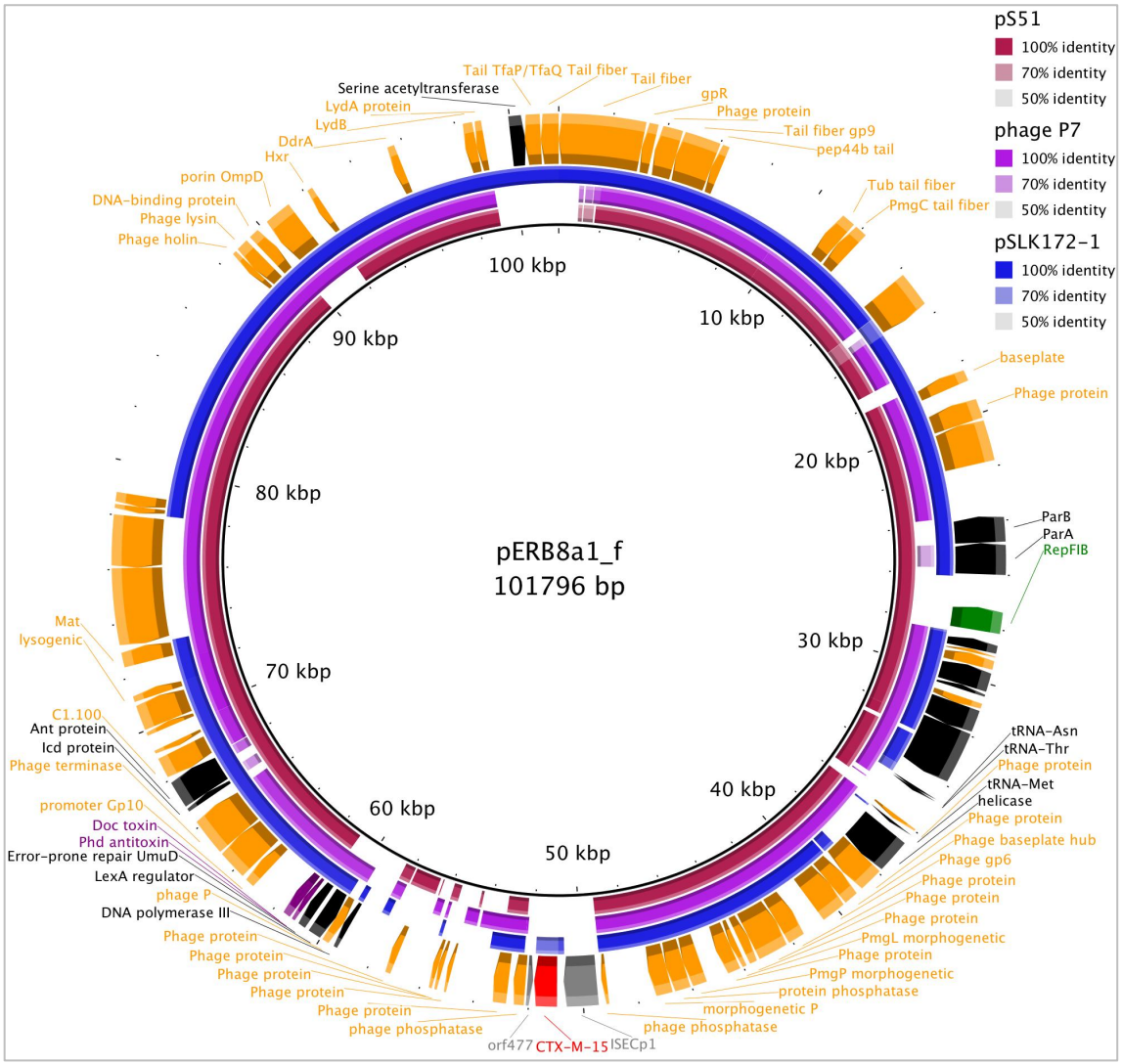


Figure 5.6i. pERB8a1_f with *BLAST* matches.

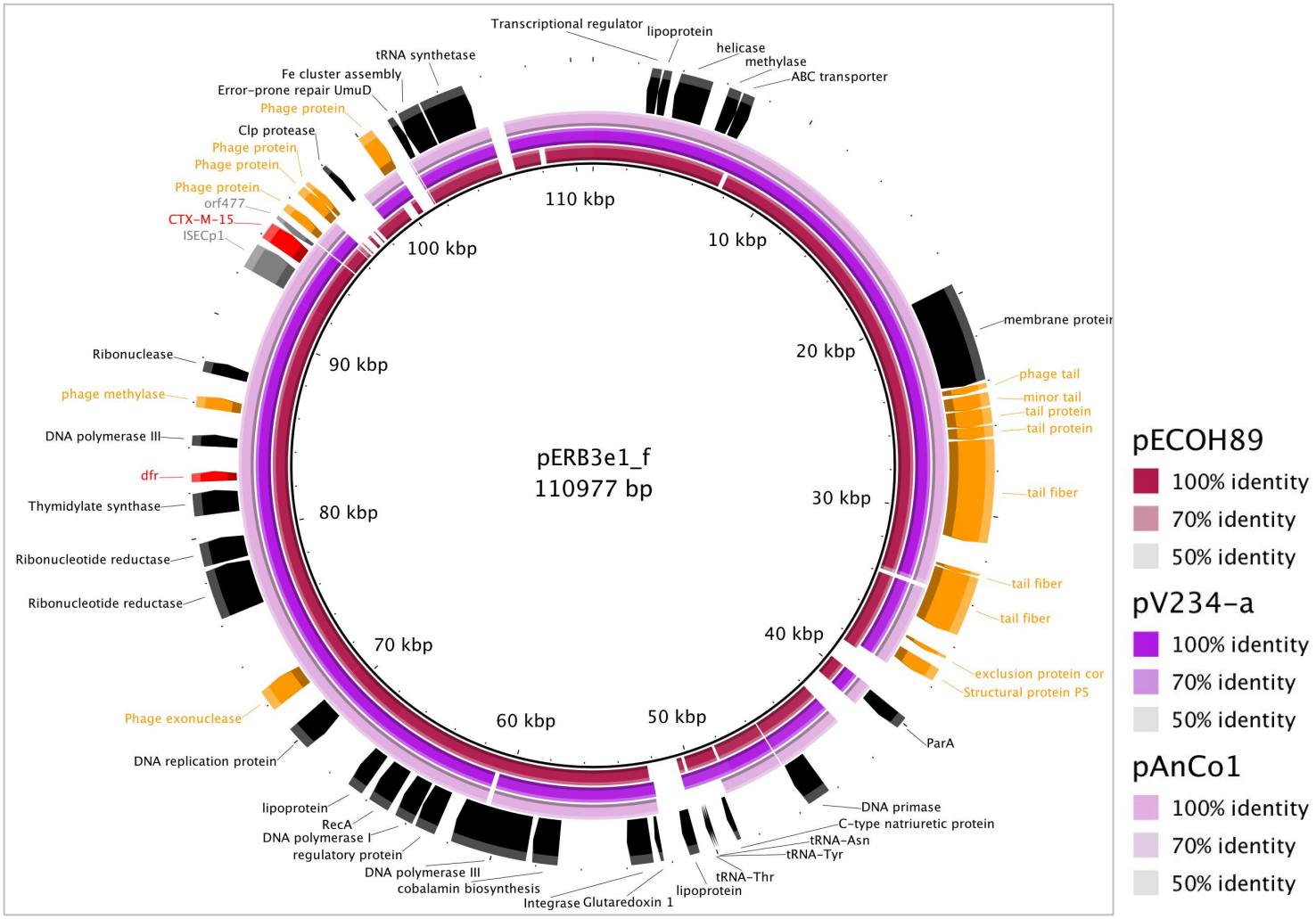


Figure 5.6j. pERB5.3e_f with *BLAST* matches.

5.4.5.1 Sequence comparisons of pERB4a2

Plasmid pERB4a2 was a 68680 bp molecule containing the IncFII replicon, with >40% of the sequence comprised of the transfer (Tra) operon (Figure 5.6a). Notably, pERB4a2 also contained two toxin-antitoxin systems: the relB/relE system, and the PsiA system; and an iron transport operon. The only antibiotic resistance gene present on pERB4a2 was *bla*$_{CTX\text{-}M\text{-}14}$. Detail from *BLA*ST matches are displayed in Table 5.5a.

5.4.5.2 Sequence comparisons of pERB3f3

The 110850 bp plasmid pERB3f3 was not found to contain a plasmid replicon after both RAST annotation and a search against the plasmid-finder database using *BLA*T and is also notable for the absence of a Tra operon and toxin-anti toxin genes. However, pERB3f3 did contain several phage genes including a large phage tail fibre protein (Figure 5.6b). The antimicrobial resistance genes carried by pERB3f3 were *bla*$_{CTX\text{-}M\text{-}15}$ and dihydrofolate reductase (dfr), which encodes trimethoprim resistance. The genetic context of the *bla* consisted of the well described 'international context': ISECp1-*bla*$_{CTX\text{-}M\text{-}15}$-orf477 (Figure 5.6b). *BLA*ST matches to 3f3 are all within a narrow size range (109-114kbp), and all 10 hits had >80% coverage of the query sequence (Table 5.5b).

5.4.5.3 Sequence comparisons of pERB9a1

The IncFII plasmid, pERB9a1 is an 80715 bp circular sequence, which includes a large transfer (Tra) operon (Figure 5.6c). Plasmid pERB9a1 also contains two toxin-antitoxin systems: the PemI/PemK system, and the RelE/StbE system (Figure 5.6c). The antimicrobial resistance genes carried by pERB9a1 are *bla*$_{CTX\text{-}M\text{-}15}$ and the plasmid-mediated quinolone-resistance

gene, qnrS1. *BLA*ST matches of the 80715 bp plasmid, pERB9a1 had relatively low coverage compared to the other circularised plasmids in this study. Only three *BLA*ST hits had query coverage >70%: pC15-1a (76%); pV035-b (73%); and pU25P002 (71%) (Figure 5.6c and Table 5.5c**)**.

Plasmid pERB9a1 has the following genes surrounding *bla*CTX-M: qnrS1-IS2-IS26-ISECp1-*bla*$_{\text{CTX-M-15}}$-orf477-Tn21-IS26. This context is also present in pV035-b from Indian wastewater (73% query cover, 100% identity); pECOCTX-M-15 from human bile *E. coli* (26% query cover, 100% identity); and pPGRT46 rom a healthy human faecal sample (26% query cover, 100% identity. It can also be seen from Figure 5.6c that p9a1 includes a Tn21 transposition unit within the Tra operon, which is not present in any of the *BLA*ST matches.

5.4.5.4 Sequence comparisons of pERB12a3

The plasmid designated pERB12a3 was a 71111 IncI1 plasmid which was predominantly composed of the transfer operon (41 kbp) (Figure 5.6d). The antimicrobial resistance content of pERB12a3 included only *bla*$_{\text{CTX-M-15}}$, within the genetic context: ParA-*bla*$_{\text{CTX-M-15}}$-orf477-Tn21 (Figure 5.4 and Figure 5.6d). Other notable plasmid genes present on pERB12a3 included a nickel transporter gene, and a gene encoding a post-segregation killing protein.

*BLA*ST matches to pERB12a3 included plasmids isolated from human carriage or clinical samples: pSH4469, from South Korea; pEK204 from the UK; pEC545_1 from Vietnam; pKHSB1 from Vietnam; pH1519-88 from Switzerland; pKPC-LKEc from Taiwan; pSKLX3330 from China; and p2474-3 from China (table of hits and BRIG figure pERB12a3). One *BLA*ST

match was pV233_b, isolated from a wastewater treatment plant in India (Figure 5.6d). The query cover for *BLA*ST hits in Figure 5.6d is 93-99%. A lower coverage in 4/10 *BLA*ST matches is due to the lack of Tn21 transposase in pV233-b, p2474-3, pSKLX3330, and pKPC-LKEc.

5.4.6 Analysis of other plasmid-like CTX-M Contigs

Contigs containing $bla_{CTX\text{-}M}$ which were found to be of plasmid origin but could not be circularised were termed 'plasmid-like' CTX-M contigs. The additional notation of '_f' (fragment) after the plasmid name was used where the plasmid could not be circularised.

5.4.6.1 pERB9c3_f

The closest *BLA*ST matches to pEB9c3_f were pHNHN21 (73% query cover; 93% identity), pM109_FII (70% query cover; 93% identity), and pHeBE7 (69% query cover; 93% identity) *BLA*ST matches showed close homology to the plasmid backbone which included the Tra operon and the IncFII replicon, but there were no *BLA*ST hits which covered the variable region which included several mobile genetic elements and antimicrobial resistance genes (Figure 5.6e).

5.4.6.2 pERB20c1_f

The plasmid-like contig pERB20c1_f, which was 116824 bp, contained a plasmid Tra operon and a 30 kbp multi-drug resistance (MDR region) (Figure 5.6f). This MDR region is likely to represent a cassette of antimicrobial resistance genes which was mobilised by one or a combination of mobile genetic elements present within the MDR region. The $bla_{CTX\text{-}M\text{-}15}$ gene is flanked by the Tn3-family transposon at each end in the arrangement: Tn3-catA2-ISECp1-

bla$_{CTX\text{-}M\text{-}15}$-orf477-Tn3, which suggests that the Tn3 transposon was involved in *bla*CTX-M and catA2 co-mobilisation in this case. Other insertion sequences present on pERB20c1_f include: IS3, IS66, IS26, IS5, IS1, and Tn1721. The hallmark of a class I integron, the intI1 gene, which encodes integrase, is also found on pERB20c1_f, in close association with aminoglycoside resistance genes (Figure 5.6f).

The MDR region of pERB20c1_f includes genes encoding resistance to five antibiotic classes: beta lactams including cephalosporins (*bla*$_{CTX\text{-}M\text{-}15}$, *bla*OXA-1, and *bla*TEM-1); chloramphenicol (CatA2); aminoglycosides (AAC(3), AAC(6')-Ib, APH(3')-Ia); lincosamides (lnu); and tetracyclines (TetA and TetR). The closest *BLA*ST plasmid matches to pERB20c1_f did not cover the MDR region of pERB20c1_f continuously (Figure 5.6f). A further *BLA*ST search with the query sequence submitted as the 30 kbp MDR region of pERB20c1_f was therefore carried out, which revealed that no sequences with coverage of above 81% for this MDR region were present in the NCBI *bla*st database.

*BLA*ST plasmid matches to pERB20c1_f were of relatively low query cover (67-73%) compared to other plasmids in this study. Plasmids with the highest coverage of pERB20c1_f were all non-published GenBank submissions and were either isolated from Chinese Poultry (pECwhn14 and pGD0503Z13) or from a Canadian wastewater treatment plant (pFEMG).

5.4.6.3 pERB8e1_f

The 73044 bp contig pERB8e1_f carried the IncFII replicon and two AMR genes: *bla*$_{CTX-M-15}$ and *bla*TEM-1 (Figure 5.6g). The genetic context of *bla*$_{CTX-M}$ is as follows: IS26-ISECp1-*bla*$_{CTX-M-15}$-orf477-Tn3-*bla*TEM-1. The *BLA*ST hits for pERB8e1_f have 67-85% query coverage.

5.4.6.4 pERB1a2_f

The plasmid-like contig pERB1a2_f was 127505 bp, and included a tra operon and an IncFII replicon. The AMR genes present in pERB1a2_f were *bla*$_{CTX-M-15}$ and QnrB. The genetic context of *bla*$_{CTX-M}$ was ParA-*bla*$_{CTX-M-15}$-orf477-Tn3. The highest query cover for the *BLA*ST plasmid matches to pERB1a2_f were collected from *E. coli* strains from the USA: pAPEC-O2-R (48%); pECO-fce (48%); and pSLy2 (47%), isolated from an avian source, a human source, and from a swine source, respectively (Figure 5.6h).

5.4.6.5 pERB8a1_f

The plasmid-like contig pERB8a1_f contained the IncFIB replicon and was found to be largely composed of phage genes (approximately 40 kbp), without any plasmid transfer operon. pERB8a1_f contained only one AMR gene, *bla*$_{CTX-M-15}$, in the following genetic context: ISECp1-*bla*$_{CTX-M-15}$-orf477.

5.4.6.6 pERB5.3e_f

The plasmid-like contig pERB5.3e_f contained an IncFII plasmid replicon, a Tra operon and a 30 kbp MDR region which included AMR genes encoding resistance to seven antimicrobial/antiseptic classes. These were beta lactams/ cephalosporins (*bla*$_{CTX\text{-}M\text{-}15}$ and *bla*OXA-1), tetracyclines (TetA and TetR), trimethoprim (dfr), aminoglycosides (AAC(6')-Ib; AAC(6')-II; and APH(3')-Ia), quaternary ammonium compounds (qacEΔ1), sulphonamides (sul2), and macrolides (mphA). The MDR region also included a chromate transport gene (chrA) (Figure 5.6j).

Mobile genetic elements present within the 30 kbp MDR region included: IS26, IS6100, Tn21, and Tn3. A class I integron was also present, with the IntI1 gene present between Tn21 and dfr (Figure 5.6j). *BLA*ST matches to pERB5.3e_f include the following plasmids isolated from human clinical *E. coli* strains: p46212 (from the UK), pEC958 (from Australia) and pEK499 (from the UK) (Table BRIG). All three plasmid matches have 100% coverage of the MDR region of pERB5.3e_f.

Table 5.6 summarises the key features of plasmids characterised in this study.

Table 5.6. Features of *bla*$_{CTX-M}$-encoding plasmids ('_f' denotes plasmid fragment, non-circularised)

Plasmid	Replicon	Tra operon present?	Phage genes present?	Toxin-antitoxin systems	AMR genes
pERB3f3*	unknown	No	Yes	none	*bla*$_{CTX-M-15}$; dfr
pERB4a2	IncFII	Yes	Yes	relBE	*bla*-CTX-M-14
pERB9a1	IncFII	Yes	Yes	PemI/PemK; relBE	*bla*$_{CTX-M-15}$; qnrS1
pERB12a3	IncI1	Yes	Yes	PndA	*bla*$_{CTX-M-15}$
pERB9c3_f	IncFII	Yes	Yes	none	*bla*$_{CTX-M-15}$; AAC(3)-IId; catA2
pERB20c1_f	unknown	Yes	No	none	*bla*$_{CTX-M-15}$; *bla*-TEM-1; CatA2 (x3); *bla*OXA-1; AAC(6')-Ib; AAC(3); Lnu; tetA/R
pERB8e1_f	IncFII	Yes	No	PemI/PemK	*bla*$_{CTX-M-15}$; *bla*TEM-1
pERB1a2_f	IncFII	Yes	No	Doc/PhD	*bla*$_{CTX-M-15}$; qnrB
pERB8a1_f*	IncFIB	No	Yes	Doc/PhD	*bla*$_{CTX-M-15}$
pERB5.3e_f	IncFII	Yes	No	PemI/PemK; Doc/PhD; VapC/VapB (x2); CcdA/CcdB	*bla*$_{CTX-M-15}$; *bla*OXA-1; mph(A); sul2; QacEΔ1; APH(3')-Ia; AAC(6')-Ib/-II dfr; tetR/A

* 'phage-like' plasmids

5.4.7 Plasmids in post travel *E. coli* strains are conjugative

5.4.7.1 Isolate screening prior to conjugation experiments

Isolates were subjected to a screening process before being put forward into conjugation experiments on filters. This was to ensure that isolates contained the appropriate characteristics to be successful donor strains in the conjugation experiment. The screening process including the number of isolates excluded and reasons for exclusion are outlined in Figure 5.7. A total of 109 isolates were subjected to initial screening, with 45 isolates excluded (Figure 5.7). The reasons for exclusion, with numbers of isolates excluded were: (1) isolates did not contain *bla*$_{\text{CTX-M}}$ (n=8); (2) Isolates rejected due to being non-lactose fermenting *E. coli* (n=33); and (3) Isolates rejected due to being resistant to rifampicin (n=4) (figure 5.7).

A representative example of lactose fermenting *E. coli* (pink colonies) and non-fermenting *E. coli* (yellow colonies), on MacConkey agar is shown in Figure 5.8a-b.

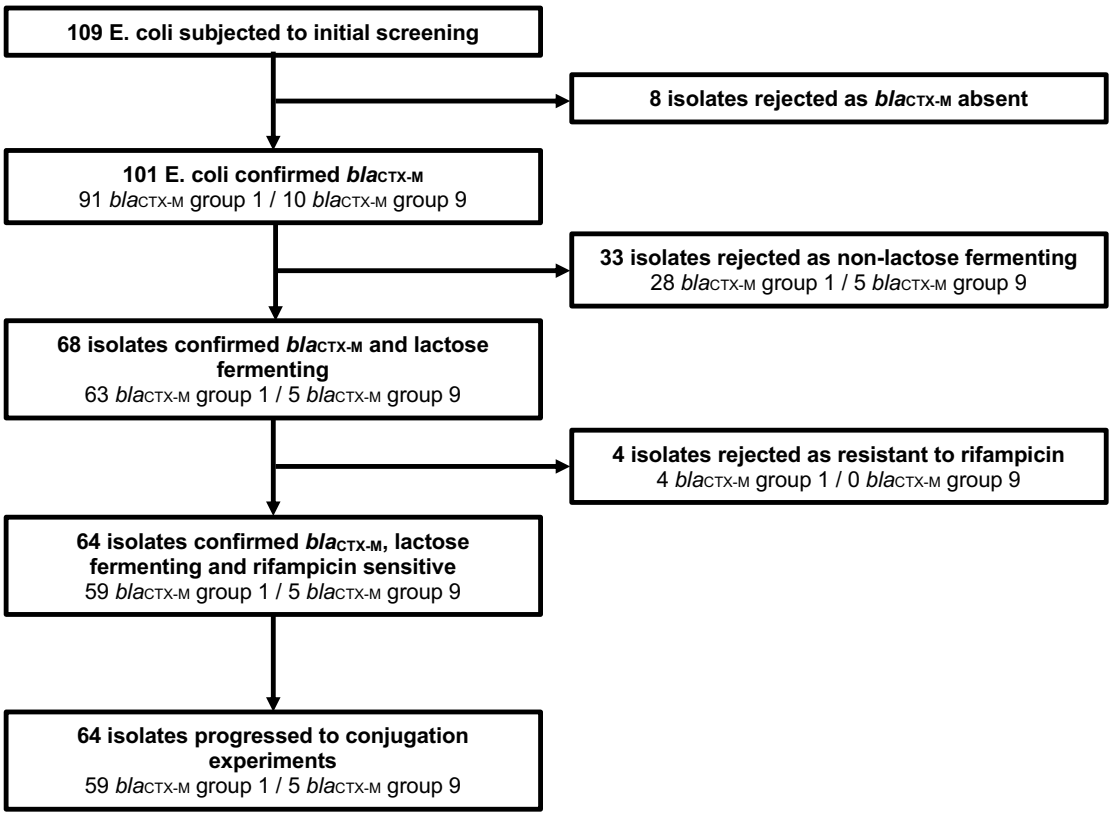


Figure 5.7. Screening process for *E. coli* isolates prior to conjugation experiments.

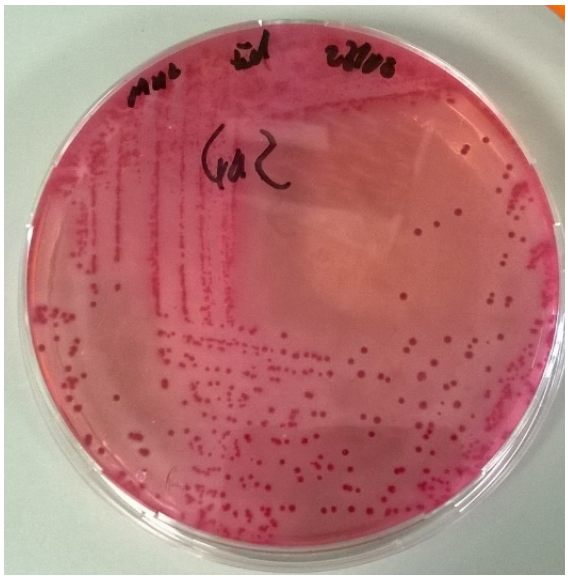


Figure 5.8a. Lactose fermenting E. coli

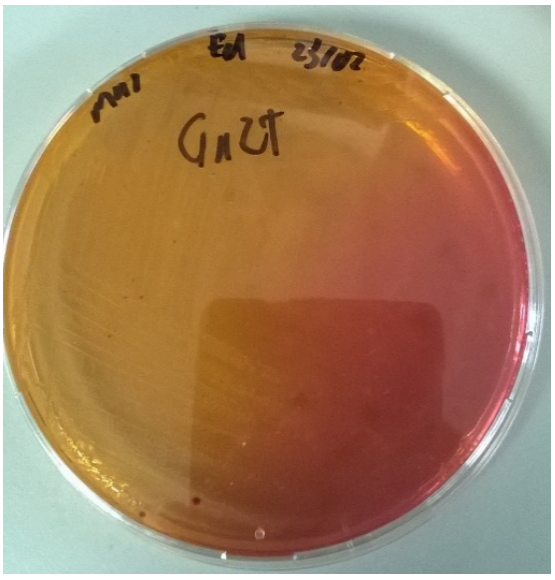


Figure 5.8b. Non-lactose fermenting E. coli

5.4.7.2 Conjugation experiments

Conjugation experiments were undertaken to investigate the transferability of plasmids carrying *bla*-$_{CTX-M}$. We found 45% of volunteers (5/11 volunteer *E. coli* tested) carried CTX-M-EC which could transfer the *bla*-$_{CTX-M}$ plasmid after overnight conjugation on filters (Table 5.7). Seven donor isolates (7/24= 29%) with unique STs had plasmids carrying *bla*$_{CTX-M}$ capable of in-vitro conjugation, under the specific conditions used in this study, with no particular ST predominating (Table 5.7). In each case where the phenotype corresponding to a transconjugant was found — non-lactose fermenting *E. coli* growing on agar supplemented with cefotaxime and rifampicin — PCR was used to confirm the presence of *bla*-$_{CTX-M}$ (Table 5.7).

Further analysis of the 19 *E. coli* STs which were not capable of *bla*$_{CTX-M}$ conjugative transfer was undertaken to determine the reason for conjugation failure. In 7/19 cases, the *bla*$_{CTX-M}$ was found to be on the chromosome, rather than on a plasmid. In 4/19 cases, *bla*$_{CTX-M}$ was located on a plasmid which lacked a transfer operon. For 1/19 STs (ST4 isolate: 9a1 and 9a3, both ST4, volunteer 19), the plasmid transfer operon was disrupted with a Tn21 transposon (Figure 5.6c and Table 5.7), which may explain the lack of conjugation for this ST. However, it is notable that isolate 9a4 (also ST4), was a successful donor of *bla*$_{CTX-M}$ by conjugation. Unfortunately, a comparison between the plasmids carried by the conjugator 9a4, and the non-conjugators 9a1 and 9a3, was not possible. This was because the 9a4 plasmid could not be fully resolved due to *bla*$_{CTX-M}$ being present on a small contig (5600 bp), and failure of growth on culture plates prior to the intended WGS of the transconjugant. Similarly, in 3/19 cases, isolates failed to conjugate when different clonal isolates of the same ST had already

successfully transferred the *bla*$_{CTX-M}$ plasmid (isolates 12a3, 4d3, and 20c4). A ST2617 isolate, (9c3), also failed as a conjugation donor of *bla*$_{CTX-M}$, despite having an intact transfer operon. In 3/19 cases, the reason for non-conjugation could not be assessed, because *bla*$_{CTX-M}$ was located on a contig <10 kbp in length (Table 5.7).

Table 5.7. Conjugation Experiment

Isolate	MLST	*bla*$_{CTX-M}$ group	Growth on experimental plates			Growth on control plates			Lactose fermentation post-conjugation	*bla*$_{CTX-M}$ containing non-lactose fermenter post conjugation	Reason for *bla*ctx-m non-conjugation
			10X	100X	1000X	10X	100X	1000X			
3a1	43	1	✓	×	×	×	×	×	Fermenter		NK: contig <10kbp
3a2	43	1	✓	✓	×	×	×	×	Fermenter		NK: contig <10kbp
3b2	38	1	✓	×	×	✓	×	×	Fermenter		chromosomal
4a2	2732	9	✓	×	×	×	×	×	Non-Fermenter	✓	
4d1	648	1	✓	×	×	✓	×	×	Fermenter		chromosomal
4d2	648	1	✓	✓	×	×	×	×	Fermenter		chromosomal
4d3	648	1	✓	×	×	×	×	×	Non-Fermenter	✓	
4d4	648	1	✓	×	×	×	×	×	Fermenter		chromosomal
4e1	69	1	✓	×	×	✓	×	×	Fermenter		chromosomal
5a1	48	1	✓	×	×	×	×	×	Fermenter		chromosomal
5b1	48	1	✓	✓	×	×	×	×	Fermenter		chromosomal
5c3	10	1	✓	×	×	×	×	×	Fermenter		Plasmid fragment: 28kbp
5e3	38	1	✓	✓	×	×	×	×	Fermenter		Chromosomal*
5e4	38	1	✓	×	×	×	×	×	Fermenter		Chromosomal*
7a1	10	1	×	×	×	×	×	×	Fermenter		*bla*$_{CTX-M}$ lost from strain
7b1	10	1	×	×	×	×	×	×	Fermenter		*bla*$_{CTX-M}$ lost from strain
7c1	200	1	✓	×	×	×	×	×	Fermenter		*bla*$_{CTX-M}$ lost from strain
8a1	448	1	✓	✓	×	✓	×	×	Fermenter		Plasmid: no tra
8b1	648	1	✓	×	×	×	×	×	Fermenter		Plasmid: no tra
8g3	38	1	✓	×	×	×	×	×	Non-Fermenter	✓	
9a1	4	1	✓	×	×	×	×	×	Fermenter		Plasmid: tra disrupted
9a3	4	1	✓	×	×	×	×	×	Non-Fermenter	×	Plasmid: tra disrupted
9a4	4	1	✓	×	×	✓	×	×	Non-Fermenter	✓	
9c3	2617	1	✓	✓	×	×	×	×	Fermenter		NK, plasmid with tra
12a2	43	1	✓	✓	✓	×	×	×	Fermenter		Unknown
12a3	43	1	✓	✓	✓	×	×	×	Non-Fermenter	✓	
12a4	43	1	✓	✓	✓	×	×	×	Fermenter		Unknown
12b1	43	1	✓	✓	×	×	×	×	Non-Fermenter	✓	
12c1	43	1	✓	✓	×	✓	×	×	Fermenter	✓	
12c2	43	1	✓	✓	✓	✓	×	×	Fermenter		Unknown
12c3	43	1	✓	×	×	✓	×	×	Fermenter		Unknown

NK, not known. Tra, transfer operon.

Table 5.7. Conjugation Experiment

Isolate	MLST	*bla*$_{CTX-M}$ group	Growth on experimental plates			Growth on control plates			Lactose fermentation post-conjugation	Growth on cef/rif macconkey's agar post-conjugation	Reason for *bla*$_{CTX-M}$ non-conjugation
			10X	100X	1000X	10X	100X	1000X			
12d1	43	1	✓	✓	✓	✓	✓	×	Non-Fermenter	✓	
12d2	43	1	✓	✓	✓	×	×	×	Non-Fermenter	✓	
12d3	43	1	✓	✓	✓	×	✓	×	Fermenter		Unknown
12d4	43	1	✓	✓	✓	×	×	×	Fermenter		Unknown
12e1	43	1	✓	✓	×	✓	×	×	Non-Fermenter	✓	
12e2	43	1	✓	✓	✓	✓	×	×	Non-Fermenter	✓	
12e3	43	1	✓	✓	×	×	×	×	Non-Fermenter	✓	
12e4	43	1	✓	✓	✓	×	×	×	Fermenter		Unknown
15a1	167	1	✓	✓	×	×	×	×	Fermenter		Plasmid: no tra
15a2	167	1	✓	×	×	×	×	×	Fermenter		Plasmid: no tra
15a3	167	1	✓	×	×	✓	×	×	Non-Ferment.	×	Plasmid: no tra
15a4	167	1	✓	×	×	×	×	×	Fermenter		Plasmid: no tra
17a1	450	1	✓	×	×	×	×	×	Non-Ferment.	×	chromosomal
17a2	450	1	✓	×	×	×	×	×	Fermenter		chromosomal
17a3	450	1	✓	×	×	×	×	×	Fermenter		chromosomal
17a4	450	1	✓	×	×	✓	×	×	Fermenter		chromosomal
19a1	43	9	✓	×	×	×	×	×	Non-Ferment.	×	NK: contig <10kbp
19a2	43	9	✓	×	×	×	×	×	Fermenter		NK: contig <10kbp
19a3	43	9	✓	✓	×	✓	×	×	Fermenter		NK: contig <10kbp
19a4	43	9	✓	×	×	×	×	×	Fermenter		NK: contig <10kbp
20a1	NT	1	✓	✓	✓	×	×	×	Non-Fermenter	✓	
20a2	NT	1	✓	×	×	×	×	×	Non-Fermenter	✓	
20a3	162	1	✓	✓	×	✓	×	×	Fermenter		Plasmid: no tra
20a4	162	1	✓	✓	×	✓	×	×	Fermenter		Plasmid: no tra
20b1	162	1	✓	✓	✓	×	×	×	Fermenter		Plasmid: no tra
20b2	162	1	✓	✓	×	✓	×	×	Fermenter		Plasmid: no tra
20b3	162	1	✓	×	×	×	×	×	Fermenter		Plasmid: no tra
20b4	162	1	✓	✓	×	×	×	×	Fermenter		Plasmid: no tra
20c2	226	1	✓	×	×	×	×	×	Non-Ferment.	×	NK
20c4	226	1	✓	×	×	×	×	×	Non-Fermenter	✓	
20e3	405	1	✓	×	×	×	×	×	Fermenter		Chromosome
20e4	405	1	✓	×	×	×	×	×	Fermenter		Chromosome

NK, not known. Tra, transfer operon.

5.4.7.3 Confirmation of plasmid transfer using pulsed field gel electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) was used to visualise the plasmid transferred in conjugation experiments. The transferred plasmid for nine isolates is displayed in Figure 5.9a and Figure 5.9b. For each isolate tested in conjugation experiments, the donor plasmid and the corresponding transconjugant (denoted with 'T' after isolate name) are displayed, showing that a matching plasmid has been transferred in each case (Figure 5.9a and Figure 5.9b**).**

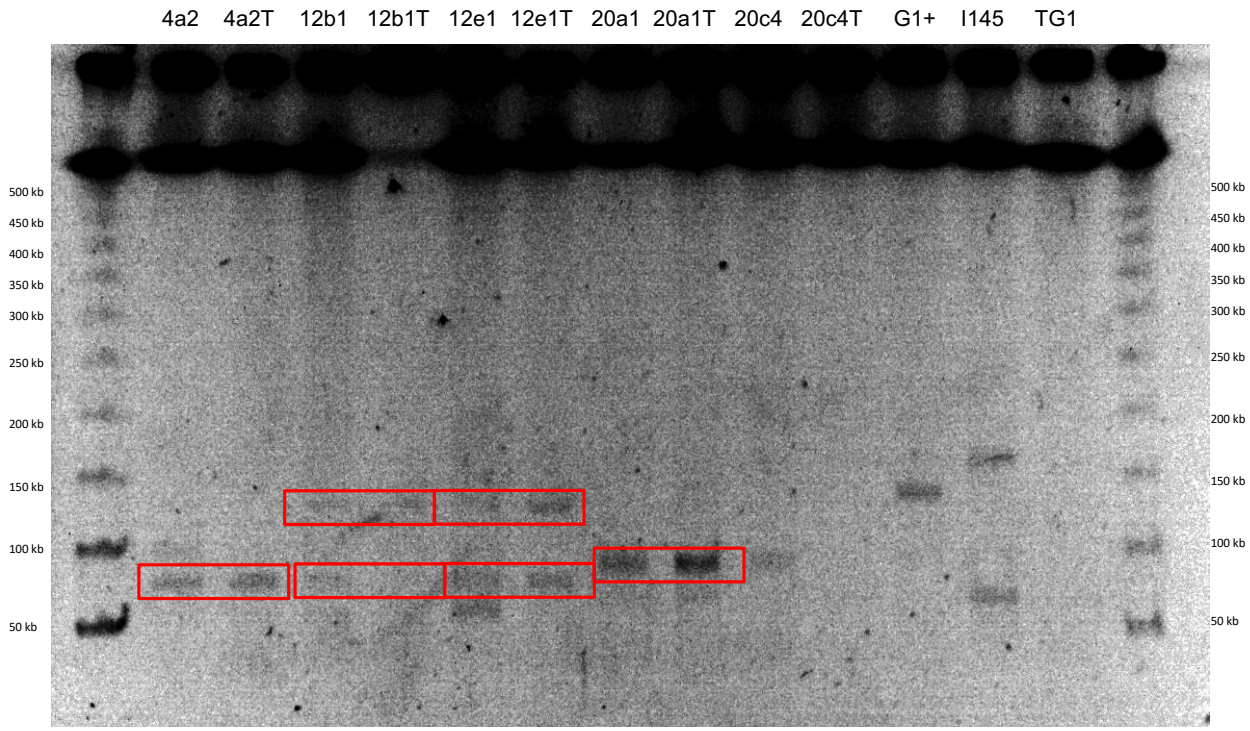


Figure 5.9a. Confirmation of plasmid transfer using PFGE gel electrophoresis. Representative donor isolates and transconjugant isolates ('T' designation) are paired in boxes. G1+ , group 1 CTX-M positive control containing pEK499. I145, *E. coli* K12 containing marker plasmids. TG1, plasmid-free recipient strain. Lambda phage sizing ladder is present at each end of the gel. Note: no visible plasmid band for 20c4T.

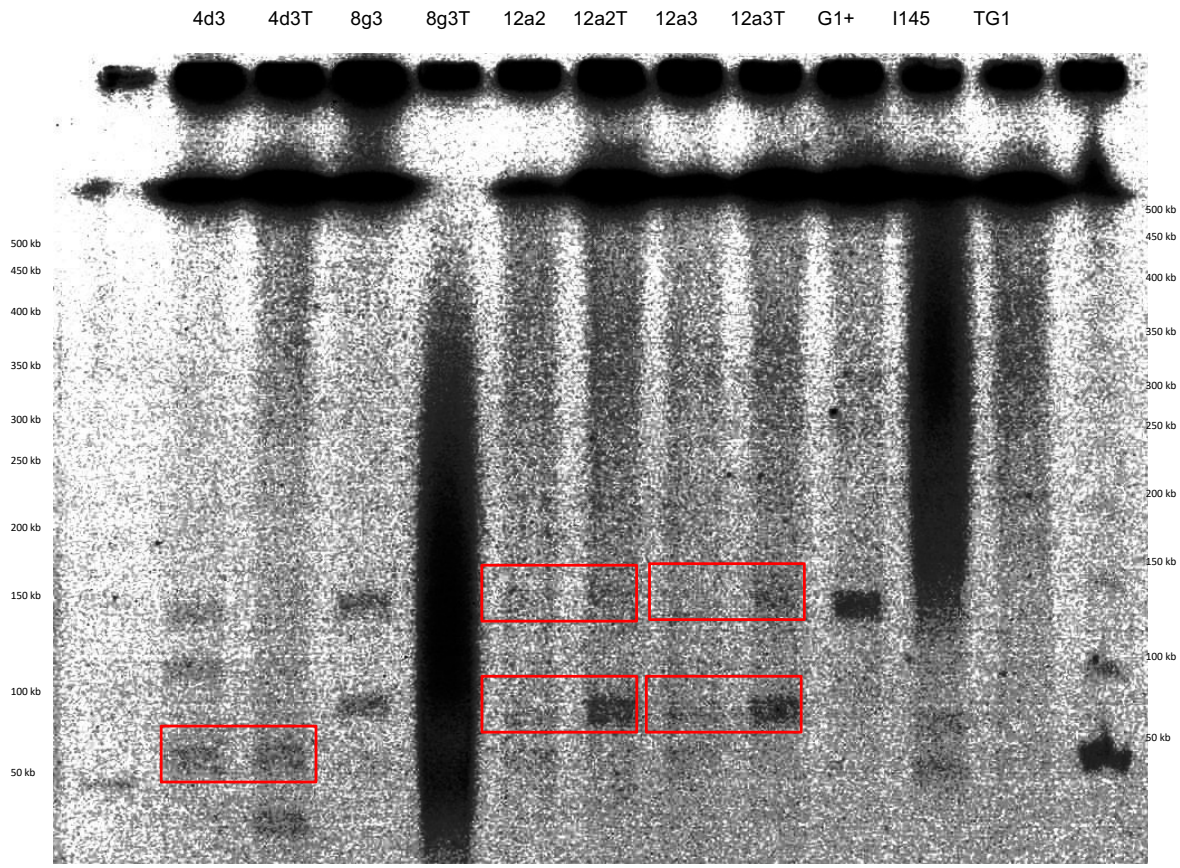


Figure 5.9b. Confirmation of plasmid transfer using PFGE gel electrophoresis. Representative donor isolates and transconjugant isolates ('T' designation) are paired in red boxes. G1+ , group 1 CTX-M positive control containing pEK499. I145, *E. coli* K12 containing marker plasmids. TG1, plasmid-free recipient strain. Lambda phage sizing ladder is present at each end of the gel. 8g3T and I145 produced a chromosomal smear, therefore plasmid bands not visible.

5.4.7.4 Minimum inhibitory concentration testing of donor and recipient isolates

Determination of minimum inhibitory concentration (MIC) of donor, recipient, and transconjugants, using the VITEK2 platform, was used to confirm the phenotypic changes which would be expected after a successful conjugation of a *bla*$_{CTX-M}$-bearing plasmid into the recipient TG1 (Table 5.8).

All transconjugants had an antibiogram consistent with cephalosporinase production. Particular phenotypes corresponded with genotypic findings after isolate screening with WGS and resistance-finder. For example, the CTX-M-14-producing transconjugant 4a2T showed high level resistance to cefotaxime, but not to ceftazidime as is typical for the CTX-M-14 enzyme. As expected, the CTX-M-15 producers had a higher-level resistance to ceftazidime (Table 5.8).

The antibiogram of 20a1 and 20a2 included high level resistance to cefoxitin, which is a hallmark of AmpC production, differentiating them from CTX-M producers (Wu *et al.*, 2005). This finding corresponded with a lack of *bla*$_{CTX-M}$ genes in these transconjugants. Instead, a plasmid carrying *bla*$_{DHA-1}$ was transferred. Isolates 20a1 and 20a2 also had resistance to nalidixic acid and trimethoprim, suggesting the presence of an MDR plasmid in these isolates. Characterisation of the specific plasmid in question was not possible due to a small contig size for 20a1 and 20a2. However, resistance gene finder was applied to the illumina sequence data which showed presence of *tetA*, *mphA*, *sul1*, *bla*$_{DHA-1}$, *QnrB4*, *aadA1/A5*, and *dfrA1*. The standard gram-negative VITEK2 antibiogram does not include testing for tetracyclines, macrolides, sulphonamides, or streptomycin, therefore these were not tested.

However, given the presence of the aforementioned resistance genes in 20a1 and 20a2, these isolates would be expected to be resistant to these additional antibiotics (see also Figure 4.3h).

Table 5.8 Minimum Inhibitory Concentration (MIC) data for recipient strain and selected transconjugants. MIC data was obtained using VITEK2. Breakpoints to determine 'resistant,' 'intermediate,' or 'sensitive,' are from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) Values in dark grey indicate 'resistant,' pale grey indicates 'intermediate' and unshaded values indicate 'sensitive'.

Antibiotic	Isolate MIC mg/L (3GC resistance genotype)						
	TG1	**4a2T (*bla*$_{CTX-M-14}$)**	**12a3T (*bla*$_{CTX-M-15}$)**	**12b1T (*bla*$_{CTX-M-15}$)**	**12e3T (*bla*$_{CTX-M-15}$)**	**20a1T (*bla*$_{DHA-1}$)**	**20a2T (*bla*$_{DHA-1}$)**
Temocillin	<4	<4	8	8	8	8	8
Ampicillin	<2	**>32**	**>32**	**>32**	**>32**	**>32**	**>32**
Amoxicillin/Clavulanic Acid	<2	8	4	4	4	**>32**	**>32**
Piperacillin/Tazobactam	<4	<4	<4	<4	<4	**16**	**32**
Cefalexin	<4	**>64**	**>64**	**>64**	**>64**	**>64**	**>64**
Cefuroxime	4	**>64**	**>64**	**>64**	**>64**	**>64**	**>64**
Cefuroxime Axetil	4	**>64**	**>64**	**>64**	**>64**	**>64**	**>64**
Cefoxitin	<4	<4	<4	<4	<4	**>64**	**32**
Cefotaxime	<1	**>64**	**>64**	**>64**	**>64**	**4**	**8**
Ceftazidime	<1	<1	***2***	***2***	***4***	**>64**	**>64**
Cefepime	<1	<1	<1	<1	<1	<1	<1
Ertapenem	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Meropenem	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Gentamicin	<1	<1	<1	<1	<1	<1	<1
Nalidixic Acid	4	8	<2	8	4	**16**	**>32**
Ciprofloxacin	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Fosfomycin	<16	<16	<16	<16	<16	<16	<16
Nitrofurantoin	<16	<16	<0.5	<16	<16	<16	<16
Trimethoprim	<0.5	<0.5	<0.5	<0.5	<0.5	**>16**	**>16**

5.4.7.5 Conjugation frequency experiments

The frequency of conjugation was determined for donor isolates which had already been shown to transfer *bla*$_{CTX-M}$ in overnight conjugation experiments. Conjugation frequencies for each isolate tested are displayed in Figure 5.10 a-b. Conjugation frequency was determined for 8 different sequence types (total isolates, n=12 plus one positive control). Donor isolate 20c4 showed conjugation frequency one log higher than all other tested strains, therefore this isolate is displayed separately with 8g3 as a comparator (Figure 5.10b).

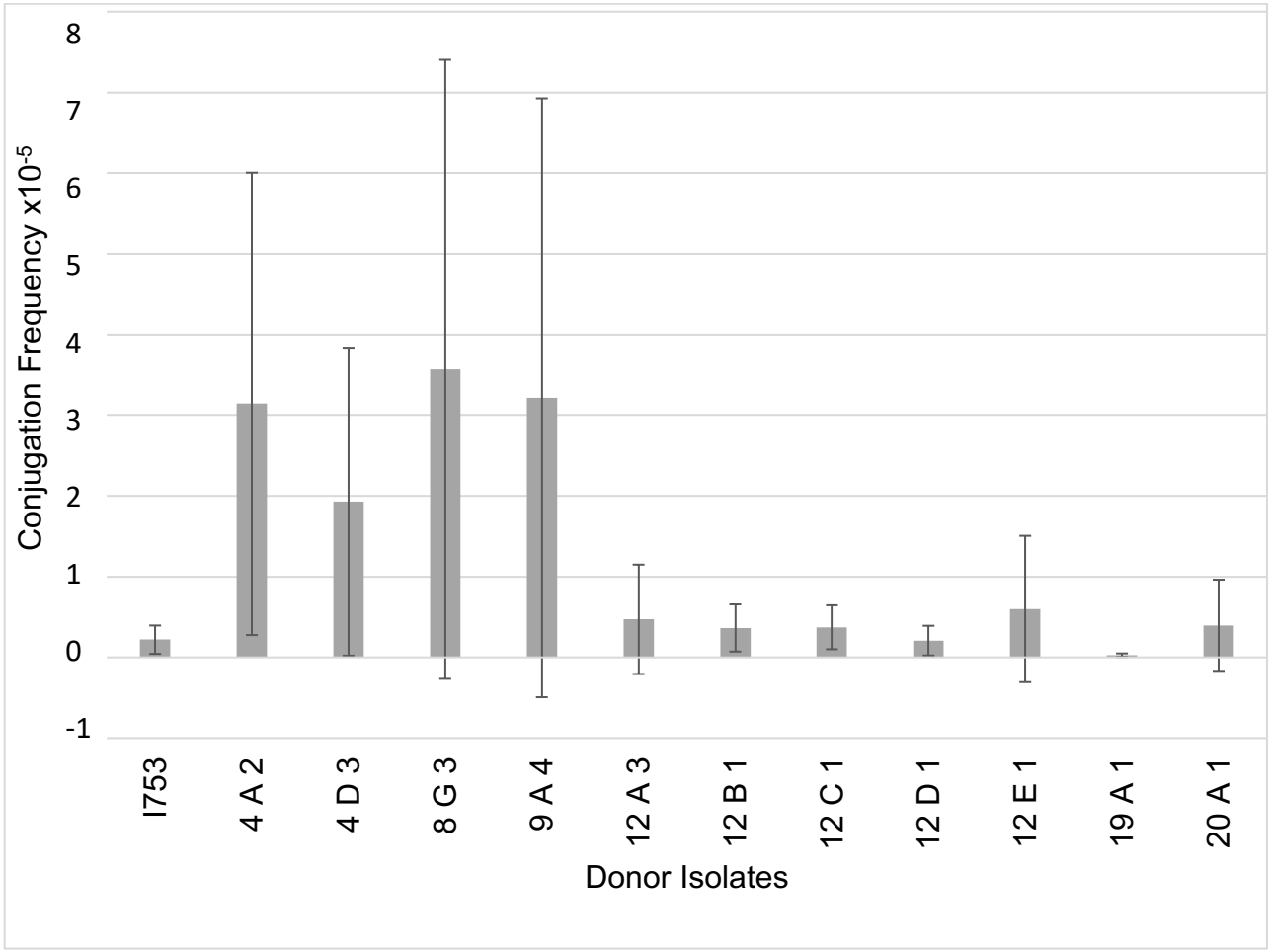


Figure 5.10a. Conjugation frequency for selected donor isolates.
Error bars show 95% confidence limits. Note that isolates 12a3, 12b1, 12c1, 12d1, 12e1 are all ST43. All other isolates are from different sequence types: 4a2 (ST2732), 4d3 (ST648), 8g3 (ST38), 8g4 (ST131), 9a4 (ST4), 19a1 (ST43), 20a1 (NT).

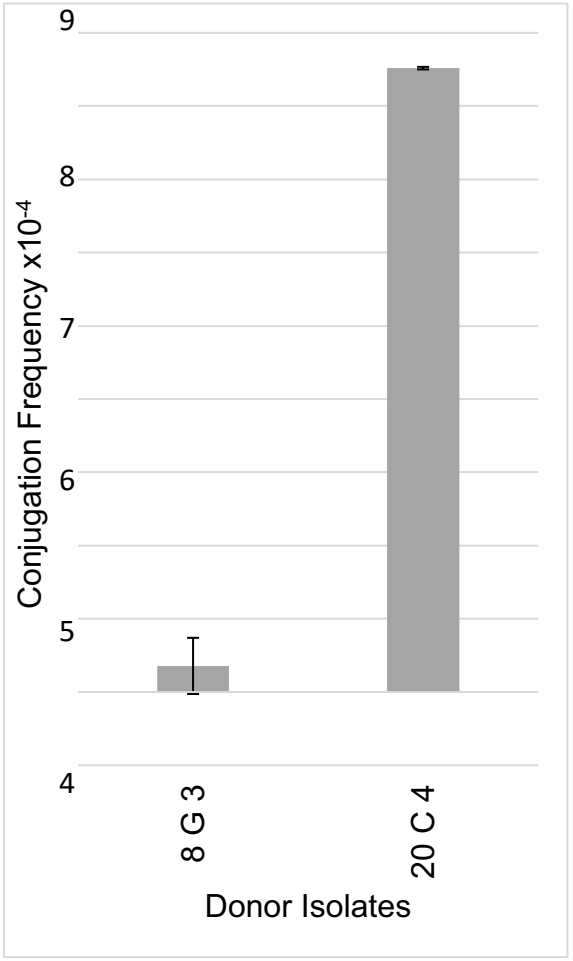


Figure 5.10b. Conjugation frequency for donor isolate 20c4 (ST226).
Isolate 8g3 is shown for comparison (see figure 5.10a).

5.5 Discussion

This chapter has focussed on the CTX-M-EC plasmids acquired by returning travellers from South Asia. The *bla*$_{CTX-M}$ containing contigs were classified as either plasmid or chromosomal in origin, and fully or partially sequenced plasmids were determined after hybrid assembly using short and long read sequencing. Particular attention was paid to the genetic context of *bla*$_{CTX-M}$ and the conjugative ability of *bla*CTX-M plasmids. Sequence comparisons with previously sequenced plasmids were undertaken using the BLAST ring image generator (BRIG).

5.5.1 MinION long read sequencing

The outputs from long read sequencing are displayed in Table 5.1 and 5.3. There were several limitations from the MinION run which must be noted. Firstly, only one flow-cell was available and only one run was carried out. Secondly, the MinION run terminated early, resulting in a slightly lower than expected number of fast5 folders. However, 9 folders each with up to 4001 files per folder were outputted successfully.

A large number of isolates were pooled before running on the MinION (n=34). This approach was used in order to maximise the plasmid content of sequence data, as all strains were CTX-M-producers. However, this did result in an average read coverage per isolate of <1. Therefore, a de-novo assembly would not be possible using this approach. Instead, a hybrid

assembly approach was used so that MinION long reads could be linked to isolates and plasmids already identified using short read sequencing.

Further work would involve repeating the MinION run after barcoding pooled isolates. This would allow a de-novo assembly using long reads only. A hybrid assembly after following this method, would allow closure/sequencing of multiple plasmids of interest.

5.5.2 Plasmid and chromosomal *bla*$_{\text{CTX-M}}$

CTX-M-contigs were found to be of plasmid origin in 22/39 (56%); chromosomal in 15/39 (38%), and not known in 2/38 cases. It is possible that the *bla*$_{\text{CTX-M}}$ has become increasingly fixed on the chromosome in *E. coli*, due to increased antibiotic selective pressure in the environment.

Chromosomal integration of *bla*$_{\text{CTX-M}}$ has been described before. The first description of this occurrence was reported in 2002, when 2/15 CTX-M-producing isolates from Guangzhou, China, had *bla*$_{\text{CTX-M}}$ on the chromosome (Chanawong *et al.*, 2002). In a collection of non-clonal clinical *E. coli* isolates collected between 1996-1999 from Barcelona, Spain, where in 1/30 isolates, *bla*$_{\text{CTX-M}}$ was found on the bacterial chromosome (García *et al.*, 2005). In a South Korean study, 21/25 isolates of Proteus mirabilis carried *bla*$_{\text{CTX-M}}$ on the chromosome (Song *et al.*, 2011). In a study including CTX-M-15-producing *E. coli* isolates from around the world, 7/32 PFGE types had the *bla*$_{\text{CTX-M-15}}$ gene integrated on the chromosome (Coque *et al.*, 2008).

5.5.3 Plasmid assembly

The unicycler hybrid assembly was found to improve the contig size compared to the original illumina assembly, in 59% of isolates. It is notable that in 5/34 isolates subjected to the unicycler assembly, the hybrid assembly approach failed because the original short read-only illumina assembly yielded contigs much larger (>1000 bp larger) compared with unicycler (Table 5.3).

In future work involving MinION long read-sequencing, the best approach for optimal read depth would be DNA quantification and barcoding strains before sequencing, allowing the completion of entire bacterial genomes after one MinION run using a de-novo assembly, followed by hybrid assembly for each strain (Bayliss *et al.*, 2017).

The use of conjugation to transfer the CTX-M plasmid from the travel-acquired *E. coli* into a laboratory *E. coli* strain was achieved for 5/11 volunteers. However, only two plasmids were successfully sequenced using this approach. This is due to the fact that after confirming successful conjugation on filters, transconjugants were frozen on beads at -80 to be sequenced at a later date – allowing batching of strains prior to illumina WGS. Unfortunately, on subculture from -80 stock, the transconjugant with the CTX-M-containing plasmid could not be grown, suggesting that storage at -80 led to the loss of the CTX-M plasmid in most cases. In future experiments it would be advisable to carry out DNA

extractions from transconjugant isolates before storage, to prevent plasmid loss from strains.

5.5.4 CTX-M-contig sequence analysis

5.5.4.1 Genetic contexts of *bla*$_{CTX-M}$

5.5.4.1.1 Context (i)

In the present study we found that the commonest genetic environment surrounding *bla*$_{CTX-M-15}$ was context (i): ISECp1-*bla*$_{CTX-M-15}$-orf477-Tn3 (Table 5.4 and Figure 5.4). The Tn1, Tn2, and Tn3 type transposons were first described associated with the *bla*$_{TEM}$ family of resistance genes in 1974 (Hedges and Jacob, 1974), and Tn3 was later found to have a strong association with *bla*TEM-1a transposition (Heffron *et al.*, 1979).

The particular association between Tn3 and *bla*$_{CTX-M-15}$ is well described. The Tn3 transposon was first described in association with *bla*$_{CTX-M-15}$ in isolates from New Delhi and Paris (Lartigue, Poirel and Nordmann, 2004). The Tn3-family of transposons are likely to play a role in the acquisition of *bla*$_{CTX-M-15}$ onto plasmids. For example, the *E. coli* plasmid pEK204 from the UK, likely evolved via a tn3-mediated transposition event, resulting in acquisition of *bla*$_{CTX-M-15}$ onto the R64 plasmid backbone (Woodford *et al.*, 2009). In addition, a specific Tn3-mediated transposition event with *bla*$_{CTX-M-15}$ led to the formation of pEC_Bactec, which was isolated from an *E. coli* strain originating from the septic joint of a horse in Belgium (Smet *et al.*, 2010).

5.5.4.1.2 Context (ii)

The finding of genetic context (ii), ISECp1-*bla*CTXM-15-orf477, on eight occasions, from seven different volunteers is not at all surprising when considering that this context has been reported on numerous occasions. It is notable that context (ii) has been widely described as "group A," and the "international genetic context" (Dhanji, Patel, *et al.*, 2011; Amos *et al.*, 2014) due to being present globally (Cantón and Coque, 2006).

Genetic context (ii) has been widely reported as the commonest context for $bla_{\text{CTX-M-15}}$. Context (ii) was found in 16/41 isolates from a UK waste-water treatment plant (Amos *et al.*, 2014); and 108/174 CTX-M-EC isolates from patients with post-travel diarrhoea in London, UK (Dhanji, Doumith, *et al.*, 2011).

5.5.4.1.3 Context (iii)

No previous published studies noting the association between ParA and $bla_{\text{CTX-M-15}}$ were found, however using a *BLA*ST search of the 1500 bp region upstream of $bla_{\text{CTX-M-15}}$ for the isolates with the ParA genetic environment, there was only one hit with both 100% query cover and 100% identity. This *BLA*ST match, part of pV150-a, was a plasmid originating from an Indian wastewater-treatment plant, deposited into the NCBI database by Akiba and colleagues (Akiba *et al.*, 2016). This is an important finding: the unusual ParA-*bla*CTX-M genetic environment which was identified from five volunteers who all travelled to India (volunteers 1, 5, 12, 16, and 20) – has also been found in the environment in India before – pointing to an epidemiological link between travellers in the present study and acquisition of CTX-M-EC in India.

Two additional *BLA*ST hits matched the 1500 bp region upstream of *bla*-$_{CTX-M}$ (the ParA-*bla*$_{CTX-M}$ region) with 100% query coverage: (1) Enterotoxigenic *E. coli*, N. Carolina, USA, 85 kbp (strain 2014EL-1345-2, unnamed plasmid, unpublished) with 99% identity to the query sequence; and (2) Enterotoxigenic *E. coli*, S. Korea, (plasmid pET20160881, unpublished), with 99% identity to the query sequence. The ParA-*bla*$_{CTX-M}$ genetic environment is therefore present in a broad range of *E. coli* including strains from environmental, and clinical isolates – thus underlying the importance of this genetic environment in the propagation of *bla*$_{CTX-M-15}$ in a variety of niches.

5.5.4.1.4 Context (iv)

Context (iv) includes the IS26-ISECp1-*bla*$_{CTX-M-15}$-orf477 sequence, which is shared by 'group B' (Dhanji, Doumith, *et al.*, 2011) and 'group N' (Amos *et al.*, 2014) genetic contexts. However, it is possible that group B and/or group N actually include a Tn3-IS26 sequence downstream of orf477, as described in the present study for context (iv). The limited 'range' of PCR primer walking used to describe group B (Dhanji, Murphy, *et al.*, 2011) and group N (Amos *et al.*, 2014), compared with the use of WGS in the present study, may account for these differences.

The position of IS26 upstream of *bla*$_{CTX-M-15}$, is well described, first being found in the formation of IS26-ISECp1-*bla*$_{CTX-M-15}$ and IS26-ISECp1-*bla*CTX-M-3 in clinical *E. coli* isolates from Paris (Eckert, Gautier and Arlet, 2006). IS26 was found in six CTX-M-EC genetic context types, across three volunteers. IS26 is an important insertion sequence, which was first

identified in association with kanamycin resistance (Terawaki, Takayasu and Akiba, 1967). In order to transfer a sequence, for example, a resistance gene, IS26 must be present at each end of the sequence to be transferred. IS26 is an important player in the spread of resistance genes in gram negative bacteria and it is likely that a number of sequential events - starting with the plasmid transfer of IS26 from an environmental bacterium - has led to the success of this IS (Toleman and Walsh, 2011).

In particular, IS26 has been implicated in the spread of clinically worrisome antibiotic resistance genes such as *bla*$_{SHV}$: IS26 mediated the transfer of *bla*$_{SHV}$ from the chromosome of *Klebsiella pneumoniae* onto plasmids, allowing dissemination throughout the Enterobacteriaceae (Ford and Avison, 2004). Furthermore, IS26 is likely to have played a role in the capture of *bla*-$_{CTX-M}$ by epidemic plasmids, and maintenance of *bla*$_{CTX-M}$ in the gene pool thereafter (Ensor *et al.*, 2006; Johnson *et al.*, 2016). (see introduction in this chapter).

Context (iv) is also present in pV035-b from Indian wastewater (Akiba *et al.*, 2016); pECOCTX-M-$_{15}$ from human bile *E. coli* (Jousset *et al.*, 2018); and pPGRT46 rom a healthy human faecal sample (Fortini *et al.*, 2015). Therefore context (iv) is likely to be a key player in the worldwide dissemination of *bla*$_{CTX-M-15}$.

5.5.4.1.5 Context (v)

Context (v) was found to be part of a wider MDR region on a plasmid, pERB5.3e_f, as displayed in Figure 5.6j and discussed in section 5.5.6.6. The plasmid pERB5.3e_f was

acquired by volunteer 5 after travel to Uzbekistan. The 30 kbp MDR region included genes encoding resistance to seven antimicrobial and antiseptic classes. This MDR region carried the hallmarks of a so-called 'clinical class I integron' and Tn21 transposon which includes the chrA chromate resistance gene, the orf encoding putative gene padR, and mph(A) (Partridge, 2011).

The *BLA*ST hits to pERB5.3e_f were all isolated from clinical E. coil isolates: p46212 from the UK (99% query coverage) (Stoesser *et al.*, 2016); pEC958 from Australia (92% query coverage) (Forde *et al.*, 2014); and pEK499 from the UK (82% query coverage) (Woodford *et al.*, 2009). The high query coverage of each of these plasmids points to pERB5.3e_f being an epidemic plasmid which is present in human *E. coli* strains in Uzbekistan, Australia and the UK. Furthermore, the MDR region is conserved between pERB5.3e_f and each of the *BLA*ST hits to this plasmid, providing evidence that this particular MDR cassette has become 'fixed' within a plasmid backbone. The MDR region of pERB5.3e_f allows antibiotic selection for plasmid propagation with any of the antibiotic, antiseptic classes with resistance genes present on the plasmid. In addition, resistance to chromate, and the presence of five toxin-anti toxin systems, allow plasmid survival in the host in the absence of antibiotic selection pressure.

The CTX-M genetic context of isolate 5.3e was subjected to a *BLA*ST search which found 100% query coverage and 100% identity to the $bla_{\text{CTX-M-15}}$ genetic context to p46212, which is a 160 kbp plasmid from an ST131 *E. coli* isolated from human urine in the UK (Stoesser *et al.*, 2016).

5.5.4.1.6 Context (vi)

Context (vi), ISECp1-*bla*$_{CTX-M-15}$-orf477-Tn3-IS3, was found in one isolate, 22a1 (Table 5.4 and Figure 5.4). No published studies include a discussion of this genetic context. However, after running a contig *BLA*ST search, this genetic context matches with 96% query coverage and 100% sequence identity to plasmid pJJ2434, isolated from a human clinical isolate from New York City (Johnson *et al.*, 2016). Strain JJ2434 (the strain bearing pJJ2434), was a pathogenic *H*30Rx ST131 *E. coli* (Johnson *et al.*, 2016), and therefore context (vi) must play an important role in the dissemination of *bla*$_{CTX-M-15}$ within the *H*30Rx clade.

5.5.4.2 ISECp1-*bla*$_{CTX-M-15}$ spacer sequence

The analysis of the ISECp1-*bla*-CTX-M spacer sequence presented in this chapter demonstrates that the 49 bp spacer does not vary between isolates which contain the ISECp1 promotor directly upstream of *bla*$_{CTX-M-15}$. The spacer sequence was identical between all twenty-three traveller isolates included in the analysis, and between other representative ISECp1-*bla*-CTX-M-containing sequences, including those originating from India and from the UK.

Dhanji and colleagues undertook a study which included 174 CTX-M-15-producing *E. coli* strains which were isolated from the faeces of returning travellers, which all contained variations of ISECp1-*bla*$_{CTX-M-15}$, with an intervening 48 bp spacer sequence (Dhanji, Patel, *et al.*, 2011). Each of the Dhanji *bla*$_{CTX-M-15}$ genetic environment sequences deposited in genbank, were downloaded and compared to the twenty-three isolates in this study (Figure

5.5). The Dhanji ISECp1-*bla*$_{CTX\text{-}M\text{-}15}$ spacer sequences, and the ISECp1-*bla*$_{CTX\text{-}M\text{-}15}$ spacer sequences from the present study were found to be identical, and all sequences, including those downloaded from genbank, were 49 bp in length. It must be noted that Dhanji et al stated that the spacer is 48 bp in length, but when each spacer sequence was analysed for this study, in each case there was 49 bp between the stop codon of ISECp1 and the start codon of *bla*$_{CTX\text{-}M\text{-}15}$. This 1bp difference may reflect differing sequencing methods between the present study (illumina short read) verses sanger sequencing in the Dhanji study.

Lartigue and colleagues also reported a 48bp spacer sequence between ISECp1 and *bla*$_{CTX\text{-}M\text{-}15}$ in clinical Enterobacteriaceae from France, Turkey and Poland (Lartigue, Poirel and Nordmann, 2004). The same study also reported that this spacer was identical between isolates with ISECp1$_{CTX\text{-}M\text{-}15}$, ISECp1-*bla*$_{CTX\text{-}M\text{-}10}$, and ISECp1-*bla*$_{CTX\text{-}M\text{-}3}$, which are each part of the group 1 CTX-Ms (Lartigue, Poirel and Nordmann, 2004). Lartigue also showed that the 79 bp upstream sequence of *bla*$_{KLUC\text{-}1}$ from *K. cryocresens* shared 79% and 77% sequence identity with the corresponding regions directly upstream of *bla*-CTX-M-3 and *bla*$_{CTX\text{-}M\text{-}15}$, respectively (Lartigue, Poirel and Nordmann, 2004).

Eckert et al described the genetic environment of eleven CTX-M-15-producing strains from clinical isolates in Paris (Eckert *et al.*, 2004). In keeping with Lartigue, Eckert also found that a 48 bp ISECp1-*bla*$_{CTX\text{-}M\text{-}15}$ spacer sequence was characteristic of all CTX-M-15 producing isolates analysed (Eckert, Gautier and Arlet, 2006).

The influence of the ISECp1-CTX-M spacer on *bla*-CTX-M expression has also been investigated (Ma, Siu and Lu, 2011). Ma et al constructed recombinant plasmids containing varying spacer sizes between ISECp1 and *bla*-CTX-M-3, showing that the highest levels of *bla* expression and higher levels of resistance to cefotaxime were reached with the shortest (42 bp) spacer (Ma, Siu and Lu, 2011).

The conserved 49bp ISECp1-$bla_{\text{CTX-M-15}}$ spacer sequence found in 23/23 isolates analysed in the present study, is not surprising, given that the same spacer sequence is found among CTX-M-15-producing isolates globally. Furthermore, the level of CTX-M expression is influenced by the spacer sequence directly upstream (Ma, Siu and Lu, 2011), therefore it would seem likely that any disruption of the 49 bp spacer would lead to reduced levels of resistance to 3GCs, and so selection pressure to carry $bla_{\text{CTX-M}}$ in this situation would be reduced.

Since the development and widespread use of whole genome sequencing, no studies have used WGS to analyse the ISECp1-$bla_{\text{CTX-M}}$ spacer. The present study is the first to use WGS to analyse the ISECp1-$bla_{\text{CTX-M-15}}$ spacer and included twenty-three diverse *E. coli* in the spacer sequence analysis. It was hypothesised that variation in the ISECp1-$bla_{\text{CTX-M-15}}$ spacer could act as a 'molecular-clock,' providing more clues to the origin of the $bla_{\text{CTX-M-15}}$ sequences in this study. The lack of any variation in the twenty-three spacer sequences means that any such molecular-clock analysis is not possible. However, further work in this area would include expanding the spacer sequence analysis to *Kluyvera* species, and to other $bla_{\text{CTX-M}}$ genotypes, building on the work of Lartigue et al, providing a large scale WGS-based

evolutionary account of the origin of the ISECp1-*bla*$_{CTX-M}$ from *Kluyvera*. In future work, a similar approach could be taken in the analysis of the spacer sequence between *bla*$_{CTX-M}$ and orf477.

5.5.5 Sequence comparisons of circularised plasmids

5.5.5.1 pERB4a2

Previously identified plasmids which had the greatest coverage of pERB4a2 were pHK01 (100%), pEG356 (100%), pSJ82 (100%) and pEC545 (97%). These plasmids have been isolated from bacterial isolates in China (pHK01 and pSJ_82) and Vietnam (pEG356 and pEC545) and contain a variety of CTX-M genotypes (Table 5.5a). A number of other plasmids share a significant plasmid backbone with pERB4a2 – including the entire Tra (transfer) operon (pTC1, pBH100, pHK23a, pVR50, pV035b and pCA08) (Figure 5.6a and table 5.5a). However, there is a notable absence of common genes between these plasmids and pERB4a2, including the iron transport operon and the maltoporin phage lambda receptor.

The genetic context of: ISECp1-*bla*$_{CTX-M-14}$-IS903; found in plasmid pERB4a2, is the most common genetic environment for *bla*$_{CTX-M-14}$, and was also found in the closely related plasmids pHK01, pEG356, pSJ_82, and pEC545.

Plasmid pERB4a2 is notable for its lack of a multi-drug resistance region – only possessing one AMR gene, *bla*$_{CTX-M-14}$. This phenomenon has been described on several occasions (Cottell *et al.*, 2011; P L Ho *et al.*, 2011), as demonstrated by the *BLA*ST matches for pERB4a2 (Figure 5.6a). Plasmid pERB4a2 does not contain other AMR genes, therefore co-selection in

the human gut via exposure to non-cephalosporin antibiotics, or to exposure to antibiotics in the environment, does not play a role in the propagation of pERB4a2. The two toxin-antitoxin systems present in pERB4a2, and the beneficial iron-transport genes are likely to contribute to the stable maintenance of this plasmid in the absence of cephalosporin-selective pressure.

The *BLA*ST match of pERB4a2 to pHK01 is a particularly interesting finding, because pHK01 is a well described 'epidemic plasmid' – with pHK01-like plasmids found across strains in animals and humans in Hong Kong and mainland China (Ho *et al.*, 2012). However, pHK01-like plasmids have not been previously reported in isolates from Sri Lanka. While CTX-M-15 are the most common ESBL in Sri Lanka, CTX-M-14-producing *E. coli* are also found in Sri Lanka (Tillekeratne *et al.*, 2016), therefore the fact that volunteer 4 acquired both genotypes is not an unexpected finding. Our study confirms that the pHK01 plasmid has become globally disseminated since being first isolated in China. A study of bovine *E. coli* plasmids isolated from the environment in Mexico also found a plasmid – pMEX01 – with homology (88% coverage) to the pHK01 isolated in China, supporting the finding in this study, that pHK01 and its homologues are globally disseminated (Soto-Alonso *et al.*, 2015).

The genetic context of $bla_{\text{CTX-M-14}}$ in pERB4a2: ISECp1-$bla_{\text{CTX-M-14}}$-IS903 has been described on numerous occasions (Zhao and Hu, 2013). It is notable that this particular genetic context was found in the first description of $bla_{\text{CTX-M-14}}$ in a plasmid, pOZ174, from China, in an isolate collected in 1998 (Chanawong *et al.*, 2002), as well as the epidemic plasmid pCT (Cottell *et al.*, 2011). The genotype $bla_{\text{CTX-M-14}}$ is globally successful, especially in China, and the

presence of a common genetic context surrounding *bla*$_{CTX\text{-}M\text{-}14}$ is likely to be an important contributor to the success of this *bla*$_{CTX\text{-}M}$ genotype (Bevan, Jones and Hawkey, 2017).

The plasmid matches to pERB4a2 come from a wide range of sources including human clinical isolates, human commensal *E. coli*, as well as animal and environmental niches (Figure 5.6a and table 5.5a). This supports both the notion of epidemic plasmid spread between humans, animals, and the environment (Carattoli, 2009; Bevan, Jones and Hawkey, 2017).

5.5.5.2 Sequence comparisons of pERB3f3

Plasmid pERB3f3 lacked a number of features which are present in other *bla*$_{CTX\text{-}M}$ carrying plasmids. Plasmid pERB3f3 lacked an identifiable plasmid replicon, toxin-antitoxin genes, and plasmid transfer genes, but did carry a number of bacteriophage genes including phage tail fibre protein, structural protein P5, putative phage protein gp42, and a cell attachment protein (Figure 5.6b). Therefore, pERB3f3 can be described as a phage-like plasmid.

Three *BLA*ST plasmid hits matching pERB3f3 included ISECp1-*bla*$_{CTX\text{-}M\text{-}15}$-orf477 (Figure 5.6b and Table 5.5b). These three hits were pECOH89, a human clinical isolate (Falgenhauer *et al.*, 2014); pV234a from Indian wastewater (Akiba *et al.*, 2016); and pAnCo1, isolated from an American bovine feed-lot (Colavecchio *et al.*, 2017). Like pERB3f3, each of pECOH89, pV234a, and pAnCo1, lacked an identifiable plasmid replicon gene, which is likely due to the fact that these plasmids are likely to have originated from a bacteriophage, rather than from Enterobacteriaceae.

Plasmid pECOH89, isolated from *E. coli* cultured from a wound swab from an inpatient in Germany, had 92% query coverage and 98% identity to pERB3f3 (Figure 5.6b and Table 5.5b), (Falgenhauer *et al.*, 2014). Plasmid pAnCo1 was isolated in *E. coli* cultured from bovine feed-lots in Colorado, USA (Colavecchio *et al.*, 2017).

Both pECOH89 and pAnCo1 are phage-like plasmids which have close homology to the bacteriophage SSU5, with pECOH89 having 109/128 (85%) of genes homologous to SSU5 genes (Falgenhauer *et al.*, 2014), and pAnCo1 having 92/130 (71%) genes homologous to SSU5 (Colavecchio *et al.*, 2017). Bacteriophage SSU5 did not appear in a list of 200 *BLA*ST hits to pERB3f3, probably because this bacteriophage has not been submitted to the NCBI *BLA*ST database.

Bacteriophage SSU5 was first identified as having a tropism for S. Typhi strains, and therefore a potential tool in phage therapy (Kim, Kim and Ryu, 2012). Gilcrease and colleagues propose that SSU5 represents a distinct supercluster of temperate bacteriophages which have an affinity for the Enterobacteriales (Gilcrease and Casjens, 2018). SSU5 is 103 kbp in length and shares >94% homology with sequences from over 650 Enterobacteriales in the NCBI database (Gilcrease and Casjens, 2018).

Plasmid pV234a was isolated from an Indian wastewater treatment plant and also shares close homology to pERB3f3. Volunteer 3 travelled widely in India (Chapter 3, Table 3.3 and

3.4) and acquired *E. coli* bearing CTX-M-15-producing plasmids, one of which was circularised in the present study (p3f3). The plasmid pERB3f3 matched with a previously sequenced plasmid – pV234a (91% coverage of query sequence, Figure 5.6b and Table 5.5b) which was isolated from an Indian wastewater treatment plant (WWTP) in Karnataka, (Akiba *et al.*, 2016) (Figure 5.11). Volunteer 3 did visit Karnataka province, albeit 240km from the particular wastewater treatment facility (Figure 5.11). Therefore, the acquisition of *E. coli* carrying pERB3f3 in the faecal microbiome of volunteer 3 provides further evidence for the role of epidemic plasmids in the dissemination of CTX-M-EC between environmental and human niches (Amos *et al.*, 2014).

Figure 5.11. Geographical relationship between WWTP in Karnataka and the nearest travel location of volunteer 3

5.5.5.3 Sequence comparisons of pERB9a1

The plasmid pERB9a1 *BLA*ST matches, pV035-b and pU25P002, were isolated from sources in India, in wastewater and human urine, respectively. Volunteer 9 travelled to Sri Lanka, but did travel back to the UK via India, which included a day in Bangalore airport. Volunteer 9 reported severe symptoms of gastroenteritis on return to the UK, and attributes this to food consumed in Bangalore. Given this history, and the similarity between p9a1 and each of pV035-b, and pU25P002, it seems likely that volunteer 9 acquired *E. coli* with $bla_{\text{CTX-M-15}}$-bearing plasmids during the brief time in Bangalore.

The 92 kbp plasmid, pC15-1a (Figure 5.6c and Table 5.5c) was isolated from an *E. coli* isolate implicated in a CTX-M-15-producing *E. coli* outbreak in Toronto, Canada (Boyd *et al.*, 2004). Plasmid pC15-1a failed to match to pERB9a1 across a 14 kbp which included the qnrS1 gene RelE/StbE toxin/antitoxin system (Figure 5.6c). The same 14 kbp segment was also missing from *BLA*ST matches pU25P002, p1002-1, pFAM22321, p48896_1, pEC743_3, and pTC_N37410PS (Figure 5.6c). Plasmids which do match with pERB9a1 for this 14 kbp region were pV035-b, $\text{pEco}_{\text{CTX-M-15}}$, and pPGRT46. The finding that 7/10 *BLA*ST matches to pERB9a1 do not match across the same 14 kbp region suggests that a transposition event may have occurred, accounting for the differences in pERB9a1 and the seven other plasmid *BLA*ST matches (Figure 5.6c). Such a transposition event – leading to the insertion of the 14 kbp segment into pERB9a1 – was probably mediated by IS26 which flanks either end of the segment (Figure 5.6c).

Plasmid pV035-b, from an Indian environmental *E. coli*, matched to 73% of pERB9a1, which included the 14kbp aforementioned segment. Plasmid pV035-b did not match to a 17 kbp

section of pERB9a1 which included an IncFII stability locus, a prophage partitioning protein, and several housekeeping genes annotated in *bla*ck in the BRIG figure (BRIG figure). A fragment of Tn21 is found at one end of this 17kbp sequence which implies that a Tn21-mediated transposition event may account for the 17kbp-difference between pERB9a1 and pV035-b.

5.5.5.4 Sequence comparisons of pERB12a3

Volunteer 12 acquired CTX-M-EC carrying pERB12a3 which has close homology to plasmids isolated from around the world. Although volunteer 12 travelled to India, apart from pV233_b isolated from an Indian wastewater treatment plant, the *BLA*ST matches to pERB12a3 were from South Korea, China, Vietnam, Taiwan, Switzerland, and the UK. The pERB12a3-like plasmids were found in *E. coli* and *Shigella sonnei* and included a variety of *bla*-$_{\text{CTX-M}}$ genotypes (Figure 5.6d and Table 5.5d), indicating that pERB12a3 is an epidemic plasmid. One of the pERB12a3-like plasmids, pV2474-3, (Figure 5.6d and Table 5.5d) was isolated from *E. coli* from a human blood culture from Jining, China, and contained *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{NDM-1}}$, and mcr-1 (Zheng *et al.*, 2016). The propensity for a pERB12a3-like plasmid to carry this combination of antimicrobial resistance genes is extremely worrisome, because a clinical isolate with high level resistance to 3GCs, carbapenems and colistin, would be very difficult to treat. This study has demonstrated that pERB12a3-like plasmids are globally disseminated, and it is fortunate that p2474-3 has not yet become an epidemic plasmid.

5.5.6 Analysis of other plasmid-like CTX-M Contigs

5.5.6.1 pERB9c3_f

The lack of *BLA*ST matches to the pERB9c3_f variable region is unsurprising given the number of mobile genetic elements in this region which included four copies of IS26, the Tn3 transposon sequences sited at three locations, as well as one copy each of IS3 and IS2 (Figure 5.6e).

The *bla*-$_{CTX-M}$ genetic environment of pERB9c3_f consisted of: IS26-Tn3-catA2-ISECp1-*bla*$_{CTX-M-15}$-orf477-Tn3, which was only found in *BLA*ST hit pHNHN21, with *bla*CTX-M-55 in place of *bla*$_{CTX-M-15}$. Plasmid pHNHN21 was isolated from an *E. coli* originating from a pigeon in China, with no publication associated with the genbank entry. Another of the plasmid matches, pM109_FII, isolated from human clinical *E. coli* from Myanmar, was a 90214 bp conjugative plasmid. Plasmid pM109_FII did not contain a *bla*$_{CTX-M}$ gene, but did contain *bla*$_{NDM-4}$ as well as AAC(3)-IId (Sugawara *et al.*, 2017). The acquisition of pERB9c3_f by volunteer 9, who travelled to Sri Lanka and India, suggests widespread dissemination of the plasmid transfer operon, which is often capable of capturing MDR regions through transposition events, which can include carbapenemase genes.

5.5.6.2 pERB20c1_f

The finding of the *Lnu* gene as part of a class I integron on pERB20c1_f is an unusual finding in Enterobacteriaceae, but has been previously described (Heir *et al.*, 2004). Heir and colleagues also found a lincosamide resistance on a class I integron structure, but in this case

the lincosamide resistance gene was linF, which was closely associated with aadA2, encoding aminogyloside resistance. The finding of the gene *lnu* on pERB20c1_f as part of an MDR cassette is a novel finding (Figure 5.6f).

5.5.6.3 pERB8e1_f

The three highest coverage *BLA*ST matches to pERB8e1_f are presented in Figure 5.6g. It is notable that the NCBI genbank submissions for the *BLA*ST matches indicated 3/3 matches originated from animal isolates in China. However, none of these genbank submissions were associated with published data. The finding of further matches of travel-acquired plasmids to plasmids of animal origin highlights the importance of environmental contamination with animal faeces.

5.5.6.4 pERB1a2_f

Figure 5.6h displays the top three coverage *BLA*ST hits for pERB1a2_f. The *BLA*ST matches come from human, avian, and swine sources. However, it must be noted that the query coverage was <50% for each hit, which limits the interpretation of these *BLA*ST matches.

5.5.6.5 pERB8a1_f

The plasmid-like contig of pERB8a1_f contained a preponderance of phage genes (Figure 5.6i), therefore pERB8a1_f is a phage-like plasmid. *BLA*ST results indicated that pERB8a1_f is closely related to the P1 supercluster of Bacteriophages, with closely related plasmids that are globally disseminated, isolated from Enterobacteriales in human clinical samples (Billard-Pomares *et al.*, 2014; Shin and Ko, 2015) and from pig faeces (Li *et al.*, 2017).

Bacteriophage P1 had 80% query coverage of pERB8a1_f (Figure 5.6i). P1 bacteriophage is a temperate phage capable of a lysogenic or lytic lifecycle, which was first described in 1951 (Bertani, 1951). In the lytic phase, P1 undergoes phage multiplication, cell lysis, and dissemination of progeny, whereas in the lysogenic phase, P1 exists as a low copy number plasmid, behaving like self-replicating plasmids of bacterial origin (Malgorzata *et al.*, 2004). In addition, bacteriophage P1 is able to integrate into the bacterial chromosome, in the form of a prophage, at one copy per chromosome. Bacteriophage P1 is known to possess plasmid maintenance systems such as the Doc/PhD toxin-antitoxin system (Malgorzata *et al.*, 2004), also present in pERB8a1_f (Figure 5.6i).

Plasmid pERB8a1_f is not the first P1 bacteriophage-like plasmid to be described carrying $bla_{\text{CTX-M-15}}$. A CTX-M-15-producing, 267645 bp phage-like plasmid, pKP12226, which had query coverage of pERB8a1_f of 81%, was isolated from a K. pneumoniae strain from the blood cultures of a patient in South Korea (Shin and Ko, 2015).

P1 bacteriophage plasmids are also widely disseminated in animal as well as human Enterobacteriales (Gilcrease and Casjens, 2018), and have a role in mobilising the mcr-1 gene (Li *et al.*, 2017). Plasmid pHYEC7-mcr1, which had a coverage of 72% of pERB8a1_f, was described in a study by Li and colleagues. The plasmid pHYEC7-mcr was isolated from an *E. coli* obtained from pig faeces in Guangdong, China, and carried *mcr-1*, encoding colistin resistance (Li *et al.*, 2017).

Billard-Pomares and colleagues described the role of P1 bacteriophages in the dissemination of ESBL genes, describing a P1 bacteriophage, RCS47, carrying the *bla*$_{SHV}$ gene in *E. coli* (Billard-Pomares *et al.*, 2014). The same study also investigated the prevalence of P1 prophages in a collection of *E. coli*, by screening a collection of 363 clinical *E. coli* isolates for *repL* using a PCR-based approach: repL is the P1 lytic replication gene and is conserved among P1 bacteriophages. The repL gene was found in 12.6% of 363 isolates screened, and the authors concluded that P1 phages are widespread in clinical *E. coli* isolates, but were not found to be significantly associated with ESBL-producing *E. coli* (Billard-Pomares *et al.*, 2014).

Other phage-like plasmids carrying *bla*CTX-M genes in the literature include pECOH89 and pAnCo1, which did not feature in any of the *BLA*ST hits to pERB8e1_f but did match with pERB3f3 as already described (Figure 5.6b).

5.5.6.6 pERB5.3e_f

The plasmid-like contig pERB5.3e_f (Figure 5.6j), was acquired by volunteer 5 after travel to Uzbekistan. The 30 kbp MDR region included genes encoding resistance to seven antimicrobial and antiseptic classes. This MDR region carried the hallmarks of a so-called 'clinical class I integron' and Tn21 transposon which includes the chrA chromate resistance gene, the orf encoding putative gene padR, and mph(A) (Partridge, 2011).

The *BLA*ST hits to pERB5.3e_f were all isolated from clinical E. coil isolates. The *BLA*ST hits included p46212 from the UK (99% query coverage) (Stoesser *et al.*, 2016); pEC958 from Australia (92% query coverage) (Forde *et al.*, 2014); and pEK499 from the UK (82% query

coverage) (Woodford *et al.*, 2009). The high query coverage of each of these plasmids points to pERB5.3e_f being an epidemic plasmid which is present in human *E. coli* strains in Uzbekistan, Australia and the UK. Furthermore, the MDR region is conserved between pERB5.3e_f and each of the *BLA*ST hits to this plasmid, providing evidence that this particular MDR cassette has become 'fixed' within a plasmid backbone. The MDR region of pERB5.3e_f allows antibiotic selection for plasmid propagation with any of the antibiotic, antiseptic classes with resistance genes present on the plasmid. In addition, resistance to chromate, and the presence of five toxin-anti toxin systems, allow plasmid survival in the host in the absence of antibiotic selection pressure (Figure 5.6j).

The CTX-M genetic context of isolate 5.3e was subjected to a *BLA*ST search which found 100% query coverage and 100% identify to the $bla_{\text{CTX-M-15}}$ genetic context to p46212, which is a 160 kbp plasmid from an ST131 *E. coli* isolated from human urine in the UK (Stoesser *et al.*, 2016).

5.5.7 Association of plasmids in this study with wastewater treatment plasmids in India

Notably, in addition to the pERB3f3 homology with pV234a, three other circularised plasmids in this study which were subjected to extensive *BLA*ST searches, also had homology to plasmids identified from the same waste-water treatment plant (WWTP) in India (Tables 5.5a, 5.5c, 5.5d). Plasmid pERB4a2 matched to pV035b (63% coverage); pERB9a1 also matched to pV035-b (73% coverage); and pERB12a3 matched to pV233-b (93% coverage).

With respect to beta-lactamase gene content, of these WWTP plasmids (Akiba *et al.*, 2016), two carried $bla_{\text{CTX-M-15}}$ (pV234a and pV035-b), and one plasmid carried *bla*-CMY-2 (pV233-b). The study by Akiba and colleagues which produced several plasmid sequences from WWTP is the only study which has published plasmid sequences from India (Akiba *et al.*, 2016). The present study is therefore valuable in providing a rare sample of 'Indian' plasmids, acquired by the travellers.

The success of similar plasmids carried by *E. coli* strains and travellers in different parts of the world highlights the continued global success of pandemic plasmid lineages. Furthermore, the various plasmids with close matches to pERB4a2, pERB3f3 , pERB9a1, and pERB12a3, are found across the world, in a variety of environmental, animal and human niches, including in *E. coli* infecting strains. The plasmids identified in this study are (1) readily acquired from the environment by travellers; and (2) being stably maintained in colonising *E. coli* after return to the UK, given the global success of similar plasmids in varied hosts.

5.5.8 Plasmid toxin-antitoxin systems

Toxin anti-toxin (TA) systems are often encoded on bacterial plasmids and are a mechanism by which the plasmid maintains itself within daughter cells. TA systems have been found to have three major functions: (1) post segregational killing (PSK); (2) abortive infection; and (3) inducing a persister-state (Harms *et al.*, 2018). Plasmid toxin-antitoxin (TA) systems were found in 7/10 plasmids presented in this chapter. One plasmid, pERB5.3e_f possessed four different toxin-antitoxin systems. (Table 5.6 and Figure 5.6j).

PSK allows so-called plasmid addiction, by prevention of the loss of a plasmid from a bacterium. The toxin and antitoxin genes are expressed leading to protein production of both components of the system. The toxin is stable and lasts longer than the unstable-antitoxin, therefore loss of a plasmid leads to rapid degradation of the anti-toxin, causing accumulation of toxin leading to host death. A typical example is plasmid F, which encodes the TA system ccdAB (Harms *et al.*, 2018). The TA system ccdAB was found to be carried by pERB5.3e_f (Figure 5.6j).

The TA system PemIK was present on three plasmids described in the present study: pERB9a1, pERB8e1_f, and pERB5.3e_f (overview table and BRIG figure). PemIK is another example of a PSK system: (also known as pemIK, homologous to mazEF) originally described in plasmid R1 (Tsuchimoto, Ohtsubo and Ohtsubo, 1988). A homologue to PemIK is the mazEF system which has been shown to provide protection from bacteriophage insult (Alawneh *et al.*, 2016). Phage immunity, also known as abortive infection, is a system whereby the TA system is activated when bacteriophages attack. For example the presence of the mazEF TA system in *E. coli*, leads to significantly reduced bacteriophage progagation compared with a parallel experiment where the mazEF system was inactivated (Alawneh *et al.*, 2016).

The *pndA* gene, which was present on plasmid pERB12a3, is a member of the hok/sok family system and can lead to PSK (Nielsen and Gerdes, 1995). The *pnd* gene encodes a protein toxin, PndA, which destroys the host cell by depolarisation of the cell membrane. The pnd

anti-toxin is the antisense RNA to pndA mRNA, and functions by binding *pnd* mRNA, preventing translation of the PndA toxin protein. PndA toxin is more stable than the anti-sense RNA, leading to cell death of plasmid-free segregants (Nielsen and Gerdes, 1995).

The RelBE TA system was found on plasmids pERB4a2 and pERB9a1. RelE is a superfamily of ribosome-dependent mRNA endonucleases which inhibit translation. RelE is one of the commonest TA systems in *E. coli* K12, with 7/13 TA system toxins in K12 belonging to the RelE family (Harms *et al.*, 2018). The RelE system is involved in the bacterial stress response: reducing amino acids in-vitro, leads to upregulation of RelE, leading to a global reduction in protein translation. This in turn, leads to reduced bacterial energy consumption, and potential upregulation of RelE-resistant cellular processes (Christensen and Gerdes, 2003). Such a response can be interpreted as an evolutionarily advantageous persister state whereby the host cell becomes dormant and therefore potentially tolerant to some antimicrobial compounds (Harms *et al.*, 2018).

The phd/doc TA was present on pERB1a2_f, pERB8a1_f, and pERB5.3e_f (overview table and BRIG figure). The phd/doc TA is typically found within P1 bacteriophages acting as a PSK inducer, or in achieving bacterial persistence in S. Typhi (Harms *et al.*, 2018).

The VapC/VapB TA system, found on pERB5.3e_f (overview table and BRIG figure), is a TA system whereby an antitoxin protein (VapB) binds a toxin protein (VapC). VapC is a site-specific tRNase which acts by cleaving tRNA anticodons between the loop and stem, leading

to bacteriostasis. This may lead to loss of expression of specific proteins, potentially playing a key role in inducing a bacterial persister state (Winther and Gerdes, 2011).

Toxin-antitoxin systems function on a spectrum between one extreme of post segregational killing, and another extreme of downregulation of protein expression leading to bacterial persistence under stressful conditions, e.g. an antibiotic-containing environment. Therefore, having a greater number and variety of TA systems, as in the case of pERB5.3e_f, which carries four different TA systems, gives the plasmid an evolutionary edge. Plasmid pERB5.3e_f probably has at least one TA system which is geared towards post segregational killing, therefore maintaining the plasmid in the host: this can be described as a parasitic or selfish plasmid (Van Melderen and De Bast, 2009). On the other hand, the variety of TA systems present on pERB5.3e_f allows the plasmid to respond to a variety of stressors, leading to downregulation of specific functions, depending on the external insult. The resulting bacterial persister state is beneficial for not only plasmid survival but host bacterial survival: the TA system is promoting a symbiotic relationship between plasmid and host.

5.5.9 Plasmids in post travel strains are conjugative

5.5.9.1 Pre-conjugation screening and identification of non-lactose fermenting *E. coli*

Prior to putting potential donor CTX-M-EC isolates forward for conjugation experiments, the lactose-fermenting ability of strains was checked by plating onto MacConkey agar. This was an essential step, as any candidate donor organisms needed to be lactose-fermenting, allowing differentiation from non-fermenting recipient (TG1).

Lactose fermenting status was chosen as a phenotypic screening method for putative transconjugants because (1) the recipient strain, TG1, was a lactose non-fermenter; and (2) wild-type *E. coli* are typically lactose fermenters (Chaudhuri and Henderson, 2012). As part of the screening of potential donor isolates before entry into conjugation experiments, 33/101 (33%) isolates were excluded because they were non-lactose fermenting *E. coli*. This was a surprising finding, as lactose-fermenting ability was originally used as one of the tests to identify *E. coli* (Edwards and Ewing, 1972).

Overall, the non-lactose fermenting *E. coli* were found across six different *E. coli* STs, from five different volunteers, and ST131 non-lactose fermenting CTX-M-EC was found in 3 volunteers. Lactose fermenting ST131 CTX-M-EC, were less common that non-fermenters, only being found in 2 volunteers: volunteer 5 and volunteer 16. The volunteer 5 lactose fermenting CTX-M-EC was cultured after the volunteer had visited Uzbekistan, and before visiting India, suggesting acquisition of the strain in Uzbekistan and not in India. The lactose fermenters present in volunteer 16 faecal cultures were actually closely related by SNP-typing to non-lactose fermenters, suggesting that the lactose fermenting gene(s) in these isolates is present on a mobile genetic element, such as a plasmid. The possibility of a lactose-fermenting strain becoming a non-fermenter after loss of the lac operon, presents an issue for in-vitro conjugation experiments using these strains. Theoretically, lactose fermenting isolates which contain $bla_{\text{CTX-M}}$, could become rifampicin-resistant via mutational resistance during the experiment, as well as becoming non-lactose fermenting. This would give a false positive (phenotypic) result in the current experiment. The only way to prove transconjugant status in this situation would be to sequence the genome of the putative

transconjugant, to confirm that the genome was that of TG1, with the addition of a $bla_{CTX\text{-}M}$ plasmid. Fortunately, in the two cases where putative transconjugants were sequenced, their status as transconjugants was confirmed with WGS.

Very few studies have described the prevalence of non-lactose fermenting *E. coli* (Chang *et al.*, 2014; Johnson *et al.*, 2018). Chang and colleagues described a 20% (33/166) prevalence of non-lactose fermenting *E. coli* in a collection of isolates causing urinary tract infection in South Korea, which included variety of *E. coli* sequence types (Chang *et al.*, 2014). Chang also found that 8/33 non-fermenting isolates were ST131 strains but found no statistical difference in ESBL carriage rate between non-lactose fermenting and lactose-fermenting *E. coli*. The ESBL genotypes were not determined (Chang *et al.*, 2014). In another study, all 427 clinical/faecal *E. coli* ST1193 isolates from a Minnesota microbiology laboratory were lactose non-fermenting (Johnson *et al.*, 2018).

The cause of the lactose-non-fermenting phenotype in *E. coli* may be due to plasmid loss (Walia *et al.*, 1987), or a problem within the lac operon, such as a deficiency in lactose permease, encoded by the LacY gene (Fernández-Castané *et al.*, 2012). Notably, a frame-shift mutation in the LacY gene has been implicated in the non-fermenting phenotype of *E. coli* ST1193 strains (Johnson *et al.*, 2018).

Further bioinformatic work stemming from the present study would involve investigation of the genetic basis of lactose non-fermenting status in the non-fermenting *E. coli* strains described above, compared with lactose fermenting strains. In particular, an investigation

could be done, into whether the lac operon is present on a plasmid or transposon, which could result in clonally indistinguishable strains having different phenotypes when grown on MacConkey agar.

5.5.9.2 Conjugation rate

In the present study, 5/11 volunteers (45%) carried CTX-M-EC where *bla*$_{CTX-M}$ could be transferred by conjugation on filters. To our knowledge, no studies have determined the conjugative ability of travel-acquired ESBL-producing *E. coli* before. However, clinical isolates from patients in Lebanon (Harajly *et al.*, 2010) and Tunisia (Mnif *et al.*, 2013) demonstrated conjugation rates of 49% and 80% respectively.

5.5.9.3 Reasons for non-conjugation

The main reason for non-conjugation was presence of *bla*$_{CTX-M}$ on the chromosome (7/19 cases) (Table 5.7). A large proportion of *bla*$_{CTX-M}$ contigs in this study were found to be chromosomal: 15/39 (38%). Assuming the traveller cohort in the present study is representative of CTX-M-EC acquisitions in India, (and provides a snapshot of CTX-M-EC which exist in the environment), the presence of *bla*$_{CTX-M}$ on the chromosome does limit the conjugation potential of *bla*$_{CTX-M}$ in these *E. coli* populations. Contrastingly, mobilisation of *bla*$_{CTX-M}$ via transposition events, allowing stable carriage on the bacterial chromosome, is an important phenomenon, which warrants further study.

A further reason for non-conjugation was that the *bla*$_{CTX-M}$ plasmid contained no conjugation machinery, therefore these strains would have the ability to create a conjugation pilus. One

of these strains, pERB8a1_f is a phage-like plasmid (see discussion section 5.5.6.5). Phage-like plasmids would not be expected to conjugate using conventional in-vitro experiments on filters. Instead, inducing pERB8a1_f transfer would likely require stimulation of a lysogenic function, allowing propagation of new bacteriophages into the extracellular environment, and thereby infecting other bacterial strains.

For isolate 9c3, which possessed an intact Tra operon, an alternative explanation is needing to explain the lack of conjugative ability. A modification in environmental conditions such as pH and/or temperature for conjugation does have an effect on the ability of some plasmids to conjugate (Hardiman *et al.*, 2016). Therefore, the modification of conjugation experiment parameters may have resulted in a higher conjugation success rate.

In Volunteer 9 plasmid pERB9a1, which contains the Tra operon, but which failed to conjugate the CTX-M plasmid in-vitro, we found a Tn21 transposon upstream of TraD (Figure 5.6c). This insertion may be the cause of the failure of pERB9a1 to conjugate. However, in order to prove this hypothesis, experiments using a transposon inserted into the Tra operon in this position would be required.

In the case of 3/19 STs which failed conjugation, different isolates of the same clonally indistinguishable STs did produce transconjugants. The failure of isolates of the same ST, from the same volunteer, to conjugate $bla_{\text{CTX-M}}$ may represent plasmid differences between clonal strains, or subtle methodological differences between experiments with different strains. Different isolates from the same ST were sometimes processed in conjugation

experiments on different days, therefore subtle changes in methodology or in room temperature could explain the differences between clonal isolates.

5.5.9.4 Conjugation experiment: limitations and suggested further work

The principle of a conjugation experiment is the use of at least 2 markers – usually antibiotic-supplemented agar – to select for (1) strains with the plasmid (cefotaxime resistance in the present study); and (2) recipient strains with a resistance phenotype which is absent from donor strains (rifampicin resistance in present study). Unfortunately, when using a chromosomally mediated resistance marker, such as rifampicin-resistance, to select for putative transconjugants, an important issue is the emergence of rifampicin-resistant donor-*E. coli*, which often grow on transconjugant agar plates. Therefore, control plates are needed in parallel, to determine if rifampicin-mutations are emerging in donor strains (Table 5.7). Phenotypically, it is not possible to differentiate rifampicin-mutant donor strains from true transconjugants. Therefore, all conjugation experiments should include a third marker to confirm phenotypically, that a colony is a transconjugant, rather than a cefotaxime-resistant donor strain which has become rifampicin-resistant.

In the present study, the lactose non-fermenting status of the recipient strain was used as a marker of transconjugant status: therefore, any non-fermenting colony growing on LB media containing rifampicin plus cefotaxime, was assumed to be a transconjugant, pending $bla_{\text{CTX-M}}$ PCR confirmation. However, this approach resulted in 33% of travel-acquired CTX-M-EC being excluded from entry into conjugation experiments due to being non-lactose fermenting *E. coli*. In further work, an approach to investigate the conjugative potential of

these isolates could not use lactose fermenting ability as a marker of transconjugant status. Instead, sodium azide could be used as a marker for successful conjugation – as resistance is rare in wild type strains. An appropriate laboratory *E. coli* which is azide-resistant, would be required for use as a conjugation recipient, as used in previous studies (Harajly *et al.*, 2010).

A further limitation of this experiment was that the conditions used in the conjugation experiments in this study were fixed and were not necessarily representative of in-vivo conditions. For example, conjugation frequencies can vary at different temperatures, depending on the plasmid under investigation. It seems plausible that different plasmids may be better able to undergo conjugative transfer at temperatures outside the human physiological norm of 37 degrees. It seems reasonable to assume that in the natural environment, plasmids and their hosts have evolved, allowing transfer more effectively at lower and high temperatures, depending on environmental conditions. Varying the temperature used would be an additional aspect to investigate in future conjugation experiments with the traveller *E. coli* strain collection.

A further limitation to our conjugation experiment methodology is that we used a laboratory *E. coli* K12 MG1655 strain (TG1) as a recipient. This strain was chosen because (1) It has been successfully used in our laboratory before as a recipient strain in conjugation experiments; and (2) TG1 is plasmid-free, allowing straight-forward plasmid sequencing by WGS of any plasmids transferred. The issue with using a laboratory recipient is that this strain is not representative of wild-type *E. coli* strains which receive plasmids in the natural environment, or in the gastrointestinal tract of humans. In future, conjugation experiments should use a

wild type recipient, to better replicate in-vivo conditions. Ultimately, a gut model should be used, to allow the best representation of gastrointestinal conditions. Models of the gastrointestinal tract for the purpose of investigating bacterial horizontal gene transfer, have been developed ex-vivo in the form of culture chemostats simulating conditions in the chicken caecum (Card *et al.*, 2017); and a porcine gut bioreactor (Peeters *et al.*, 2017). Chemostat-based models of the human faecal microbiome have also been developed (Patrick De Boever, Deplancke and Verstraete, 2000; Cinquin *et al.*, 2004; McDonald *et al.*, 2013; Crowther *et al.*, 2014), however no studies have investigated the conjugative potential of strains using such a model. There is certainly scope to develop an in-vitro chemostat-based model for bacterial conjugation, with a view to then investigating the effect of external modifications such as antibiotic concentrations and putative inhibitors of conjugation.

5.5.9.5 Determination of Conjugation Frequency

Among eight discrete CTX-M-EC sequence types, there was notable variation in conjugation frequencies (Figure 5.10a-b). These differences are likely to be due to different plasmids with different conjugative abilities being present in each strain. However, it must be noted that these differences were not statistically significant in most cases, as indicated by the overlapping confidence intervals (Figure 5.10a-b). This was due to a low number of replicate experiments carried out (three per isolate tested) due to time limitations.

Conjugation rates for $bla_{\text{CTX-M}}$ genes in the present study were comparable to Amos et al who tested 51 isolates (from >9 sequence types), which all had conjugation frequencies

between 10^{-3} and 10^{-7} (Amos *et al.*, 2014). The frequencies of conjugation in the present study are also comparable to data from Cottell and colleaugues who showed that the frequency of transfer of the *bla*$_{\text{CTX-M-14}}$ plasmid, pCT, varies between 10^{-2} and 10^{-6} (Cottell, Webber and Piddock, 2012).

In one case, for isolate 20c4 which carried the AmpC gene *bla*$_{\text{DHA-}}$1, the conjugation frequency was significantly higher (around x1 log difference) compared to all other strains (CTX-M-producers) (Figure 5.10a-b). The in-vitro conjugal transfer of *bla*$_{\text{DHA-1}}$ has been described in previous studies using clinical isolates from South Korea (Yoon *et al.*, 2011) and India (Ingti *et al.*, 2017). However, the conjugation frequency of a *bla*$_{\text{DHA-1}}$ plasmid is not described in the current literature. It is likely that the plasmid carrying *bla*$_{\text{DHA-1}}$ in isolate 20c4 was particularly well-adapted to transfer under the experimental conditions used in the conjugation experiment.

The reason that conjugation frequency differed so much for strain 20c4 compared to the *bla*$_{\text{CTX-M}}$ strains, may be reflective of a more efficient transfer operon present in the 20c4 plasmid. Alternatively, 20c4 may possess greater 'fitness' than the CTX-M-producing strains tested, allowing the donor 20c4 to reach higher concentrations on the conjugation plates, thus leading to higher rates of conjugation. Further work to investigate these possibilities would involve a detailed comparison of the plasmid transfer operons between the transconjugants, as well as competition experiments between the strains in question.

5.5.10 PFGE as an additional check on plasmid transfer

PFGE was utilised as an additional check on plasmid transfer, and also provided an estimate of plasmid size. This was an especially useful method in the pre-WGS era to confirm plasmid conjugation had occurred and has been utilised for this purpose in the past (Chanawong *et al.*, 2002).

5.5.11 Confirming transfer using VITEK sensitivity testing

Undertaking phenotypic sensitivity testing of transconjugants is important, because this confirms the potential clinical impact of the dissemination of AMR via horizontal gene transfer (HGT). The phenotypic and genotypic characteristics of transconjugant strains demonstrates the transfer of MDR cassettes via plasmid HGT (see Chapter 4 section 4.3.6).

In turn, the demonstration of HGT of MDR cassettes in vitro provides weight to phenomenon of antibiotic co-selection leading to propagation of strains which contain MDR cassettes. There are two key situations where this phenomenon is important: (1) human therapeutics, e.g. the wide unregulated use of fluoroquinolones in human medicine (Hawkey, 2015); and (2) environmental contamination with MDR organisms from a variety of sources including human and animal waste (Wellington *et al.*, 2013). The problem of environmental contamination with MDR-Enterobacteriaceae from humans and animals is confounded by further environmental contamination with antibiotics from the human/animal food chains, as well as the wastewater from antibiotic manufacture (Wellington *et al.*, 2013). In Hyderabad, India, a study assessed the impact of environmental contamination from antibiotic manufacture on AMR-gram negative bacteria in the environment (Lübbert *et al.*,

2017). Lubbert and colleagues found a strong association between antibiotic contamination and AMR at all their sampling sites. Notably, 23/23 sites sampled in Hyderabad had gram negative bacteria which were ESBL and carbapenemase-producers (Lübbert *et al.*, 2017).

The poor standards of basic sanitation in countries like India are an additional factor which in part explains the high level of environmental contamination with CTX-M-EC in South Asia and in turn, the high acquisition rates among travellers from the West who visit these countries (Bevan, Jones and Hawkey, 2017).

5.5.12 Overview of phage-like plasmids

In the present study, we found that 6/10 plasmids (and plasmid-like contigs) contained genes of bacteriophage origin. In the case of 2/10 plasmids, pERB8e1_f and pERB3f3, which matched to bacteriophage P1-like plasmids and bacteriophage SSU5-like plasmids, respectively (Figures 5.6b and 5.6g). These are the two bacteriophage superclusters which target Enterobacteriales: P1-like bacteriophages and SSU5-like bacteriophages, which may both co-exist as circularised plasmids with the host bacterium (Gilcrease and Casjens, 2018). Both types of bacteriophage have been found in Enterobacteriales carrying ESBLs (Billard-Pomares *et al.*, 2014; Falgenhauer *et al.*, 2014; Shin and Ko, 2015; Akiba *et al.*, 2016; Colavecchio *et al.*, 2017) and mcr-1 (Li *et al.*, 2017).

Given the small sample size of plasmids, this suggests that phage-like plasmids play a key role in the dissemination of $bla_{\text{CTX-M}}$ genes globally – perhaps equalling the role of epidemic plasmids of bacterial origin. Further work is needed in interrogating isolate sequence

repositories for phage-like plasmids, including all strains sequenced in the present study. This will further the understanding the role of bacteriophage-mediated spread of AMR in gram negative bacteria, which will help inform strategies to combat the spread of multi-resistant strains.

5.5.13 The challenge of plasmid assembly

The experiments described in this chapter support the notion that plasmid assembly is a challenge – whether this is done by conjugation or by whole genome sequencing using a hybrid assembly approach. Due to the presence of multiple repetitive sequences, short read illumina sequencing is not well suited to the assembly and circularisation of large plasmids (>50 kbp) (Arredondo-Alonso *et al.*, 2017). There are several options to allow plasmid sequence completion: (1) plasmid DNA isolation and short read sequencing without chromosomal DNA; (2) plasmidome network analysis after short read sequencing; (3) short read sequencing of a transconjugant with the plasmid of interest; (4) long read sequencing plus short read sequencing followed by hybrid assembly.

Each option for plasmid sequencing has particular limitations. For option (1), the isolation of low-copy number plasmid DNA in sufficiently high concentrations requires high volumes of bacterial culture, and is highly labour intensive even for the sequencing of a single plasmid (Cottell *et al.*, 2011). Option (2) requires utilisation of a tailor-made bioinformatic pipeline, 'Plasmid Constellation Network' (PLACNET) (Lanza *et al.*, 2014); and option (3), the transconjugant analysis, is limited by donor suitability issues and plasmid instability in the recipient *E. coli*. Option (4): hybrid assembly, relies on achieving good read depth of long

sequence reads (Ashton *et al.*, 2015). In the present study we used options (3) and (4) to resolve plasmid sequences, as these methods were well-suited to the resources available for this project.

5.6 Conclusion

The first hypothesis presented in this chapter, that *bla*$_{CTX-M}$ genes are primarily plasmid-mediated was confirmed, with 56% of CTX-M-EC isolates with *bla*$_{CTX-M}$ found on a plasmid, rather than on the bacterial chromosome.

A further hypothesis was that travel acquired *bla*$_{CTX-M}$ containing plasmids are more closely related to Indian plasmids, than to UK plasmids. This is rather a challenging hypothesis to investigate, as relatively few CTX-M plasmids from the Indian subcontinent have been sequenced. There was homology between travel acquired CTX-M plasmids and Indian plasmids, for example with the Indian wastewater treatment plasmid, pV234a. However, this study has also demonstrated that travel-acquired CTX-M plasmids are not specific to the Indian subcontinent – there are close homologues found from around the world.

The genetic context of *bla*$_{CTX-M}$ is key to its global spread in *E. coli* and other Enterobacteriales. Six main types of *bla*$_{CTX-M}$ genetic context were described in this chapter, all with homologues in previously sequenced, geographically widespread Enterobacteriales. A specific epidemiological link to India was found for context (iii): the only previous description of this context (which included ParA-*bla*$_{CTX-M}$-) was found in *BLA*ST hits from previously sequenced Indian waste-water plasmids. This finding serves to re-enforce the importance of human travel in the dissemination of *bla*$_{CTX-M}$ in a novel genetic context.

A strong promotor sequence is provided by ISECp1 and the highly conserved ISECp1-*bla*$_{CTX-M-15}$ spacer sequence is likely to be important in maintaining *bla*$_{CTX-M}$ expression and propagation. This was contrary to the hypothesis that there may be geographic variation in the ISECp1-*bla*$_{CTX-M-15}$ spacer sequence. An additional level of *bla*$_{CTX-M}$ propagation are the plasmid-borne TA systems. TA systems have been shown to play an important role in plasmid maintenance, for the mutual benefit of the plasmid, and the host bacterium.

The sources of the plasmids described in this chapter were global clinical, animal, and environmental strains with epidemic plasmids crossing into each of these niches. Identifying CTX-M-EC plasmids from the faecal microbiome of returning travellers is an innovative approach to sampling environmental *E. coli* from the Indian subcontinent. This approach also provides a new insight into the promiscuity of epidemic plasmids. Two main classes of plasmid identified have been identified: those of bacterial origin, and those of phage-like origin. A limitation of the approach used is reliance on sequence comparisons with previously annotated plasmids, already submitted to the NCBI database. Unknown or unannotated plasmids are inevitably missed.

After confirming that CTX-M-EC acquired by travellers possess the conjugation machinery for horizontal gene transfer (HGT), it would be expected that HGT occurs in vivo – i.e. within the human faecal microbiome, or in the natural environment. HGT by plasmid conjugation does occur in the human gut, although reports are limited to case reports (Gona *et al.*, 2014) and self-administration of bacterial cultures (Smith, 1969). Measuring the in-vivo transfer rates of CTX-M-EC plasmids in the human gut would be possible , as this approach has been used

before (Smith, 1969), although there are potential ethical issues with conducting a similar experiment in the 21st century.

We used SNP typing of non-CTX-M-EC pre-travel strains versus CTX-M-EC post travel strains to look for instances where pre-existing host flora acquired a plasmid with $bla_{CTX\text{-}M}$ and found no evidence of this occurrence (see Chapter 4, section 4.3.7). We also looked for common plasmids shared between different *E. coli* strains in the same faecal sample but did not find any.

This study supports the notion of globally successful conjugative epidemic plasmids in the spread of CTX-M-EC – plasmids are readily acquired by our healthy volunteers– and are carried on return to the UK, providing further opportunities for onward dissemination.

Chapter 6: Conclusion

This study has provided analysis and discussion of the molecular epidemiology of CTX-M-EC in the faecal microbiome of returning travellers. The world-wide prevalence of ESBL-producing Enterobacteriaceae continues to increase in all regions, and *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-14}}$ continue to be globally predominant, although other *bla*$_{\text{CTX-M}}$ genes such as *bla*$_{\text{CTX-M-27}}$ and *bla*$_{\text{CTX-M -55}}$ are becomingly increasingly prevalent in genotype surveys. There is little doubt that a combination of human factors has increased the propagation of *bla*$_{\text{-CTX-M}}$. Antibiotic overuse in humans and animals, increased global migration and population density, and inadequate access to basic sanitation in developing countries promote the spread of CTX-M-EC (Bevan, Jones and Hawkey, 2017). Attempts must be made to mitigate these risks through preventative strategies, such as improving access to latrines in developing countries, better antimicrobial stewardship in humans and animals, environmental controls, and targeted evidence-based ESBL screening strategies (O'Neil, 2016).

The evolution of globally successful clones of CTX-M-EC is important, and ExPEC CTX-M-producing strains are dominated by the ST131 lineage (Roer *et al.*, 2017; Harris *et al.*, 2018). Colonisation of CTX-M-EC after travel was found to be due to the polyclonal acquisition of novel CTX-M-EC strains, which colonised the faecal microbiome for up to a year after travel (Bevan *et al.*, 2018). The present study is the first to report that pre-travel *E. coli* strains are not completely displaced from the faecal microbiome by CTX-M-EC, but are maintained as a

minority population throughout travel and become detectable again as a majority population after travel (Bevan *et al.*, 2018). Therefore, non-pathogenic, non-CTX-M-EC strains were identified which have the ability to out-compete CTX-M-EC after return to the UK.

Further work could involve the use of these non-CTX-M strains as part of a strategy to decolonise individuals who have CTX-M-EC in the faecal microbiome. Such undesirable AMR bacteria are not limited to CTX-M-EC, but also include CPE and *mcr* carrying strains. Non-pathogenic *E. coli*, such as the Nissle 1917 strain have been formulated as a drug (Mutaflor®), which is used in Germany as an effective treatment for preventing relapses of inflammatory bowel disease (Rembacken *et al.*, 1999; Sonnenborn and Schulze, 2009). However, Nissle fails to displace AMR *E. coli* from the faecal microbiome in humans (Tannock *et al.*, 2011) and in swine (Mourand *et al.*, 2017). Further work is required to determine the microbiological properties of the non-CTX-M-EC in the present study, which allow persistent colonisation and could support the development of an effective AMR-displacing probiotic.

Case reports suggest that faecal microbiota transplantation (FMT) is an effective means to de-colonise selected patients who are faecally colonised with of AMR bacteria (Singh *et al.*, 2014; Lagier *et al.*, 2015; Wei *et al.*, 2015). However, the microbiological follow-up of these patients did not exceed three months, raising the possibility of persisting minority AMR strains over a longer time period after FMT. Several trials are underway to assess the effectiveness of FMT for decolonisation of MDR Enterobacteriaceae (Manges, Steiner and Wright, 2016). After further strain characterisation and screening for pathogenicity factors,

the pre-travel non-CTX-M-EC identified in the present study (Bevan *et al.*, 2018), could be utilised as part of FMT to decolonise patients.

The data presented in Chapter 5 reinforces the importance of epidemic plasmid spread in the global dissemination of CTX-M-EC. It is notable that no instances of horizontal gene transfer (HGT) of the $bla_{CTX\text{-}M}$ plasmid from a travel-acquired strain onto a pre-existing host strain were found. Therefore, if HGT occurs, it is most likely to be at a low frequency, as it was not detected in the present study.

There are two lines of evidence from this study which suggest that HGT is possible in CTX-M-EC-colonised individuals. Firstly, epidemic plasmids are found carried by CTX-M-EC in returning travellers, which have sequence matches to plasmids isolated from different ecological niches from around the world (Chapter 5, See Tables 5.5a-c). Plasmid matches come from different *E. coli* STs. For example, pERB4a2 was isolated from an *E. coli* ST2732 strain, whereas the *BLA*ST match pHK01 has been found in multiple different STs (P. L. Ho *et al.*, 2012). Similarly, pERB12a3 (from an ST43 strain) matches to pEK204 (ST131 strain) (Woodford *et al.*, 2009), and pERB3f3 (from an ST405 strain) matches to pM160133_p1 (from an ST1485 strain) (Gilrane *et al.*, 2017). Secondly, plasmids in this study can be transferred by conjugation in-vitro (Chapter 5, Table 5.7), implying that horizontal gene transfer also occurs in vivo, in the human faecal microbiome.

Characterising the role of $bla_{CTX\text{-}M}$-encoding plasmids from CTX-M-EC acquired by travellers will help to support the development of novel strategies to displace these plasmids from the

host strain, such as plasmid curing (Hale *et al.*, 2010) and conjugation inhibition (Ripoll-rozada *et al.*, 2016).

Studying CTX-M-EC acquisition in healthy volunteers helps inform future studies in hospital patients and is an important surrogate for future spread of carbapenemase genes. *E. coli* is the single most common cause of bacteraemia in the UK. Determining the mechanism of spread of resistance genes in *E. coli* underpins infection prevention and control practices, decreasing excess mortality, and allows the preservation of existing classes of antibiotics for future generations.

Further work

- Determine the pathogenicity factors and colonisation ability of travel-acquired CTX-M-EC and pre-travel non-CTX-M-EC and compare these to the *E. coli* Nissle strain.
- Utilise non-pathogenic, non-CTX-M-EC in an in-vivo setting in healthy human volunteers known to be colonised with CTX-EC. In a new traveller study, pre-travel resident non-CTX-M-EC could be provided to volunteers to consume after travel, aiming to speed up de-colonisation of CTX-M-EC.
- Provide further characterisation of $bla_{\text{CTX-M}}$-encoding plasmids using repeat long read sequencing after strain barcoding. Identify plasmids which are amenable to plasmid curing and undertake a pilot study using plasmid curing in healthy volunteers colonised with CTX-M-EC.

REFERENCES

Abernethy, J. *et al.* (2017) 'Epidemiology of Escherichia coli bacteraemia in England: results of an enhanced sentinel surveillance programme', *Journal of Hospital Infection*. Elsevier Ltd, 95(4), pp. 365–375. doi: 10.1016/j.jhin.2016.12.008.

Adeolu, M. *et al.* (2016) 'Genome-based phylogeny and taxonomy of the "Enterobacteriales": Proposal for enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morgane', *International Journal of Systematic and Evolutionary Microbiology*, 66(12), pp. 5575–5599. doi: 10.1099/ijsem.0.001485.

Adler, A. *et al.* (2012) 'Transmission dynamics of ESBL-producing Escherichia coli clones in rehabilitation wards at a tertiary care centre', *Clinical Microbiology and Infection*. European Society of Clinical Infectious Diseases, 18(12), pp. E497–E505. doi: 10.1111/j.1469-0691.2012.03999.x.

Afgan, E. *et al.* (2015) 'Genomics Virtual Laboratory: A practical bioinformatics workbench for the cloud', *PLoS ONE*, 10(10), pp. 1–20. doi: 10.1371/journal.pone.0140829.

Ahmed, S. A. *et al.* (2012) 'Genomic Comparison of Escherichia coli O104:H4 Isolates from 2009 and 2011 Reveals Plasmid, and Prophage Heterogeneity, Including Shiga Toxin Encoding Phage stx2', *PLoS ONE*, 7(11). doi: 10.1371/journal.pone.0048228.

Akiba, M. *et al.* (2016) 'Distribution and Relationships of Antimicrobial Resistance Determinants among Extended-Spectrum-Cephalosporin-Resistant or Carbapenem-Resistant Escherichia coli Isolates from Rivers and Sewage Treatment Plants in India', *Antimicrob Agent Chemother*, 60(5), pp. 2972–2980. doi: 10.1128/AAC.01950-15.Address.

Alawneh, A. M. *et al.* (2016) 'An ADP-ribosyltransferase Alt of bacteriophage T4 negatively regulates the Escherichia coliMazF toxin of a toxin-antitoxin module', *Molecular Microbiology*, 99(1), pp. 188–198. doi: 10.1111/mmi.13225.

Alikhan, N. F. *et al.* (2011) 'BLAST Ring Image Generator (BRIG): Simple prokaryote genome comparisons', *BMC Genomics*. BioMed Central Ltd, 12(1), p. 402. doi: 10.1186/1471-2164-12-402.

Amos, G. C. a *et al.* (2014) 'Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment.', *The Journal of antimicrobial chemotherapy*, 69(7), pp. 1785–91. doi: 10.1093/jac/dku079.

Angelin, M. *et al.* (2015) 'Risk factors for colonization with extended-spectrum beta-lactamase producing Enterobacteriaceae in healthcare students on clinical assignment abroad: A prospective study', *Travel Medicine and Infectious Disease*, 13(3), pp. 223–229. doi: 10.1016/j.tmaid.2015.04.007.

Arcilla, M. S. *et al.* (2015) 'Dissemination of the mcr-1 colistin resistance gene', *The Lancet Infectious Diseases*. Elsevier Ltd, 3099(15), pp. 1–2. doi: 10.1016/S1473-3099(15)00532-0.
Arcilla, M. S. *et al.* (2017) 'Import and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae by international travellers (COMBAT study): a prospective, multicentre cohort study', *The Lancet Infectious Diseases*. Elsevier Ltd, 17(1), pp. 78–85. doi: 10.1016/S1473-3099(16)30319-X.

Arredondo-Alonso, S. *et al.* (2017) 'On the (im)possibility of reconstructing plasmids from whole-genome short-read sequencing data', *Microbial Genomics*, 3. doi: 10.1099/mgen.0.000128.

Arumugam, M. *et al.* (2011) 'Enterotypes of the human gut microbiome', *Nature*, 473(7346), pp. 174–180. doi: 10.1038/nature09944.

Ashton, P. M. *et al.* (2015) 'MinION nanopore sequencing identifies the position and structure of a bacterial antibiotic resistance island', *Nature Biotechnology*, 33(3), p. 296. doi: 10.1038/nbt.3103.

Asir, J. *et al.* (2015) 'Simultaneous gut colonisation and infection by ESBL-producing Escherichia coli in hospitalised patients', *Australasian Medical Journal*, 8(6), pp. 200–207.
Ausubel, F. M. *et al.* (2003) *Current Protocols in Molecular Biology*, *Molecular Biology*. doi: 10.1002/mrd.1080010210.

Azam, M., Jan, A. T. and Haq, Q. M. R. (2016) 'blaCTX-M-152, a Novel Variant of CTX-M-group-25, Identified in a Study Performed on the Prevalence of Multidrug Resistance among Natural Inhabitants of River Yamuna, India', *Frontiers in Microbiology*, 7(February), pp. 1–13. doi: 10.3389/fmicb.2016.00176.

Aziz, R. K. *et al.* (2008) 'The RAST Server: Rapid annotations using subsystems technology', *BMC Genomics*, 9, pp. 1–15. doi: 10.1186/1471-2164-9-75.

Bajaj, P. *et al.* (2015) 'Distribution and molecular characterization of genes encoding CTX-M and AmpC β-lactamases in Escherichia coli isolated from an Indian urban aquatic environment.', *The Science of the total environment*, 505, pp. 350–6. doi: 10.1016/j.scitotenv.2014.09.084.

Banerjee, R. and Johnson, J. R. (2014) 'A new clone sweeps clean: The enigmatic emergence of Escherichia coli sequence type 131', *Antimicrobial Agents and Chemotherapy*, 58(9), pp. 4997–5004. doi: 10.1128/AAC.02824-14.

Bankevich, A. *et al.* (2012) 'SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing', *Journal of Computational Biology*, 19(5), pp. 455–477. doi: 10.1089/cmb.2012.0021.

Barreto Miranda, I. *et al.* (2016) 'High carriage rate of ESBL-producing Enterobacteriaceae at presentation and follow-up among travellers with gastrointestinal complaints returning from India and Southeast Asia', *Journal of travel medicine*, 23(2), p. tav024. doi: 10.1093/jtm/tav024.

Bauernfeind, A. *et al.* (1992) 'A new plasmidic cefotaximase from patients infected with Salmonella typhimurium', *Infection*, 20(3), pp. 158–163.

Bauernfeind, A., Grimm, H. and Schweighart, S. (1990) 'A new plasmidic cefotaximase in a clinical isolate of Escherichia coli.', *Infection*, 18(5), pp. 294–298. doi: 10.1007/BF01647010.

Bayliss, S. C. *et al.* (2017) 'The use of Oxford Nanopore native barcoding for complete genome assembly', *GigaScience*, 6(3), pp. 1–6. doi: 10.1093/gigascience/gix001.

Beatson, S. A. *et al.* (2015) 'Molecular analysis of asymptomatic bacteriuria Escherichia coli strain VR50 reveals adaptation to the urinary tract by gene acquisition', *Infection and Immunity*, 83(5), pp. 1749–1764. doi: 10.1128/IAI.02810-14.

de Been, M. *et al.* (2014) 'Dissemination of Cephalosporin Resistance Genes between Escherichia coli Strains from Farm Animals and Humans by Specific Plasmid Lineages', *PLoS Genetics*, 10(12), p. doi:10.1371/journal.pgen.1004776. doi: 10.1371/journal.pgen.1004776.

Bengtsson-Palme, J. *et al.* (2015) 'The human gut microbiome as a transporter of antibiotic resistance genes between continents', *Antimicrobial Agents and Chemotherapy*, 59(10), p. AAC.00933-15. doi: 10.1128/AAC.00933-15.

Bernard, H. *et al.* (1992) 'A novel plasmid-mediated extended-spectrum beta lactamase not derived from TEM- or SHV-type enzymes', *J Antimicrob Chemother*, 29, pp. 590–592.

Bertani, G. (1951) 'Studies on lysogenesis: I. The mode of phage liberation by lysogenic Escherichia coli', *J Bacteriol*, 62(3), pp. 293–300.

Bevan, E. R. *et al.* (2018) 'Acquisition and Loss of CTX-M-Producing and Non-Producing *E. coli* in the Fecal Microbiome of Travelers to South Asia', *mBio*, 9, pp. e02408-18.

Bevan, E. R., Jones, A. M. and Hawkey, P. M. (2017) 'Global epidemiology of CTX-M β-lactamases: Temporal and geographical shifts in genotype', *Journal of Antimicrobial Chemotherapy*, 72(8), pp. 2145–2155. doi: 10.1093/jac/dkx146.

Bhattacharya, A. *et al.* (2018) 'Estimating the incidence and 30-day all-cause mortality rate of

Escherichia coli bacteraemia in England by 2020/21', *Journal of Hospital Infection*. The Healthcare Infection Society, 98(3), pp. 228–231. doi: 10.1016/j.jhin.2017.09.021.

Biedenbach, D. J. *et al.* (2014) 'Antimicrobial susceptibility and extended-spectrum beta-lactamase rates in aerobic gram-negative bacteria causing intra-abdominal infections in Vietnam: Report from the Study for Monitoring Antimicrobial Resistance Trends (SMART 2009-2011)', *Diagnostic Microbiology and Infectious Disease*. Elsevier Inc., 79(4), pp. 463–467. doi: 10.1016/j.diagmicrobio.2014.05.009.

Billard-Pomares, T. *et al.* (2014) 'Characterization of a P1-like bacteriophage carrying an SHV-2 extended-spectrum β-lactamase from an Escherichia coli strain', *Antimicrobial Agents and Chemotherapy*, 58(11), pp. 6550–6557. doi: 10.1128/AAC.03183-14.

Birgy, A. *et al.* (2016) 'ESBL-producing Escherichia coli ST131 versus non-ST131: evolution and risk factors of carriage among French children in the community between 2010 and 2015.', *The Journal of antimicrobial chemotherapy*, 71(10), pp. 2949–56. doi: 10.1093/jac/dkw219.

Blanc, V. *et al.* (2014) 'Prevalence of day-care centre children (France) with faecal CTX-M-producing Escherichia coli comprising O25b:H4 and O16:H5 ST131 strains.', *The Journal of antimicrobial chemotherapy*, 69(5), pp. 1231–7. doi: 10.1093/jac/dkt519.

Blattner, F. R. *et al.* (1997) 'The complete genome sequence of Escherichia coli K-12', *Science*, 277(5331), pp. 1453–1462. doi: 10.1126/science.277.5331.1453.

Bo, G. (2000) 'Giuseppe Brotzu and the discovery of cephalosporins', *Clinical Microbiology and Infection*, 6(S3), pp. 6–8. doi: 10.1111/j.1469-0691.2000.tb02032.x.

Van Boeckel, T. P. *et al.* (2015) 'Global trends in antimicrobial use in food animals.', *Proceedings of the National Academy of Sciences of the United States of America*, 112(18), pp. 5649–5654. doi: 10.1073/pnas.1503141112.

Bonnet, R. *et al.* (2003) 'Effect of D240G substitution in a novel ESBL CTX-M-27.', *The Journal of antimicrobial chemotherapy*, 52(1), pp. 29–35. doi: 10.1093/jac/dkg256.

Boyd, D. a. *et al.* (2004) 'Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extended-spectrum beta-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada', *Antimicrobial Agents and Chemotherapy*, 48(10), pp. 3758–3764. doi: 10.1128/AAC.48.10.3758-3764.2004.

Boyd, E. F. and Hartl, D. L. (1998) 'Chromosomal regions specific to pathogenic isolates of Escherichia coli have a phylogenetically clustered distribution', *Journal of Bacteriology*, 180(5), pp. 1159–1165.

Brenner, D. J. *et al.* (1972) 'Polynucleotide sequence divergence among strains of Escherichia

coli and closely related organisms.', *Journal of Bacteriology*, 109(3), pp. 933–965. doi: .

Brettin, T. *et al.* (2015) 'RASTtk: A modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes', *Scientific Reports*, 5. doi: 10.1038/srep08365.

Burton, H. S. and Abraham, E. P. (1951) 'Isolation of antibiotics from a species of Cephalosporium. Cephalosporins P1, P2, P3, P4 and P5', *Biochemical Journal*, 50(2), pp. 168–174. doi: 10.1042/bj0500168.

Bush, K. and Jacoby, G. A. (2010) 'Updated functional classification of beta-lactamases', *Antimicrobial Agents and Chemotherapy*, 54(3), pp. 969–976. doi: 10.1128/AAC.01009-09.

Cabezón, E. *et al.* (2014) 'Towards an integrated model of bacterial conjugation', *FEMS Microbiology Reviews*, 39(1), p. n/a-n/a. doi: 10.1111/1574-6976.12085.

Cantón, R. and Coque, T. M. (2006) 'The CTX-M β-lactamase pandemic', *Current Opinion in Microbiology*, 9(5), pp. 466–475. doi: 10.1016/j.mib.2006.08.011.

Cantón, R., González-Alba, J. M. and Galán, J. C. (2012) 'CTX-M enzymes: Origin and diffusion', *Frontiers in Microbiology*, 3, p. doi: 10.3389/fmicb.2012.00110. doi: 10.3389/fmicb.2012.00110.

Carattoli, A. *et al.* (2005) 'Identification of plasmids by PCR-based replicon typing.', *Journal of microbiological methods*, 63(3), pp. 219–28. doi: 10.1016/j.mimet.2005.03.018.

Carattoli, A. (2009) 'Resistance plasmid families in Enterobacteriaceae.', *Antimicrobial agents and chemotherapy*, 53(6), pp. 2227–38. doi: 10.1128/AAC.01707-08.

Carattoli, A. (2013) 'Plasmids and the spread of resistance', *International journal of medical microbiology : IJMM*. Elsevier GmbH., 303(6–7), pp. 298–304. doi: 10.1016/j.ijmm.2013.02.001.

Card, R. M. *et al.* (2017) 'An In Vitro Chicken Gut Model Demonstrates Transfer of a Multidrug Resistance Plasmid from Salmonella to Commensal Escherichia coli.', *mBio*, 8(4), pp. e00777-17. doi: 10.1128/mBio.00777-17.

Castanheira, M. *et al.* (2013) 'Prevalence of ??-lactamase-encoding genes among Enterobacteriaceae bacteremia isolates collected in 26 U.S. Hospitals: Report from the SENTRY antimicrobial surveillance program (2010)', *Antimicrobial Agents and Chemotherapy*, 57(7), pp. 3012–3020. doi: 10.1128/AAC.02252-12.

Castellani, A. S. and Chalmers, A. J. (1919) *Manual of Tropical Medicine*. 3rd edn. London: Bailliere, Tindall & Cox.

Cavaco, L. M. *et al.* (2008) 'Selection and persistence of CTX-M-producing Escherichia coli in the intestinal flora of pigs treated with amoxicillin, ceftiofur, or cefquinome', *Antimicrobial Agents and Chemotherapy*, 52(10), pp. 3612–3616. doi: 10.1128/AAC.00354-08.

CDC (2013) *Antibiotic Resistance Threats*, *U .S .Centers for Disease Control and Prevention*. doi: CS239559-B.

Chain, E. *et al.* (1940) 'Penicillin As a Chemotherapeutic Agent', *The Lancet*, 236(6104), pp. 226–228. doi: 10.1016/S0140-6736(01)08728-1.

Chanawong, A. *et al.* (2002) 'Three Cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among Enterobacteriaceae in the People's Republic of China', *Antimicrobial agents and chemotherapy*, 46(3), pp. 630–637. doi: 10.1128/AAC.46.3.630.

Chang, J. *et al.* (2014) 'Prevalence and characteristics of lactose non-fermenting Escherichia coli in urinary isolates', *Journal of Infection and Chemotherapy*. Elsevier Ltd, 20(11), pp. 738–740. doi: 10.1016/j.jiac.2014.07.005.

Chaudhuri, B. N. *et al.* (2011) 'Incidence of ESBL producers amongst Gram-negative bacilli isolated from intra-abdominal infections across India (Based on SMART study, 2007 data)', *Journal of Association of Physicians of India*, 59(5).

Chaudhuri, R. R. and Henderson, I. R. (2012) 'The evolution of the Escherichia coli phylogeny', *Infection, Genetics and Evolution*. Elsevier B.V., 12(2), pp. 214–226. doi: 10.1016/j.meegid.2012.01.005.

Chen, Y. H. *et al.* (2011) 'Antimicrobial susceptibility profiles of aerobic and facultative Gram-negative bacilli isolated from patients with intra-abdominal infections in the Asia-Pacific region according to currently established susceptibility interpretive criteria', *Journal of Infection*. Elsevier Ltd, 62(4), pp. 280–291. doi: 10.1016/j.jinf.2011.02.009.

Chen, Y. T. *et al.* (2014) 'KPC-2-encoding plasmids from escherichia coli and Klebsiella pneumoniae in Taiwan', *Journal of Antimicrobial Chemotherapy*, 69(3), pp. 628–631. doi: 10.1093/jac/dkt409.

Christensen, S. K. and Gerdes, K. (2003) 'RelE toxins from Bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA', *Molecular Microbiology*, 48(5), pp. 1389–1400. doi: 10.1046/j.1365-2958.2003.03512.x.

Cinquin, C. *et al.* (2004) 'Immobilization of infant fecal microbiota and utilization in an in vitro colonic fermentation model', *Microbial Ecology*, 48(1), pp. 128–138. doi: 10.1007/s00248-003-2022-7.

Clermont, O. *et al.* (2013) 'The Clermont Escherichia coli phylo-typing method revisited:

Improvement of specificity and detection of new phylo-groups', *Environmental Microbiology Reports*, 5(1), pp. 58–65. doi: 10.1111/1758-2229.12019.

Clermont, O., Bonacorsi, S. and Bingen, E. (2000) 'Rapid and simple determination of the Escherichia coli phylogenetic group', *Applied and Environmental Microbiology*, 66(10), pp. 4555–4558. doi: 10.1128/AEM.66.10.4555-4558.2000.Updated.

Colavecchio, A. *et al.* (2017) 'Complete Genome Sequences of Two Phage-Like Plasmids Carrying the CTX- M-15 Extended-Spectrum beta-Lactamase Gene', *Genome announcements*, 5(19), pp. 198–199.

Connor, T. R. *et al.* (2016) 'CLIMB (the Cloud Infrastructure for Microbial Bioinformatics): an online resource for the medical microbiology community', *Microbial Genomics*, 2(9). doi: 10.1099/mgen.0.000086.

Conza, J. Di *et al.* (2002) 'Novel Class 1 Integron (InS21) Carrying bla CTX-M-2 in Salmonella enterica Serovar Infantis', *Antimicrob Agent Chemotherapy*, 46(7), pp. 2257–2261. doi: 10.1128/AAC.46.7.2257.

Coque, T. M. *et al.* (2008) 'Dissemination of clonally related Escherichia coli strains expressing extended-spectrum β-lactamase CTX-M-15', *Emerging Infectious Diseases*, 14(2), pp. 195–200. doi: 10.3201/eid1402.070350.

Cottell, J. L. *et al.* (2011) 'Complete sequence and molecular epidemiology of IncK epidemic plasmid encoding blaCTX-M-14.', *Emerging infectious diseases*, 17(4), pp. 645–52. doi: 10.3201/eid1704.101009.

Cottell, J. L., Webber, M. a and Piddock, L. J. V (2012) 'Persistence of transferable extended-spectrum-β-lactamase resistance in the absence of antibiotic pressure.', *Antimicrobial agents and chemotherapy*, 56(9), pp. 4703–6. doi: 10.1128/AAC.00848-12.

Crowther, G. S. *et al.* (2014) 'Development and validation of a chemostat gut model to study both planktonic and biofilm modes of growth of Clostridium difficile and human microbiota', *PLoS ONE*, 9(2). doi: 10.1371/journal.pone.0088396.

Dale, A. P. and Woodford, N. (2015) 'Extra-intestinal pathogenic Escherichia coli (ExPEC): Disease, carriage and clones', *Journal of Infection*. Elsevier Ltd, 71(6), pp. 615–626. doi: 10.1016/j.jinf.2015.09.009.

Dallenne, C. *et al.* (2010) 'Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae', *Journal of Antimicrobial Chemotherapy*, 65(3), pp. 490–495. doi: 10.1093/jac/dkp498.

Darling, A. E., Mau, B. and Perna, N. T. (2010) 'Progressivemauve: Multiple genome alignment with gene gain, loss and rearrangement', *PLoS ONE*, 5(6). doi:

10.1371/journal.pone.0011147.

Datta, N. and Hedges, R. W. (1971) 'Compatibility groups among fi-R factors [11]', *Nature*, 234(5326), pp. 222–223. doi: 10.1038/234222a0.

Datta, N. and Hughes, V. M. (1983) 'Plasmids of the same Inc groups in Enterobacteria before and after the medical use of antibiotics.', *Nature*, pp. 616–617. doi: 10.1038/306616a0.

Datta, N. and Richmond, M. H. (1966) 'The purification and properties of a penicillinase whose synthesis is mediated by an R-factor in Escherichia coli', *Biochem J*, 98, pp. 204–209. doi: 10.1042/bj0980204.

Davies, S. C. (2013) 'Infections and the Rise of Antimicrobial Resistance', *Annual Report of the Chief Medical Officer - Volume Two (2011)*, Chapter 5., pp. 73–86.

Dawkins, R. (1976) *The Selfish Gene*. London: Paladin.

Decousser, J. W., Poirel, L. and Nordmann, P. (2001) 'Characterization of a chromosomally encoded extended-spectrum class A beta-lactamase from Kluyvera cryocrescens', *Antimicrobial Agents and Chemotherapy*, 45(12), pp. 3595–3598. doi: 10.1128/AAC.45.12.3595-3598.2001.

Denisuik, A. J. *et al.* (2013) 'Molecular epidemiology of extended-spectrum -lactamase-, AmpC -lactamase- and carbapenemase-producing Escherichia coli and Klebsiella pneumoniae isolated from Canadian hospitals over a 5 year period: CANWARD 2007-11', *Journal of Antimicrobial Chemotherapy*, 68(suppl 1), pp. i57–i65. doi: 10.1093/jac/dkt027.

Desjardins, P. *et al.* (1995) 'Sex in Escherichia coli does not disrupt the clonal structure of the population: evidence from random amplified polymorphic DNA and restriction-fragment-length polymorphism', *Journal of Molecular Evolution*, 41(4), pp. 440–448. doi: 10.1007/BF00160315.

Dhanji, H., Murphy, N. M., *et al.* (2011) 'Isolation of fluoroquinolone-resistant O25b:H4-ST131 Escherichia coli with CTX-M-14 extended-spectrum β-lactamase from UK river water.', *The Journal of antimicrobial chemotherapy*, 66(3), pp. 512–6. doi: 10.1093/jac/dkq472.

Dhanji, H., Doumith, M., *et al.* (2011) 'Molecular epidemiology of fluoroquinolone-resistant ST131 Escherichia coli producing CTX-M extended-spectrum beta-lactamases in nursing homes in Belfast, UK.', *The Journal of antimicrobial chemotherapy*, 66(2), pp. 297–303. doi: 10.1093/jac/dkq463.

Dhanji, H., Patel, R., *et al.* (2011) 'Variation in the genetic environments of bla(CTX-M-15) in Escherichia coli from the faeces of travellers returning to the United Kingdom.', *The Journal of antimicrobial chemotherapy*, 66(5), pp. 1005–12. doi: 10.1093/jac/dkr041.

Diestra, K. *et al.* (2009) 'Characterization of plasmids encoding blaESBL and surrounding genes in Spanish clinical isolates of Escherichia coli and Klebsiella pneumoniae.', *The Journal of antimicrobial chemotherapy*, 63(1), pp. 60–6. doi: 10.1093/jac/dkn453.

Doi, Y. *et al.* (2010) 'Extended-spectrum and CMY-type beta-lactamase-producing Escherichia coli in clinical samples and retail meat from Pittsburgh, USA and Seville, Spain', *Clinical Microbiology and Infection*, 16(1), pp. 33–38. doi: 10.1111/j.1469-0691.2009.03001.x.

Domagk, G. (1935) 'Ein Beitrag zur Chemotherapie der bakterillen Infektionen. (for English translation see Brock TD Milestones in Microbiology [1961]. American Society for Microbiology, Washington DC)', *Deutsch Med Wochenschrift*, 61, pp. 250–253.

Doumith, M. *et al.* (2012) 'Characterization of plasmids encoding extended-spectrum β-lactamases and their addiction systems circulating among Escherichia coli clinical isolates in the UK.', *The Journal of antimicrobial chemotherapy*, 67(4), pp. 878–85. doi: 10.1093/jac/dkr553.

Duggar, B. M. (1948) 'Product of the continuing search for new antibiotics', *Ann NY Acad Sci*, 51, pp. 177–181.

ECDC (2018) *European Centre for Disease Prevention and Control: Surveillance of antimicrobial resistance in Europe - Annual report of the European Antimicrobial Resistance Surveillance Network EARS-Net) 2017*, *Surveillance of antimicrobial resistance in Europe*. Stockholm: ECDC. doi: 10.2900/296939.

Eckert, C. *et al.* (2004) 'Dissemination of CTX-M-Type β -Lactamases among Clinical Isolates of Enterobacteriaceae in Paris , France Dissemination of CTX-M-Type ᴺʟ -Lactamases among Clinical Isolates of Enterobacteriaceae in Paris , France', 48(4), pp. 1249–1255. doi: 10.1128/AAC.48.4.1249.

Eckert, C., Gautier, V. and Arlet, G. (2006) 'DNA sequence analysis of the genetic environment of various blaCTX-M genes.', *The Journal of antimicrobial chemotherapy*, 57(1), pp. 14–23. doi: 10.1093/jac/dki398.

Edwards, P. R. and Ewing, W. H. (1972) *Identification of Enterobacteriaceae*. 3rd edn. Minneapolis: Burgess.

Eherlich, J. *et al.* (1947) 'Chloromycetin, a New Antibiotic From a Soil Actinomycete', *Science*, 106, p. 417.

Ehrlich, P. (1913) 'Address in pathology, on chemiotherapy', *British Medical Journal*, 2(2746), pp. 353–359. doi: 10.1136/bmj.2.2746.353.

Eisen, D. (2010) 'Tetracycline', in *Kuchers' The Use of Antibiotics*, pp. 843–850.
Elwell, L. P. and Shipley, P. L. (1980) 'Plasmid-mediated factors associated with virulence of bacteria to animals', *Annu.Rev.Microbiol.*, 34, pp. 465–496. doi: 10.1146/annurev.mi.34.100180.002341.

Ensor, V. M. *et al.* (2006) 'Occurrence, prevalence and genetic environment of CTX-M beta-lactamases in Enterobacteriaceae from Indian hospitals', *Journal of Antimicrobial Chemotherapy*, 58, pp. 1260–1263. doi: 10.1093/jac/dkl422.

Escherich, T. (1885) 'Die Darmbakterien des Neugeborenen und Säuglings', *Fortschr. Med.*, 3, pp. 547–554.

Escherich, T. (1988) 'The Intestinal Bacteria of the Neonate and Breast-Fed Infant', *Reviews of Infectious Diseases*, 10(6), pp. 1220–1225. doi: 10.1093/clinids/10.6.1220.

EUCAST (2018) *EUCAST clinical breakpoints*, *European Committee on Antimicrobial Susceptibility Testing*. Available at: http://www.eucast.org/clinical_breakpoints/.

Falgenhauer, L. *et al.* (2014) 'Complete Genome Sequence of Phage-Like Plasmid pECOH89, Encoding CTX-M-15.', *Genome announcements*, 2(2), pp. 356–14. doi: 10.1128/genomeA.00356-14.

Fekete, P. Z. *et al.* (2012) 'DNA sequence analysis of the composite plasmid pTC conferring virulence and antimicrobial resistance for porcine enterotoxigenic Escherichia coli', *International Journal of Medical Microbiology*, 302(1), pp. 4–9. doi: 10.1016/j.ijmm.2011.07.003.

Fernández-Castané, A. *et al.* (2012) 'Evidencing the role of lactose permease in IPTG uptake by Escherichia coli in fed-batch high cell density cultures', *Journal of Biotechnology*. Elsevier B.V., 157(3), pp. 391–398. doi: 10.1016/j.jbiotec.2011.12.007.

Fernandez, A. *et al.* (2007) 'Interspecies spread of CTX-M-32 extended-spectrum beta-lactamase and the role of the insertion sequence IS1 in down-regulating blaCTX-M gene expression', *Journal of Antimicrobial Chemotherapy*, 59(5), pp. 841–847. doi: 10.1093/jac/dkm030.

Fick, J. *et al.* (2009) 'Contamination of Surface, Ground, and Drinking Water From Pharmaceutical Production', *Environmental Toxicology and Chemistry*, 28(12), pp. 2522–2527.

Fleming, A. (1929) 'On The antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae', *British Journal of Experimental Pathology*, 10(3), pp. 226–236. doi: 10.1093/clinids/2.1.129.

Ford, P. J. and Avison, M. B. (2004) 'Evolutionary mapping of the SHV β-lactamase and

evidence for two separate IS26-dependent blaSHV mobilization events from the Klebsiella pneumoniae chromosome', *Journal of Antimicrobial Chemotherapy*, 54(1), pp. 69–75. doi: 10.1093/jac/dkh251.

Forde, B. M. *et al.* (2014) 'The complete genome sequence of escherichia coli EC958: A high quality reference sequence for the globally disseminated multidrug resistant *E. coli* O25b:H4-ST131 clone', *PLoS ONE*, 9(8). doi: 10.1371/journal.pone.0104400.

Fortini, D. *et al.* (2015) 'A novel plasmid carrying blaCTX-M-15 identified in commensal Escherichia coli from healthy pregnant women in Ibadan, Nigeria', *Journal of Global Antimicrobial Resistance*, 3(1), pp. 9–12. doi: 10.1016/j.jgar.2014.12.002.

García, A. *et al.* (2005) 'Characterization of the highly variable region surrounding the blaCTX-M-9gene in non-related Escherichia coli from Barcelona', *Journal of Antimicrobial Chemotherapy*, 56(5), pp. 819–826. doi: 10.1093/jac/dki345.

Geyer, C. N. *et al.* (2015) 'Evaluation of CTX-M steady-state mRNA, mRNA half-life and protein production in various STs of *Escherichia coli*', *Journal of Antimicrobial Chemotherapy*, p. dkv388. doi: 10.1093/jac/dkv388.

Gibb, A. P. and Crichton, M. (2000) 'Cefpodoxime screening of Escherichia coli and Klebsiella spp. By Vitek for detection of organisms producing extended-spectrum beta-lactamases', *Diagnostic Microbiology and Infectious Disease*, 38(4), pp. 255–257. doi: 10.1016/S0732-8893(00)00203-0.

Gilcrease, E. B. and Casjens, S. R. (2018) 'The genome sequence of Escherichia coli tailed phage D6 and the diversity of Enterobacteriales circular plasmid prophages', *Virology*. Elsevier Inc., 515(November 2017), pp. 203–214. doi: 10.1016/j.virol.2017.12.019.

Gilrane, V. L. *et al.* (2017) 'Complete Genome Sequence of a Colistin-Resistant Escherichia coli Strain Harboring mcr-1 on an IncHI2 Plasmid in the United States.', *Genome announcements*, 5(42), pp. 10–11. doi: 10.1128/genomeA.01095-17.

Gniadkowski, M. *et al.* (1998) 'Cefotaxime-resistant Enterobacteriaceae isolates from a hospital in Warsaw, Poland: Identification of a new CTX-M-3 cefotaxime-hydrolyzing beta-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme', *Antimicrobial Agents and Chemotherapy*, 42(4), pp. 827–832.

Gogarten, M., Gogarten, J. and Olendzenski, L. (2009) *Horizontal Gene Transfer: Genomes in Flux*. Humana Press. doi: 10.1007/978-1-60327-853-9.

Gona, F. *et al.* (2014) 'In vivo multiclonal transfer of blaKPC-3 from Klebsiella pneumoniae to Escherichia coli in surgery patients', *Clinical Microbiology and Infection*. European Society of Clinical Infectious Diseases, 20(10), pp. O633–O635. doi: 10.1111/1469-0691.12577.

Gonullu, N. *et al.* (2008) 'Dissemination of CTX-M-15 beta-lactamase genes carried on Inc FI and FII plasmids among clinical isolates of Escherichia coli in a University Hospital in Istanbul, Turkey', *Journal of Clinical Microbiology*, 46(3), p. 1110. doi: 10.1128/JCM.01974-07.

Goren, M. G. *et al.* (2010) 'Transfer of Carbapenem-Resistant Plasmid from *Klebsiella pneumoniae* ST258 to *Escherichia coli* in Patient', *Emerging Infectious Diseases*, 16(6), pp. 1014–1017. doi: 10.3201/eid1606.091671.

Gurevich, A. *et al.* (2013) 'QUAST: Quality assessment tool for genome assemblies', *Bioinformatics*, 29(8), pp. 1072–1075. doi: 10.1093/bioinformatics/btt086.

Hadfield, J. *et al.* (2017) 'Phandango: an interactive viewer for bacterial population genomics', *Bioinformatics*, pp. 1–2. doi: 10.1093/bioinformatics/btx610.

Hale, L. *et al.* (2010) 'An efficient stress-free strategy to displace stable bacterial plasmids.', *BioTechniques*, 48(3), pp. 223–8. doi: 10.2144/000113366.

Hamburger, M. *et al.* (1951) 'Streptomycin-resistant coliform bacilli in stools of tuberculous patients treated with intramuscular streptomycin', *J Lab Clin Med.*, 37(1), pp. 64–72.

Hammerum, A. M. *et al.* (2014) 'Characterization of extended-spectrum β-lactamase (ESBL)-producing Escherichia coli obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third- or fourth-generation cephalosporins.', *The Journal of antimicrobial chemotherapy*, 69(10), pp. 2650–7. doi: 10.1093/jac/dku180.

Harada, K., Nakai, Y. and Kataoka, Y. (2012) 'Mechanisms of resistance to cephalosporin and emergence of O25b-ST131 clone harboring CTX-M-27 beta-lactamase in extraintestinal pathogenic Escherichia coli from dogs and cats in Japan', *Microbiology and Immunology*, 56(7), pp. 480–485. doi: 10.1111/j.1348-0421.2012.00463.x.

Harajly, M. *et al.* (2010) 'Frequency of conjugative transfer of plasmid-encoded ISEcp1 - blaCTX-M-15 and aac(6')-Ib-cr genes in Enterobacteriaceae at a tertiary care center in Lebanon - role of transferases', *Annals of Clinical Microbiology and Antimicrobials*, 9, pp. 1–7. doi: 10.1186/1476-0711-9-19.

Hardiman, C. A. *et al.* (2016) 'Horizontal Transfer of Carbapenemase-Encoding Plasmids and', *Antimicrobial Agents and Chemotherapy*, 60(8), pp. 4910–4919. doi: 10.1128/AAC.00014-16.Address.

Harms, A. *et al.* (2018) 'Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology', *Molecular Cell*. Elsevier Inc., 70(5), pp. 768–784. doi: 10.1016/j.molcel.2018.01.003.

Harris, P. N. A. *et al.* (2018) 'Effect of piperacillin-tazobactam vs meropenem on 30-day mortality for patients with e coli or Klebsiella pneumoniae bloodstream infection and ceftriaxone resistance', *JAMA - Journal of the American Medical Association*, 320(10), pp.

984–994. doi: 10.1001/jama.2018.12163.

Hattem, J. M. Van *et al.* (2016) 'Prolonged carriage and potential onward transmission of carbapenemase-producing Enterobacteriaceae in Dutch travelers', *Future Microbiol.*, 11, pp. 857–64.

Hawkey, P. M. (2008) 'Prevalence and clonality of extended-spectrum β-lactamases in Asia', *Clinical Microbiology and Infection*. European Society of Clinical Microbiology and Infectious Diseases, 14, pp. 159–165. doi: 10.1111/j.1469-0691.2007.01855.x.

Hawkey, P. M. (2015) 'Multidrug-resistant Gram-negative bacteria: a product of globalization', *Journal of Hospital Infection*. Elsevier Ltd, 89(4), pp. 241–247. doi: 10.1016/j.jhin.2015.01.008.

Hawkey, P. M. (2018) 'The 2017 Garrod Lecture: Genes, guts and globalization', *Journal of Antimicrobial Chemotherapy*, 73, pp. 2589–2600. doi: 10.1093/jac/dky277.

Hawkey, P. M. *et al.* (2018) 'Treatment of infections caused by multidrug-resistant gram-negative bacteria: Report of the British society for antimicrobial chemotherapy/healthcare infection society/british infection association joint working party', *Journal of Antimicrobial Chemotherapy*, 73(March 2015), pp. iii2–iii78. doi: 10.1093/jac/dky027.

Hawkey, P. M. and Jones, A. M. (2009) 'The changing epidemiology of resistance', *Journal of Antimicrobial Chemotherapy*, 64(SUPPL.1). doi: 10.1093/jac/dkp256.

Hawser, S. *et al.* (2011) 'Antimicrobial susceptibility of intra-abdominal Gram-negative bacilli from Europe: SMART Europe 2008', *European Journal of Clinical Microbiology and Infectious Diseases*, 30(2), pp. 173–179. doi: 10.1007/s10096-010-1066-0.

Hawser, S. P. *et al.* (2010) 'Incidence and Antimicrobial Susceptibility of Escherichia coli and Klebsiella pneumoniae with extended-spectrum ??-lactamases in community- and hospital-associated intra-abdominal infections in Europe: Results of the 2008 Study for Monitoring Antimicrobia', *Antimicrobial Agents and Chemotherapy*, 54(7), pp. 3043–3046. doi: 10.1128/AAC.00265-10.

Hawser, S. P. *et al.* (2012) 'Low frequency of ertapenem-resistant intra-abdominal isolates of Escherichia coli from Latin America: susceptibility, ESBL-occurrence, and molecular characterisation (SMART 2008-2009).', *Journal of chemotherapy (Florence, Italy)*, 24(1), pp. 6–11. doi: 10.1179/1120009X12Z.0000000003.

He, L. *et al.* (2013) 'Complete nucleotide sequence of pHN7A8, an F33:A-:B-type epidemic plasmid carrying bla CTX-M-65, fosA3 and rmtB from China', *J Antimicrob Chemother*, 68, pp. 46–50. doi: 10.1093/jac/dks369.

Hedges, A. J., Howe, K. and Linton, A. H. (1977) 'Statistical Considerations in the Sampling of

Escherichia coli from Intestinal Sources for Serotyping', *Journal of Applied Bacteriology*, 43(2), pp. 271–280. doi: 10.1111/j.1365-2672.1977.tb00751.x.

Hedges, R. and Jacob, A. (1974) 'Transposition of ampicillin resistance from RP4 to other replicons', *Molecular and General Genetics MGG*, 132(1), pp. 31–40.

Heffron, F. *et al.* (1979) 'DNA sequence analysis of the transposon Tn3: Three genes and three sites involved in transposition of Tn3', *Cell*, 18(4), pp. 1153–1163. doi: 10.1016/0092-8674(79)90228-9.

Heir, E. *et al.* (2004) 'Prevalence and characterization of integrons in blood culture Enterobacteriaceae and gastrointestinal Escherichia coli in Norway and reporting of a novel class I integron-located lincosamide resistance gene', *Annals of Clinical Microbiology and Antimicrobials*, 3, pp. 1–9. doi: 10.1186/1476-0711-3-12.

Ho *et al.* (2012) 'Dissemination of pHK01-like incompatibility group IncFII plasmids encoding CTX-M-14 in Escherichia coli from human and animal sources.', *Veterinary microbiology*, 158(1–2), pp. 172–9. doi: 10.1016/j.vetmic.2012.02.004.

Ho, P L *et al.* (2011) 'Complete sequencing of the FII plasmid pHK01, encoding CTX-M-14, and molecular analysis of its variants among Escherichia coli from Hong Kong.', *The Journal of antimicrobial chemotherapy*, 66(4), pp. 752–6. doi: 10.1093/jac/dkr010.

Ho, P. L. *et al.* (2011) 'Extensive dissemination of CTX-M-producing Escherichia coli with multidrug resistance to "critically important" antibiotics among food animals in Hong Kong, 2008-10', *Journal of Antimicrobial Chemotherapy*, 66(4), pp. 765–768. doi: 10.1093/jac/dkq539.

Ho, P. L. *et al.* (2012) 'Predominance of pHK01-like incompatibility group FII plasmids encoding CTX-M-14 among extended-spectrum beta-lactamase-producing Escherichia coli in Hong Kong, 1996-2008.', *Diagnostic microbiology and infectious disease*. Elsevier Inc., 73(2), pp. 182–6. doi: 10.1016/j.diagmicrobio.2012.03.009.

Ho, P. L. *et al.* (2013) 'Plasmid-mediated fosfomycin resistance in Escherichia coli isolated from pig', *Veterinary Microbiology*, 162(2–4), pp. 964–967. doi: 10.1016/j.vetmic.2012.09.023.

Holmes, A. H. *et al.* (2016) 'Understanding the mechanisms and drivers of antimicrobial resistance', *The Lancet*, 387(10014), pp. 176–187. doi: 10.1016/S0140-6736(15)00473-0.

Holt, K. E. *et al.* (2013) 'Tracking the establishment of local endemic populations of an emergent enteric pathogen.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(43), pp. 17522–7. doi: 10.1073/pnas.1308632110.

Hsueh, P.-R. *et al.* (2010) 'Epidemiology and antimicrobial susceptibility profiles of aerobic

and facultative Gram-negative bacilli isolated from patients with intra-abdominal infections in the Asia-Pacific region: 2008 results from SMART (Study for Monitoring Antimicrobial Resistan', *International journal of antimicrobial agents*. Elsevier B.V., 36(5), pp. 408–14. doi: 10.1016/j.ijantimicag.2010.07.002.

Humeniuk, C. *et al.* (2002) 'β-lactamases of Kluyvera ascorbata, probable progenitors of some plasmid-encoded CTX-M types', *Antimicrobial Agents and Chemotherapy*, 46(9), pp. 3045–3049. doi: 10.1128/AAC.46.9.3045-3049.2002.

Hunter, P. A. *et al.* (2010) 'Antimicrobial-resistant pathogens in animals and man: Prescribing, practices and policies', *Journal of Antimicrobial Chemotherapy*, 65(SUPPL.1), pp. 3–17. doi: 10.1093/jac/dkp433.

Hussain, A. *et al.* (2012) 'Multiresistant uropathogenic Escherichia coli from a region in India where urinary tract infections are endemic: Genotypic and phenotypic characteristics of sequence type 131 isolates of the CTX-M-15 extended-spectrum-β- lactamase-producing lineage', *Antimicrobial Agents and Chemotherapy*, 56, pp. 6358–6365. doi: 10.1128/AAC.01099-12.

Ingti, B. *et al.* (2017) 'Occurrence of blaDHA-1mediated cephalosporin resistance in Escherichia coli and their transcriptional response against cephalosporin stress: A report from India', *Annals of Clinical Microbiology and Antimicrobials*. BioMed Central, 16(1), pp. 1–8. doi: 10.1186/s12941-017-0189-x.

Jiang, H.-X. *et al.* (2014) 'Multiple transmissible genes encoding fluoroquinolone and third-generation cephalosporin resistance co-located in non-typhoidal Salmonella isolated from food-producing animals in China.', *International journal of antimicrobial agents*. Elsevier B.V., 43(3), pp. 242–7. doi: 10.1016/j.ijantimicag.2013.12.005.

Johnson, J. R. *et al.* (2010) 'Escherichia coli sequence type ST131 as the major cause of serious multidrug-resistant *E. coli* infections in the United States.', *Clinical Infectious Diseases*, 51(3), pp. 286–294. doi: 10.1086/653932.

Johnson, T. J. *et al.* (2016) 'Separate F-Type Plasmids Have Shaped the Evolution of the H 30 Subclone of Escherichia coli Sequence Type 131', *clinical science and epidemiology*, 1(4), pp. 1–15. doi: 10.1128/mSphere.00121-16.Editor.

Johnson, T. J. *et al.* (2018) 'Phylogenomic analysis of extraintestinal pathogenic Escherichia coli ST1193, an emerging multidrug-resistant clonal group', *Antimicrobial Agents and Chemotherapy*. doi: 10.1128/AAC.01913-18.

Jousset, A. B. *et al.* (2018) 'CTX-M-15-Producing Shewanella Species Clinical Isolate Expressing OXA-535, a Chromosome-Encoded OXA-48 Variant, Putative Progenitor of the Plasmid-Encoded OXA-436', *Antimicrobial Agents and Chemotherapy*, 62(1), pp. 1–11.

Kantele, A. *et al.* (2015) 'Antimicrobials increase travelers' risk of colonization by extended-spectrum betalactamase-producing enterobacteriaceae', *Clinical Infectious Diseases*, 60(6), pp. 837–846. doi: 10.1093/cid/ciu957.

Karanika, S. *et al.* (2016) 'Fecal Colonization with Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae and Risk Factors among Healthy Individuals: A Systematic Review and Meta-Analysis.', *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 63(3), pp. 310–318. doi: 10.1093/cid/ciw283.

Karfunkel, D. *et al.* (2013) 'The emergence and dissemination of CTX-M-producing Escherichia coli sequence type 131 causing community-onset bacteremia in Israel.', *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*, 32(4), pp. 513–21. doi: 10.1007/s10096-012-1765-9.

Karim, A. *et al.* (2001) 'Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEcp1', *FEMS Microbiology Letters*, 201, pp. 237–241. doi: 10.1016/S0378-1097(01)00276-2.

Kauffmann, F. (1943) 'Ueber neue thermolabile Kerperantigene der Colibakterien', *Acta Pathol. Microbiol. Scand.*, 20, pp. 21–44.

Kennedy, K. and Collignon, P. (2010) 'Colonisation with Escherichia coli resistant to "critically important" antibiotics: a high risk for international travellers.', *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*, 29(12), pp. 1501–6. doi: 10.1007/s10096-010-1031-y.

Kim, J. *et al.* (2011) 'Characterization of IncF plasmids carrying the blaCTX-M-14 gene in clinical isolates of Escherichia coli from Korea.', *The Journal of antimicrobial chemotherapy*, 66(6), pp. 1263–8. doi: 10.1093/jac/dkr106.

Kim, J. S. *et al.* (2014) 'Complete nucleotide sequence of the IncI1 plasmid pSH4469 encoding CTX-M-15 extended-spectrum β-lactamase in a clinical isolate of Shigella sonnei from an outbreak in the Republic of Korea', *International Journal of Antimicrobial Agents*. Elsevier B.V., 44(6), pp. 533–537. doi: 10.1016/j.ijantimicag.2014.08.007.

Kim, M., Kim, S. and Ryu, S. (2012) 'Complete Genome Sequence of Bacteriophage SSU5 Specific for Salmonella enterica serovar Typhimurium Rough Strains', *Journal of Virology*, 86(19), pp. 10894–10894. doi: 10.1128/JVI.01796-12.

Kim, S. Y. *et al.* (2016) 'Prevalence and characteristics of Escherichia coli sequence type 131 and its H30 and H30Rx subclones: A multicenter study from Korea', *Diagnostic Microbiology and Infectious Disease*. Elsevier Inc., 84(2), pp. 97–101. doi: 10.1016/j.diagmicrobio.2015.10.016.

Kliebe, C. *et al.* (1985) 'Evolution of plasmid-coded resistance to broad-spectrum cephalosporins', *Antimicrobial Agents and Chemotherapy*, 28(2), pp. 302–307. doi: 10.1128/AAC.28.2.302.

Knudsen, P. K. *et al.* (2018) 'Transfer of a bla CTX-M-1-carrying plasmid between different Escherichia coli strains within the human gut explored by whole genome sequencing analyses', *Scientific Reports*, 8(1), p. 280. doi: 10.1038/s41598-017-18659-2.

Kuenzli, E. *et al.* (2014) 'High colonization rates of extended-spectrum β-lactamase (ESBL)-producing Escherichia coli in Swiss Travellers to South Asia– a prospective observational multicentre cohort study looking at epidemiology, microbiology and risk factors', *BMC Infectious Diseases*, 14(1), pp. 528–537. doi: 10.1186/1471-2334-14-528.

Kumar, S., Stecher, G. and Tamura, K. (2016) 'MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets', *Molecular biology and evolution*, 33(7), pp. 1870–1874. doi: 10.1093/molbev/msw054.

Kuroda, H. *et al.* (2012) 'Molecular characteristics of extended-spectrum β-lactamase-producing Escherichia coli in Japan: emergence of CTX-M-15-producing *E. coli* ST131', *Diagnostic Microbiology and Infectious Disease*. Elsevier Inc., 74(2), pp. 201–203. doi: 10.1016/j.diagmicrobio.2012.06.011.

Lagier, J. C. *et al.* (2015) 'Faecal microbiota transplantation for stool decolonization of OXA-48 carbapenemase-producing Klebsiella pneumoniae', *Journal of Hospital Infection*. The Healthcare Infection Society, 90(2), pp. 173–174. doi: 10.1016/j.jhin.2015.02.013.
Lanza, V. F. *et al.* (2014) 'Plasmid Flux in Escherichia coli ST131 Sublineages, Analyzed by Plasmid Constellation Network (PLACNET), a New Method for Plasmid Reconstruction from Whole Genome Sequences.', *PLoS genetics*, 10(12), p. e1004766. doi: 10.1371/journal.pgen.1004766.

Lartigue, M.-F., Poirel, L. and Nordmann, P. (2004) 'Diversity of genetic environment of bla(CTX-M) genes.', *FEMS microbiology letters*, 234(2), pp. 201–7. doi: 10.1016/j.femsle.2004.01.051.

Laube, H. *et al.* (2014) 'Transmission of ESBL/AmpC-producing Escherichia coli from broiler chicken farms to surrounding areas', *Veterinary Microbiology*, 172(3–4), pp. 519–527. doi: 10.1016/j.vetmic.2014.06.008.

Lavollay, M. *et al.* (2006) 'Clonal dissemination of a CTX-M-15 beta-lactamase-producing Eschenchia coli strain in the Paris Area, Tunis, and Bangui', *Antimicrobial Agents and Chemotherapy*, 50(7), pp. 2433–2438. doi: 10.1128/AAC.00150-06.

Laxminarayan, R. *et al.* (2013) 'Antibiotic resistance-the need for global solutions', *The Lancet Infectious Diseases*, 13(12), pp. 1057–1098. doi: 10.1016/S1473-3099(13)70318-9.

Lazarus, B. *et al.* (2015) 'Do human extraintestinal Escherichia coli infections resistant to expanded-spectrum cephalosporins originate from food-producing animals? A systematic review', *Clinical Infectious Diseases*, 60(3), pp. 439–452. doi: 10.1093/cid/ciu785.

Lecky, D. M. *et al.* (2017) 'Informing future research for carriage of multiresistant Gram-negative bacteria : problems with recruiting to an English stool sample community prevalence study', *BMJ Open*, pp. 1–7. doi: 10.1136/bmjopen-2017-017947.

Lee, M. Y. *et al.* (2011) 'High prevalence of CTX-M-15-producing Klebsiella pneumoniae isolates in Asian countries: Diverse clones and clonal dissemination', *International Journal of Antimicrobial Agents*, 38, pp. 160–163. doi: 10.1016/j.ijantimicag.2011.03.020.

Lemon, J. K. *et al.* (2017) 'Rapid nanopore sequencing of plasmids and resistance gene detection in clinical isolates', *Journal of Clinical Microbiology*, 55(12), pp. 3530–3543. doi: 10.1128/JCM.01069-17.

Li, H. and Durbin, R. (2010) 'Fast and accurate long-read alignment with Burrows-Wheeler transform', *Bioinformatics*, 26(5), pp. 589–595. doi: 10.1093/bioinformatics/btp698.

Li, J.-J. *et al.* (2015) 'Complete nucleotide sequences of bla(CTX-M)-harboring IncF plasmids from community-associated Escherichia coli strains in the United States.', *Antimicrobial agents and chemotherapy*, 59(6), pp. 3002–7. doi: 10.1128/AAC.04772-14.

Li, R. *et al.* (2017) 'Complete genetic analysis of plasmids carrying mcr-1 and other resistance genes in an Escherichia coli isolate of animal origin', *The Journal of antimicrobial chemotherapy*, 72(3), pp. 696–699. doi: 10.1093/jac/dkw509.

Li, S. *et al.* (2015) 'Spread of extended-spectrum beta-lactamase-producing Escherichia coli from a swine farm to the receiving river', *Environmental Science and Pollution Research*, 22(17), pp. 13033–13037. doi: 10.1007/s11356-015-4575-7.

Livermore, D. M. *et al.* (2006) 'CTX-M: changing the face of ESBLs in Europe', *Journal of Antimicrobial Chemotherapy*, 59(2), pp. 165–174. doi: 10.1093/jac/dkl483.

Livermore, D. M. (2008) 'Defining an extended-spectrum b -lactamase', *Clinical microbiology and infection*, 14, pp. 3–10.

Livermore, D. M. and Hawkey, P. M. (2005) 'CTX-M: Changing the face of ESBLs in the UK', *Journal of Antimicrobial Chemotherapy*, 56(3), pp. 451–454. doi: 10.1093/jac/dki239.

Lob, S. H. *et al.* (2015) 'Trends in susceptibility of Escherichia coli from intra-abdominal infections to ertapenem and comparators in the United States according to data from the SMART program, 2009 to 2013', *Antimicrobial Agents and Chemotherapy*, 59(6), pp. 3606–3610. doi: 10.1128/AAC.05186-14.

Lübbert, C. *et al.* (2015) 'Colonization with extended-spectrum beta-lactamase-producing and carbapenemase-producing Enterobacteriaceae in international travelers returning to Germany', *International Journal of Medical Microbiology*, 305(1), pp. 148–156. doi: 10.1016/j.ijmm.2014.12.001.

Lübbert, C. *et al.* (2017) 'Environmental pollution with antimicrobial agents from bulk drug manufacturing industries in Hyderabad, South India, is associated with dissemination of extended-spectrum beta-lactamase and carbapenemase-producing pathogens', *Infection*. Springer Berlin Heidelberg, 45(4), pp. 479–491. doi: 10.1007/s15010-017-1007-2.

Ma, J. *et al.* (2012) 'Characterization of extended-spectrum β-lactamase genes found among Escherichia coli isolates from duck and environmental samples obtained on a duck farm', *Applied and Environmental Microbiology*, 78(10), pp. 3668–3673. doi: 10.1128/AEM.07507-11.

Ma, L., Siu, L. K. and Lu, P. L. (2011) 'Effect of spacer sequences between bla CTX-M and ISE cp1 on bla CTX-M expression', *Journal of Medical Microbiology*, 60(12), pp. 1787–1792. doi: 10.1099/jmm.0.033910-0.

Maclaren, G. and Shann, F. (2010) 'Choramphenicol and Thiamphenicol', in Grayson, M. (ed.) *Kuchers' The Use of Antibiotics*. sixth. London: ASM Press, pp. 1008–1030.

Maiden, M. C. J. *et al.* (1998) 'Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms', *Proceedings of the National Academy of Sciences*, 95(6), pp. 3140–3145. doi: 10.1073/pnas.95.6.3140.

Malgorzata, L. *et al.* (2004) 'Genome of Bacteriophage P1', *Journal of Bacteriology*, 186(21), pp. 7032–7068. doi: 10.1128/JB.186.21.7032.

Manges, A. R., Steiner, T. S. and Wright, A. J. (2016) 'Fecal microbiota transplantation for the intestinal decolonization of extensively antimicrobial-resistant opportunistic pathogens: A review', *Infectious Diseases*, 48(8), pp. 587–592. doi: 10.1080/23744235.2016.1177199.

Maravić, A. *et al.* (2015) 'Prevalence and diversity of extended-spectrum-β-lactamase-producing Enterobacteriaceae from marine beach waters', *Marine Pollution Bulletin*, 90(1–2), pp. 60–67. doi: 10.1016/j.marpolbul.2014.11.021.

Marcadé, G. *et al.* (2009) 'Replicon typing of plasmids in Escherichia coli producing extended-spectrum beta-lactamases.', *The Journal of antimicrobial chemotherapy*, 63(1), pp. 67–71. doi: 10.1093/jac/dkn428.

Mata, C. *et al.* (2010) 'In vivo transmission of a plasmid coharbouring blaDHA-1 and qnrB genes between Escherichia coli and Serratia marcescens', *FEMS Microbiology Letters*, 308(1), pp. 24–28. doi: 10.1111/j.1574-6968.2010.01980.x.

Mata, C. *et al.* (2011) 'Association of blaDHA-1 and qnrB genes carried by broad-host-range plasmids among isolates of Enterobacteriaceae at a Spanish hospital', *Clinical Microbiology and Infection*, 17(10), pp. 1514–1517. doi: 10.1111/j.1469-0691.2011.03539.x.

Matsumura, Y. *et al.* (2015) 'CTX-M-27- and CTX-M-14-producing, ciprofloxacin-resistant Escherichia coli of the H30 subclonal group within ST131 drive a Japanese regional ESBL epidemic', *Journal of Antimicrobial Chemotherapy*, 70, pp. 1639–49. doi: 10.1093/jac/dkv017.

McDonald, J. A. K. *et al.* (2013) 'Evaluation of microbial community reproducibility, stability and composition in a human distal gut chemostat model', *Journal of Microbiological Methods*. Elsevier B.V., 95(2), pp. 167–174. doi: 10.1016/j.mimet.2013.08.008.

McNulty, C. A. M. *et al.* (2018) 'CTX-M ESBL-producing Enterobacteriaceae: estimated prevalence in adults in England in 2014', *Journal of Antimicrobial Chemotherapy*, 73, pp. 1368–1388. doi: 10.1093/jac/dky007.

Meinersmann, R. J. *et al.* (2016) 'Complete Genome Sequence of a Colistin Resistance mcr-1 Gene-Bearing Escherichia coli Strain from the United States Richard', *Genome announcements*, 4(5), pp. 2015–2016. doi: 10.1128/genomeA.00898-16.Copyright.

Van Melderen, L. and De Bast, M. S. (2009) 'Bacterial toxin-Antitoxin systems: More than selfish entities?', *PLoS Genetics*, 5(3). doi: 10.1371/journal.pgen.1000437.

Micenková, L. *et al.* (2014) 'Characterization of Human Uropathogenic ESBL-Producing Escherichia coli in the Czech Republic: Spread of CTX-M-27-Producing Strains in a University Hospital.', *Microbial drug resistance (Larchmont, N.Y.)*, 20(6), pp. 610–7. doi: 10.1089/mdr.2014.0013.

Milkman, R. (1973) 'Electrophoretic variation in escherichia coli from natural sources', *Science*, 182(4116), pp. 1024–1026. doi: 10.1126/science.182.4116.1024.

Mizuno, Y. *et al.* (2016) 'Extended-spectrum β-lactamase-producing Enterobacteriaceae colonisation in long-term overseas business travellers', *Travel Medicine and Infectious Disease*. Elsevier Ltd, 14(6), pp. 561–567. doi: 10.1016/j.tmaid.2016.09.012.

Mnif, B. *et al.* (2013) 'Molecular epidemiology of extended-spectrum beta-lactamase-producing Escherichia coli in Tunisia and characterization of their virulence factors and plasmid addiction systems', *BMC Microbiology*, 13(1), p. 147. doi: 10.1186/1471-2180-13-147.

Modi SR, Lee H, Spina CS, and C. J. (2013) 'Antibiotic Treatment Expands the Resistance Reservoir and Ecological Network of the Phage Metagenome', *Nature*, 499(7457), pp. 219–222. doi: 10.1016/bs.mcb.2015.01.016.Observing.

Mourand, G. *et al.* (2017) 'Escherichia coli Probiotic Strain ED1a in Pigs Has a Limited Impact on the Gut Carriage of Extended Spectrum beta- Lactamase-Producing *E. coli*', *Antimicrobial Agents and Chemotherapy*, 61.

Nahid, F., Zahra, R. and Sandegren, L. (2017) 'A blaOXA-181-harbouring multi-resistant ST147 Klebsiella pneumoniae isolate from Pakistan that represent an intermediate stage towards pan-drug resistance', *PLoS ONE*, 12, pp. 1–9. doi: 10.1371/journal.pone.0189438.

Nakane, K., Kawamura, K. and Goto, K. (2016) 'Long-Term Colonization by blaCTX-M - Harboring Escherichia coli in Healthy Japanese People Engaged in Food Handling', *Applied and Environmental Microbiology*, 82(6), pp. 1818–1827. doi: 10.1128/AEM.02929-15.Editor.

Naseer, U. *et al.* (2012) 'Fecal colonization of VIM-1-producing Klebsiella pneumoniae and in vivo transfer of multidrug-resistant IncN plasmid in a renal transplant patient', *Diagnostic Microbiology and Infectious Disease*. Elsevier Inc., 72(4), pp. 363–366. doi: 10.1016/j.diagmicrobio.2011.12.010.

Neuwirth, C. *et al.* (2001) 'Evidence of in vivo transfer of a plasmid encoding the extended-spectrum beta-lactamase TEM-24 and other resistance factors among different members of the family Enterobacteriaceae', *Journal of Clinical Microbiology*, 39(5), pp. 1985–1988. doi: 10.1128/JCM.39.5.1985-1988.2001.

NHS Improvement (2017) *Ambition to halve healthcare associated Gram-negative blood stream infections by March 2021*.

Nhu, N. T. K. *et al.* (2010) 'The sudden dominance of blaCTX-Mharbouring plasmids in Shigella spp. circulating in Southern Vietnam', *PLoS Neglected Tropical Diseases*, 4(6). doi: 10.1371/journal.pntd.0000702.

Nicolas-Chanoine, M. H. *et al.* (2008) 'Intercontinental emergence of Escherichia coli clone O25:H4-ST131 producing CTX-M-15', *Journal of Antimicrobial Chemotherapy*, 61(2), pp. 273–281. doi: 10.1093/jac/dkm464.

Nicolas-Chanoine, M. H., Bertrand, X. and Madec, J. Y. (2014) 'Escherichia coli st131, an intriguing clonal group', *Clinical Microbiology Reviews*, 27(3), pp. 543–574. doi: 10.1128/CMR.00125-13.

Nielsen, A. K. and Gerdes, K. (1995) 'Mechanism of post-segregational killing by hok-homologue pnd of plasmid R483: Two translational control elements in the pnd mRNA', *Journal of Molecular Biology*, 249(2), pp. 270–282. doi: 10.1006/jmbi.1995.0296.

Nordahl Petersen, T. *et al.* (2015) 'Meta-genomic analysis of toilet waste from long distance flights; a step towards global surveillance of infectious diseases and antimicrobial resistance.', *Scientific reports*. Nature Publishing Group, 5(April), p. 11444. doi: 10.1038/srep11444.

Nordmann, P. *et al.* (2011) 'Does broad-spectrum β-lactam resistance due to NDM-1 herald the end of the antibiotic era for treatment of infections caused by Gram-negative bacteria?', *Journal of Antimicrobial Chemotherapy*, 66(4), pp. 689–692. doi: 10.1093/jac/dkq520.

Nordmann, P., Naas, T. and Poirel, L. (2011) 'Global spread of carbapenemase producing Enterobacteriaceae', *Emerging Infectious Diseases*, 17(10), pp. 1791–1798. doi: http://dx.doi.org/10.3201/eid1710.110655.

Notredame, C., Higgins, D. G. and Heringa, J. (2000) 'T-coffee: A novel method for fast and accurate multiple sequence alignment', *Journal of Molecular Biology*, 302(1), pp. 205–217. doi: 10.1006/jmbi.2000.4042.

O'Neil, J. (2016) *Tackling Drug-Resistant Infections Globally : Final Report and Recommendations, The Review on Antimicrobial Resistance*.

Ochman, H. and Selander, R. K. (1984) 'Standard reference strains of Escherichia coli from natural populations', *Journal of Bacteriology*, 157(2), pp. 690–693. doi: 10.1152/jn.01312.2007.

Ogawara, H. (1981) 'Antibiotic resistance in pathogenic and producing bacteria, with special reference to beta-lactam antibiotics.', *Microbiological reviews*, 45(4), pp. 591–619.

Oliver, A. *et al.* (2005) 'CTX-M-10 linked to a phage-related element is widely disseminated among Enterobacteriaceae in a Spanish Hospital', *Antimicrobial Agents and Chemotherapy*, 49(4), pp. 1567–1571. doi: 10.1128/AAC.49.4.1567-1571.2005.

Olson, A. B. *et al.* (2005) 'Identification of a progenitor of the CTX-M-9 group of extended-spectrum β-lactamases from Kluyvera georgiana isolated in Guyana', *Antimicrobial Agents and Chemotherapy*, 49(5), pp. 2112–2115. doi: 10.1128/AAC.49.5.2112-2115.2005.

Orskov, I. *et al.* (1977) 'Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*', *Bacteriological Reviews*, 41(3), pp. 667–710.

Osawa, K. *et al.* (2015) 'Molecular Characteristics of Extended-Spectrum β-Lactamase-Producing Escherichia coli in a University Teaching Hospital.', *Microbial drug resistance (Larchmont, N.Y.)*, 21(2), pp. 130–9. doi: 10.1089/mdr.2014.0083.

Ostholm-Balkhed, A. *et al.* (2013) 'Travel-associated faecal colonization with ESBL-producing Enterobacteriaceae: incidence and risk factors.', *The Journal of antimicrobial chemotherapy*, 68(9), pp. 2144–53. doi: 10.1093/jac/dkt167.

Overbeek, R. *et al.* (2014) 'The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST)', *Nucleic Acids Research*, 42(D1), pp. 206–214. doi: 10.1093/nar/gkt1226.

Overdevest, I. *et al.* (2011) 'Extended-spectrum β-lactamase genes of Escherichia coli in chicken meat and humans, The Netherlands.', *Emerging infectious diseases*, 17(7), pp. 1216–22. doi: 10.3201/eid1707.110209.

Paltansing, S. *et al.* (2013) 'Extended-spectrum beta-lactamase-producing Enterobacteriaceae among travelers from the Netherlands.', *Emerging infectious diseases*, 19(8), pp. 1206–1213. doi: 10.3201/eid.1908.130257.

Partridge, S. R. (2011) 'Analysis of antibiotic resistance regions in Gram-negative bacteria', *FEMS Microbiology Reviews*, 35(5), pp. 820–855. doi: 10.1111/j.1574-6976.2011.00277.x.

Partridge, S. R. *et al.* (2012) 'pEl1573 carrying bla IMP-4, from Sydney, Australia, is closely related to other IncL/M plasmids', *Antimicrobial Agents and Chemotherapy*, 56(11), pp. 6029–6032. doi: 10.1128/AAC.01189-12.

Patrick De Boever, Deplancke, B. and Verstraete, W. (2000) 'Fermentation by Gut Microbiota Cultured in a Simulator of the Human Intestinal Microbial Ecosystem Is Improved by Supplementing a Soygerm Powder', *The Journal of Nutrition*, 130(10), pp. 2599–2606. doi: 10.1093/jn/130.10.2599.

Peeters, L. *et al.* (2017) 'Selection and transfer of an IncI1-tet(A) plasmid of *E. coli* in an ex vivo model of the porcine cecum at doxycycline concentrations caused by cross-contaminated feed', *ARPN Journal of Engineering and Applied Sciences*, 12(10), pp. 3218–3221. doi: 10.1111/ijlh.12426.

Pehrsson, E. C. *et al.* (2016) 'Interconnected microbiomes and resistomes in low-income human habitats', *Nature*. Nature Publishing Group, 533(7602), pp. 212–216. doi: 10.1038/nature17672.

Peirano, G. *et al.* (2012) 'Molecular epidemiology over an 11-year period (2000 to 2010) of extended-spectrum ??-lactamase-producing Escherichia coli causing bacteremia in a centralized Canadian region', *Journal of Clinical Microbiology*, 50(2), pp. 294–299. doi: 10.1128/JCM.06025-11.

Peirano, G. *et al.* (2014) 'Characteristics of Escherichia coli sequence type 131 isolates that produce extended-spectrum beta-lactamases: Global distribution of the H30-Rx sublineage', *Antimicrobial Agents and Chemotherapy*, 58(7), pp. 3762–3767. doi: 10.1128/AAC.02428-14.

Peirano, G. *et al.* (2017) 'Rates of colonization with extended-spectrum β-lactamase-producing Escherichia coli in Canadian travellers returning from South Asia: a cross-sectional assessment.', *CMAJ open*, 5(4), pp. E850–E855. doi: 10.9778/cmajo.20170041.

Pires, J. *et al.* (2016) 'Polyclonal Intestinal Colonization with Extended-Spectrum Cephalosporin-Resistant Enterobacteriaceae upon Traveling to India', *Frontiers in*

Microbiology, 7(July), p. 1069. doi: 10.3389/fmicb.2016.01069.

Pitout, J. D. D. *et al.* (2009) 'Molecular characteristics of extended-spectrum-beta-lactamase-producing Escherichia coli isolates causing bacteremia in the Calgary Health Region from 2000 to 2007: emergence of clone ST131 as a cause of community-acquired infections.', *Antimicrobial agents and chemotherapy*, 53(7), pp. 2846–51. doi: 10.1128/AAC.00247-09.

Platell, J. L. *et al.* (2011) 'Multidrug-resistant extraintestinal pathogenic Escherichia coli of sequence type ST131 in animals and foods', *Veterinary Microbiology*. Elsevier B.V., 153(1–2), pp. 99–108. doi: 10.1016/j.vetmic.2011.05.007.

Poirel, L., Decousser, J. W. and Nordmann, P. (2003) 'Insertion sequence ISEcp1B is involved in expression and mobilization of a blaCTX-M β-lactamase gene', *Antimicrobial Agents and Chemotherapy*, 47(9), pp. 2938–2945. doi: 10.1128/AAC.47.9.2938-2945.2003.

Poirel, L., Ka, P. and Nordmann, P. (2002) 'Chromosome-Encoded Ambler Class A beta-Lactamase of Kluyvera georgiana , a Probable Progenitor of a Subgroup of CTX-M', 46(12), pp. 4038–4040. doi: 10.1128/AAC.46.12.4038.

Price, L. B., Johnson, J. R. and Aziz, M. (2013) 'The Epidemic of Extended-Spectrum- β -Lactamase-Producing Escherichia coli ST131 Is Driven by a Single Highly Pathogenic Subclone, H 30-Rx', *mBio*, 4(7), pp. 377–13. doi: 10.1128/mBio.00377-13.Editor.

Public Health England (2013) *Acute trust toolkit for the early detection management and control of CPE*.

Public Health England (2018) *English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) Report 2018*, *Public Health England*. doi: 2014362.

Rafiq, Z., Sam, N. and Vaidyanathan, R. (2016) 'Whole genome sequence of Klebsiella pneumoniae U25, a hypermucoviscous, multidrug resistant, biofilm producing isolate from India', *Memorias do Instituto Oswaldo Cruz*, 111(2), pp. 144–146. doi: 10.1590/0074-02760150423.

Randall, L. *et al.* (2014) 'Detection of antibiotic residues and association of cefquinome residues with the occurrence of Extended-Spectrum β-Lactamase (ESBL)-producing bacteria in waste milk samples from dairy farms in England and Wales in 2011', *Research in Veterinary Science*. Elsevier Ltd, 96(1), pp. 15–24. doi: 10.1016/j.rvsc.2013.10.009.

Ranjan, A. *et al.* (2017) 'Comparative Genomics of *Escherichia coli* Isolated from Skin and Soft Tissue and Other Extraintestinal Infections', *mBio*, 8(4), pp. e01070-17. doi: 10.1128/mBio.01070-17.

Rao, L. *et al.* (2014) 'Increasing prevalence of extended-spectrum cephalosporin-resistant Escherichia coli in food animals and the diversity of CTX-M genotypes during 2003–2012',

Veterinary Microbiology. Elsevier B.V., 172(3–4), pp. 534–541. doi: 10.1016/j.vetmic.2014.06.013.

Rembacken, B. *et al.* (1999) 'Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: A randomised trial', *Lancet*, 354(9179), pp. 635–639. doi: 10.1016/S0140-6736(98)06343-0.

Reuland, E A *et al.* (2016) 'Prevalence and risk factors for carriage of ESBL-producing Enterobacteriaceae in Amsterdam', *J Antimicrob Chemother*, 71, pp. 1076–1082. doi: 10.1093/jac/dkv441.

Reuland, E. A. *et al.* (2016) 'Travel to Asia and traveller's diarrhoea with antibiotic treatment are independent risk factors for acquiring ciprofloxacin-resistant and extended spectrum β-lactamase-producing Enterobacteriaceae—a prospective cohort study', *Clinical Microbiology and Infection*. Elsevier Ltd, 22(8), pp. 731.e1-731.e7. doi: 10.1016/j.cmi.2016.05.003.
Ripoll-rozada, J. *et al.* (2016) 'Type IV traffic ATPase TrwD as molecular target to inhibit bacterial conjugation', *Molecular Microbiology*, 100(5), pp. 912–921. doi: 10.1111/mmi.13359.

Robinson, T. P. *et al.* (2016) 'Animal production and antimicrobial resistance in the clinic', *The Lancet*. Elsevier Ltd, 387(10014), pp. e1–e3. doi: 10.1016/S0140-6736(15)00730-8.
Rodrigues, C. *et al.* (2016) 'An update on faecal carriage of ESBL-producing Enterobacteriaceae by Portuguese healthy humans: Detection of the H30 subclone of B2-ST131 Escherichia coli producing CTX-M-27', *Journal of Antimicrobial Chemotherapy*, 71(4), pp. 1120–1122. doi: 10.1093/jac/dkv443.

Rodriguez, M. M. *et al.* (2010) 'Novel chromosome-encoded CTX-M-78 beta-lactamase from a Kluyvera georgiana clinical isolate as a putative origin of CTX-M-25 subgroup', *Antimicrobial Agents and Chemotherapy*, 54(7), pp. 3070–3071. doi: 10.1128/AAC.01615-09.

Rodríguez, M. M. *et al.* (2004) 'Chromosome-encoded CTX-M-3 from Kluyvera ascorbata: A possible origin of plasmid-borne CTX-M-1-derived cefotaximases', *Antimicrobial Agents and Chemotherapy*, 48(12), pp. 4895–4897. doi: 10.1128/AAC.48.12.4895-4897.2004.

Roer, L. *et al.* (2017) 'WGS-based surveillance of third-generation cephalosporin-resistant Escherichia coli from bloodstream infections in Denmark', *Journal of Antimicrobial Chemotherapy*, 72(7), pp. 1922–1929. doi: 10.1093/jac/dkx092.

Rolinson, G. N. and Stevens, S. (1961) 'Microbiological Studies on a New Broad-Spectrum Penicillin, "Penbritin"', *British Medical Journal*, 2(5246), pp. 191–196. doi: 10.1136/bmj.2.5246.191.

Roy, S. *et al.* (2015) 'Insight into neonatal septicaemic Escherichia coli from India with respect to phylogroups, serotypes, virulence, extended-spectrum-β-lactamases and association of ST131 clonal group', *Epidemiology and Infection*, 143(15), pp. 3266–3276. doi:

10.1017/S0950268815000539.

Ruppé, E. *et al.* (2015) 'High Rate of Acquisition but Short Duration of Carriage of Multidrug-Resistant Enterobacteriaceae after Travel to the Tropics', *Clinical Infectious Diseases*, 61(4), pp. 593–600. doi: 10.1093/cid/civ333.

Rutherford, K. *et al.* (2000) 'Artemis: Sequence visualization and annotation', *Bioinformatics*, 16(10), pp. 944–945. doi: 10.1093/bioinformatics/16.10.944.

van Schaik, W. (2015) 'The human gut resistome', *Royal Society Publisher*, 370, p. 20140087. doi: 10.1098/rstb.2014.0087.

Schatz, A., Bugie, E. and Waksman, S. (1944) 'Streptomycin, a Substance Exhibiting Antibiotic Activity Against Gram- Positive and Gram-Negative Bacteria', *Experimental Biology and Medicine*, 55(1), pp. 66–69. doi: 10.3181/00379727-55-14461.

Seemann, T. (2014) 'Prokka: Rapid prokaryotic genome annotation', *Bioinformatics*, 30(14), pp. 2068–2069. doi: 10.1093/bioinformatics/btu153.

Seemann, T. (2017a) *abricate*, *GitHub, Inc.* Available at: https://github.com/tseemann/abricate.

Seemann, T. (2017b) *mlst*, *GitHub, Inc.* Available at: https://github.com/tseemann/mlst.

Seemann, T. (2017c) *snippy*, *GitHub, Inc.* Available at: https://github.com/tseemann/snippy.

Seemann, T. (2017d) *snp-dists*, *GitHub, Inc.* Available at: https://github.com/tseemann/snp-dists.

Sekizuka, T. *et al.* (2017) 'Elucidation of quantitative structural diversity of remarkable rearrangement regions, shufflons, in IncI2 plasmids.', *Scientific reports*. Springer US, 7(1), p. 928. doi: 10.1038/s41598-017-01082-y.

Selander, R. K. *et al.* (1987) 'Population genetics of pathogenic bacteria', *Microbial Pathogenesis*, 3(1), pp. 1–7. doi: 10.1016/0882-4010(87)90032-5.

Selander, R. K. and Levin, B. R. (1980) 'Genetic diversity and structure in Escherichia coli populations', *Science*, 210(4469), pp. 545–547. doi: 10.1126/science.6999623.

Sennati, S. *et al.* (2016) 'pHN7A8-related multiresistance plasmids (*bla* $_{\text{CTX-M-65}}$, *fosA3* and *rmtB*) detected in clinical isolates of *Klebsiella pneumoniae* from Bolivia: intercontinental plasmid dissemination?', *Journal of Antimicrobial Chemotherapy*, 71(6), pp. 1732–1734. doi: 10.1093/jac/dkv506.

Shahid, M. (2010) 'Citrobacter spp. simultaneously harboring blaCTX-M, bla TEM, blaSHV,

blaampC, and insertion sequences IS26 and orf513: An evolutionary phenomenon of recent concern for antibiotic resistance', *Journal of Clinical Microbiology*, 48(5), pp. 1833–1838. doi: 10.1128/JCM.01467-09.

Shin, J. and Ko, K. S. (2015) 'A Plasmid bearing the blaCTX-M-15 gene and phage P1-like sequences from a sequence type 11 Klebsiella pneumoniae isolate', *Antimicrobial Agents and Chemotherapy*, 59(10), pp. 6608–6610. doi: 10.1128/AAC.00265-15.

Singh, R. *et al.* (2014) 'Donor feces infusion for eradication of Extended Spectrum beta-Lactamase producing Escherichia coli in a patient with end stage renal disease', *Clinical Microbiology and Infection*. European Society of Clinical Infectious Diseases, 20(11), pp. O977–O978. doi: 10.1111/1469-0691.12683.

Smet, A. *et al.* (2010) 'Complete nucleotide sequence of CTX-M-15-plasmids from clinical Escherichia coli isolates: Insertional events of transposons and insertion sequences', *PLoS ONE*, 5(6), pp. 1–8. doi: 10.1371/journal.pone.0011202.

Smith, D. H. (1967) 'R factors mediate resistance to mercury, nickel, and cobalt', *Science*, 156(3778), pp. 1114–1116. doi: 10.1126/science.156.3778.1114.

Smith, H. W. (1969) 'Transfer of antibiotic resistance from animal and human strains of Escherichia coli to resident *E. coli* in the alimentary tract of man.', *The Lancet*, 1(7607), pp. 1174–6. doi: 10.1136/vr.85.2.31.

Song, W. *et al.* (2011) 'Chromosome-encoded AmpC and CTX-M extended-spectrum β-lactamases in clinical isolates of Proteus mirabilis from Korea', *Antimicrobial Agents and Chemotherapy*, 55(4), pp. 1414–1419. doi: 10.1128/AAC.01835-09.

Sonnenborn, U. and Schulze, J. (2009) 'The non-pathogenic Escherichia coli strain Nissle 1917-features of a versatile probiotic', *Microbial Ecology in Health and Disease*, 21(3–4), pp. 122–158. doi: 10.3109/08910600903444267.

Soto-Alonso, G. *et al.* (2015) 'Isolation of a conjugative F-like plasmid from a multidrug-resistant Escherichia coli strain CM6 using tandem shock wave-mediated transformation', *Journal of Microbiological Methods*. Elsevier B.V., 114, pp. 1–8. doi: 10.1016/j.mimet.2015.04.007.

Springer, B. *et al.* (2001) 'Mechanisms of Streptomycin Resistance: Selection of Mutations in the 16S rRNA Gene Conferring Resistance', *Antimicrobial Agents and Chemotherapy*, 45(10), pp. 2877–2884. doi: 10.1128/AAC.45.10.2877.

Stecher, B. *et al.* (2012) 'Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae.', *Proceedings of the National Academy of Sciences of the United States of America*, 109(4), pp. 1269–74. doi: 10.1073/pnas.1113246109.

Stoesser, N. *et al.* (2016) 'Evolutionary history of the global emergence of the Escherichia coli epidemic clone ST131', *mBio*, 7(2), pp. 1–15. doi: 10.1128/mBio.02162-15.Invited.

Stokes, M. O. *et al.* (2012) 'Detection and characterization of pCT-like plasmid vectors for blaCTX-M-14 in Escherichia coli isolates from humans, turkeys and cattle in England and Wales.', *The Journal of antimicrobial chemotherapy*, 67(7), pp. 1639–44. doi: 10.1093/jac/dks126.

Subirats, J. *et al.* (2016) 'Metagenomic analysis reveals that bacteriophages are reservoirs of antibiotic resistance genes', *International Journal of Antimicrobial Agents*. Elsevier B.V., 48(2), pp. 163–167. doi: 10.1016/j.ijantimicag.2016.04.028.

Sugawara, Y. *et al.* (2017) 'Genetic characterization of blaNDM-harboring plasmids in carbapenem-resistant Escherichia coli from Myanmar', *PLoS ONE*, 12(9), pp. 1–15. doi: 10.1371/journal.pone.0184720.

Sutherland, R., Croydon, E. A. P. and Rolinson, G. N. (1972) 'Amoxycillin : A new Semi-synthetic Penicillin', *British Medical Journal*, 3, pp. 13–16.

Tängdén, T. *et al.* (2010) 'Foreign travel is a major risk factor for colonization with Escherichia coli producing CTX-M-type extended-spectrum β-lactamases: A prospective study with Swedish volunteers', *Antimicrobial Agents and Chemotherapy*, 54(9), pp. 3564–3568. doi: 10.1128/AAC.00220-10.

Tannock, G. W. *et al.* (2011) 'Testing probiotic strain Escherichia coli Nissle 1917 (Mutaflor) for its ability to reduce carriage of multidrug-resistant *E. coli* by elderly residents in long-term care facilities', *Journal of Medical Microbiology*, 60(3), pp. 366–370. doi: 10.1099/jmm.0.025874-0.

Tausova, D. *et al.* (2012) 'Escherichia coli with extended-spectrum β-lactamase and plasmid-mediated quinolone resistance genes in great cormorants and mallards in central europe', *Journal of Antimicrobial Chemotherapy*, 67(5), pp. 1103–1107. doi: 10.1093/jac/dks017.

Terawaki, Y., Takayasu, H. and Akiba, T. (1967) 'Thermosensitive replication of a kanamycin resistance factor', *Journal of Bacteriology*, 94, pp. 687–690.

Teunis, P. F. M. *et al.* (2018) 'Time to acquire and lose carriership of ESBL/ pAmpC producing *E. coli* in humans in the Netherlands', *PLoS ONE*, 3, pp. 1–14. doi: 10.1371/journal.pone.0193834.

Tham, J. *et al.* (2010) 'Extended-spectrum beta-lactamase-producing Escherichia coli in patients with travellers ' diarrhoea', 46(September 2009), pp. 275–280. doi: 10.3109/00365540903493715.

Thomas, C. M. and Nielsen, K. M. (2005) 'Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria', *Nature Reviews Microbiology*, 3(9), pp. 711–721. doi: 10.1038/nrmicro1234.

Tillekeratne, L. G. *et al.* (2016) 'Extended-spectrum ss-Lactamase-producing Enterobacteriaceae as a Common Cause of Urinary Tract Infections in Sri Lanka', *Infect Chemother*, 48(3), pp. 160–165. doi: 10.3947/ic.2016.48.3.160.

Toleman, M. A. and Walsh, T. R. (2011) 'Combinatorial events of insertion sequences and ICE in Gram-negative bacteria', *FEMS Microbiology Reviews*, 35(5), pp. 912–935. doi: 10.1111/j.1574-6976.2011.00294.x.

Treangen, T. J. *et al.* (2014) 'The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes', *Genome Biology*, 15(11), p. 524. doi: 10.1186/s13059-014-0524-x.

Tsuchimoto, S., Ohtsubo, H. and Ohtsubo, E. (1988) 'Stable Maintenance of Resistance Plasmid R100', *Journal of Bacteriology*, 170(4), pp. 1461–1466.

Vading, M. *et al.* (2016) 'Frequent acquisition of low-virulence strains of ESBL-producing Escherichia coli in travellers', *Journal of Antimicrobial Chemotherapy*, 71, pp. 3548–3555. doi: 10.1093/jac/dkw335.

Valenza, G. *et al.* (2015) 'Prevalence and genetic diversity of extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli in nursing homes in Bavaria, Germany', *Veterinary Microbiology*. Elsevier B.V., (15), p. doi: 10.1016/j.vetmic.2015.10.008. doi: 10.1016/j.vetmic.2015.10.008.

Valverde, A. *et al.* (2015) 'CTX-M-15-non-ST131 Escherichia coli isolates are mainly responsible of faecal carriage with ESBL-producing Enterobacteriaceae in travellers, immigrants and those visiting friends and relatives', *Clinical Microbiology and Infection*. Elsevier, 21(3), pp. 252.e1-252.e4. doi: 10.1016/j.cmi.2014.09.021.

Vandeputte, D. *et al.* (2015) 'Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates', *Gut*, 65, pp. 57–62. doi: 10.1136/gutjnl-2015-309618.

Vlieghe, E. R. *et al.* (2015) 'Prevalence and distribution of beta-lactamase coding genes in third-generation cephalosporin-resistant Enterobacteriaceae from bloodstream infections in Cambodia', *European Journal of Clinical Microbiology and Infectious Diseases*, 34(6), pp. 1223–1229. doi: 10.1007/s10096-015-2350-9.

Walia, S. K. *et al.* (1987) 'Characterization of Self-Transmissible Plasmids Determining Lactose Fermentation and Multiple Antibiotic Resistance in Clinical Strains of Klebsiella pneumoniae Lactose fermentation is an important dif- ferential test in diagnostic bacteriology . The path',

Plasmid, 12, pp. 3–12.

Walsh, T. R. *et al.* (2011) 'Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: An environmental point prevalence study', *The Lancet Infectious Diseases*. Elsevier Ltd, 11(5), pp. 355–362. doi: 10.1016/S1473-3099(11)70059-7.

Walsh, T. R., Toleman, M. A. and Jones, R. N. (2007) 'Comment on: Occurrence, prevalence and genetic environment of CTX-M beta-lactamases in Enterobacteriaceae from Indian hospitals', *Journal of Antimicrobial Chemotherapy*, 59, pp. 799–820. doi: 10.1093/jac/dkl532.
Wang, J. *et al.* (2014) 'Nucleotide sequences of 16 transmissible plasmids identified in nine multidrug-resistant Escherichia coli isolates expressing an ESBL phenotype isolated from food-producing animals and healthy humans', *Journal of Antimicrobial Chemotherapy*, 69(10), pp. 2658–2668. doi: 10.1093/jac/dku206.

Wang, S. *et al.* (2016) 'Antimicrobial Resistance and Molecular Epidemiology of Escherichia coli Causing Bloodstream Infections in Three Hospitals in Shanghai, China', *Plos One*, 11(1), p. doi: 10.1371/journal.pone.0147740. doi: 10.1371/journal.pone.0147740.

Watanabe, T. and Fukasawa, T. (1961) 'Episome-mediated transfer of drug resistance in Enterobacteriaceae. I. Transfer of resistance factors by conjugation.', *Journal of bacteriology*, 81, pp. 669–678.

Wei, Y. *et al.* (2015) 'Fecal microbiota transplantation restores dysbiosis in patients with methicillin resistant Staphylococcus aureus enterocolitis', *BMC Infectious Diseases*. BMC Infectious Diseases, 15(1), pp. 1–8. doi: 10.1186/s12879-015-0973-1.

Weisenberg, S. A. *et al.* (2012) 'Extended Spectrum Beta-Lactamase-Producing Enterobacteriaceae in International Travelers and Non-Travelers in New York City', *PLoS ONE*, 7(9), pp. 2010–2013. doi: 10.1371/journal.pone.0045141.

Wellington, E. M. H. *et al.* (2013) 'The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria.', *The Lancet infectious diseases*. Elsevier Ltd, 13(2), pp. 155–65. doi: 10.1016/S1473-3099(12)70317-1.

WHO (2015) *Global action plan on antimicrobial resistance: Geneva*, *World Health Organization, 2015*. Available at: https://www.who.int/antimicrobial-resistance/global-action-plan/en/ (Accessed: 2 December 2018).

WHO (2016) *World Bank Improved Sanitation Facilities (% population with access)*, *WHO/UNICEF Joint Monitoring Programme (JMP) for Water Supply and Sanitation*. Available at: http://data.worldbank.org/indicator/SH.STA.ACSN (Accessed: 5 July 2016).

WHO (2017) *Global Antimicrobial Resistance Surveillance System (GLASS) report: Early implementation 2016-2017*. Geneva. doi: 10.1016/S1473-3099(18)30060-4.

Wick, R. R. *et al.* (2017) 'Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads', *PLOS Computational Biology*, 13(6), p. e1005595. doi: 10.1371/journal.pcbi.1005595.

Wickramasinghe, N. H. *et al.* (2012) 'High community faecal carriage rates of CTX-M ESBL-producing Escherichia coli in a specific population group in Birmingham, UK.', *The Journal of antimicrobial chemotherapy*, 67(5), pp. 1108–13. doi: 10.1093/jac/dks018.

von Wintersdorff, C. J. H. *et al.* (2014) 'High rates of antimicrobial drug resistance gene acquisition after international travel, The Netherlands.', *Emerging infectious diseases*, 20(4), pp. 649–57. doi: 10.3201/eid.2004.131718.

Winther, K. S. and Gerdes, K. (2011) 'Enteric virulence associated protein VapC inhibits translation by cleavage of initiator tRNA', *Proceedings of the National Academy of Sciences*, 108(18), pp. 7403–7407. doi: 10.1073/pnas.1019587108.

Woerther, P.-L. *et al.* (2010) 'Emergence and dissemination of extended-spectrum beta-lactamase-producing Escherichia coli in the community: lessons from the study of a remote and controlled population.', *The Journal of infectious diseases*, 202(4), pp. 515–523. doi: 10.1086/654883.

Woerther, P. L. *et al.* (2013) 'Trends in human fecal carriage of extended-spectrum beta-lactamases in the community: Toward the globalization of CTX-M', *Clinical Microbiology Reviews*, 26(4), pp. 744–758. doi: 10.1128/CMR.00023-13.

Woodford, N. *et al.* (2009) 'Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M enzymes in three major Escherichia coli lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone', *Antimicrobial Agents and Chemotherapy*, 53(10), pp. 4472–4482. doi: 10.1128/AAC.00688-09.

Woodford, N., Turton, J. F. and Livermore, D. M. (2011) 'Multiresistant Gram-negative bacteria: The role of high-risk clones in the dissemination of antibiotic resistance', *FEMS Microbiology Reviews*, 35, pp. 736–755. doi: 10.1111/j.1574-6976.2011.00268.x.

Wu, L. T. *et al.* (2005) 'Identification of a novel cephalosporinase (DHA-3) in Klebsiella pneumoniae isolated in Taiwan', *Clinical Microbiology and Infection*, 11, pp. 893–897. doi: 10.1111/j.1469-0691.2005.01252.x.

Xia, S. *et al.* (2014) 'Dominance of CTX-M-Type Extended-Spectrum β-Lactamase (ESBL)-Producing Escherichia coli Isolated from Patients with Community-Onset and Hospital-Onset Infection in China', *PLoS ONE*, 9(7), p. doi: 10.1371/journal.pone.0100707. doi: 10.1371/journal.pone.0100707.

Yagi, T. *et al.* (2000) 'A preliminary survey of extended-spectrum beta-lactamases (ESBLs) in

clinical isolates of Klebsiella pneumoniae and Escherichia coli in Japan.', *FEMS microbiology letters*, 184(1), pp. 53–6. doi: S0378-1097(00)00016-1 [pii].

Yaita, K. *et al.* (2014) 'Epidemiology of extended-spectrum β-lactamase producing Escherichia coli in the stools of returning Japanese travelers, and the risk factors for colonization', *PLoS ONE*, 9(5), pp. 1–5. doi: 10.1371/journal.pone.0098000.

Yang, L. *et al.* (2017) 'Characterization of a P1-like bacteriophage carrying CTX-M-27 in Salmonella spp. resistant to third generation cephalosporins isolated from pork in China', *Scientific Reports*. Nature Publishing Group, 7(December 2016), p. 40710. doi: 10.1038/srep40710.

Yang, Y. *et al.* (2012) 'Quantification and characterization of β-lactam resistance genes in 15 sewage treatment plants from East Asia and North America', *Applied Microbiology and Biotechnology*, 95(5), pp. 1351–1358. doi: 10.1007/s00253-011-3810-5.

Yano, H. *et al.* (2013) 'Molecular characteristics of extended-spectrum β-lactamases in clinical isolates from Escherichia coli at a Japanese tertiary hospital.', *PloS one*, 8(5), p. doi: 10.1371/journal.pone.0064359. doi: 10.1371/journal.pone.0064359.

Yoon, Y. K. *et al.* (2011) 'Molecular analysis of a prolonged spread of Klebsiella pneumoniae co-producing DHA-1 and SHV-12 β-lactamases', *Journal of microbiology (Seoul, Korea)*, 49(3), pp. 363–368. doi: 10.1007/s12275-011-0491-9.

Zankari, E. *et al.* (2012) 'Identification of acquired antimicrobial resistance genes', *Journal of Antimicrobial Chemotherapy*, 67(11), pp. 2640–2644. doi: 10.1093/jac/dks261.

Zhang, J. *et al.* (2014) 'Nationwide high prevalence of CTX-M and an increase of CTX-M-55 in Escherichia coli isolated from patients with community-onset infections in Chinese county hospitals', *BMC infectious diseases*, 14, p. 659.

Zhang, Q. Q. *et al.* (2015) 'Comprehensive evaluation of antibiotics emission and fate in the river basins of China: Source analysis, multimedia modeling, and linkage to bacterial resistance', *Environmental Science and Technology*, 49(11), pp. 6772–6782. doi: 10.1021/acs.est.5b00729.

Zhang, W. H. *et al.* (2016) 'CTX-M-27 producing Salmonella enterica serotypes typhimurium and Indiana are prevalent among food-producing animals in China', *Frontiers in Microbiology*, 7(MAR), pp. 1–11. doi: 10.3389/fmicb.2016.00436.

Zhao, W.-H. and Hu, Z.-Q. (2012) 'Epidemiology and genetics of CTX-M extended-spectrum β-lactamases in Gram-negative bacteria', *Critical Reviews in Microbiology*, 39(May 2012), pp. 1–23. doi: 10.3109/1040841X.2012.691460.

Zhao, W. H. and Hu, Z. Q. (2013) 'Epidemiology and genetics of CTX-M extended-spectrum β-

lactamases in Gram-negative bacteria', *Critical Reviews in Microbiology*, 39(1), pp. 79–101. doi: 10.3109/1040841X.2012.691460.

Zheng, B. *et al.* (2016) 'Coexistence of MCR-1 and NDM-1 in Clinical Escherichia coli Isolates', *Clinical Infectious Diseases*, 63(10), pp. 1393–1395. doi: 10.1093/cid/ciw553.

Zheng, H. *et al.* (2012) 'Prevalence and characterisation of CTX-M β-lactamases amongst Escherichia coli isolates from healthy food animals in China.', *International journal of antimicrobial agents*. Elsevier B.V., 39(4), pp. 305–10. doi: 10.1016/j.ijantimicag.2011.12.001.

Zhong, Y.-M. *et al.* (2015) 'Emergence and spread of O16-ST131 and O25b-ST131 clones among faecal CTX-M-producing Escherichia coli in healthy individuals in Hunan Province, China', *Journal of Antimicrobial Chemotherapy*, 70(8), pp. 2223–2227. doi: 10.1093/jac/dkv114.

Zhu, W. H. *et al.* (2009) 'Complete nucleotide sequence of pCTX-M360, an intermediate plasmid between pEL60 and pCTX-M3, from a multidrug-resistant Klebsiella pneumoniae strain isolated in China', *Antimicrobial Agents and Chemotherapy*, 53(12), pp. 5291–5293. doi: 10.1128/AAC.00032-09.

Zhuo, C. *et al.* (2013) 'Epidemic Plasmid Carrying blaCTX-M-15 in Klebsiella penumoniae in China', *PLoS ONE*, 8(1), pp. 1–8. doi: 10.1371/journal.pone.0052222.

Zurfluh, K. *et al.* (2013) 'Characteristics of extended-spectrum β-lactamase- and carbapenemase-producing Enterobacteriaceae isolates from rivers and lakes in Switzerland', *Applied and Environmental Microbiology*, 79(9), pp. 3021–3026. doi: 10.1128/AEM.00054-13.

APPENDIX 1

Table S1. *E. coli* Isolates before and after travel with corresponding sample collection time points and MLSTs. Normal font: non-CTX-M-EC. Bold: CTX-M-EC.

***E. coli* Isolate**	**Faecal sample time-point (pre or post-travel)**	**MLST**	**Phylogenetic group**
1.1a	10 days pre	131	B2
1.1b		131	B2
1.1c		131	B2
1.1d		131	B2
1.1e		NS	
1.1f		NS	
1.2a	5 days pre	131	B2
1.2b		131	B2
1.2c		131	B2
1.2d		NS	
1.2e		NS	
1.2f		NS	
1a1	4 days post	349*	D
1a2		349	D
1a3		349	D
1a4		349	D
1b1	19 days post	394	D
1c1	31 days post	69	D
1.e.1	122 days post	131	B2
1.e.2		131	B2
1.e.3		131	B2
1.e.4		131*	B2
3.1a	28 days pre	69	D
3.1b		941	B2
3.1c		941	B2
3.1d		69	D
3.1e		69	D
3.1f		69	D
3.2a	14 days pre	69	D
3.2b		69	D
3.2c		69	D
3.2d		69	D

3.2e		69	D
3.2f		2008	NG
3.3a	3 days pre	69	D
3.3b		69	D
3.3c		69	D
3.3d		69	D
3.3e		69	D
3.3f		69	D
3a1	7 days post	43	A
3a2		43	A
3b2	7 days post	38	D
3b3		1193	B2
3b4		1193	B2
3c1	28 days post	38	D
3c3		1193	B2
3d1	56 days post	1193	B2
3d2		1193	B2
3d3		1193	B2
3d4		1193	B2
3.e.1	84 days post	405	D
3.e.2		405	D
3.e.3		38	D
3.e.4		405	D
3f1	274 days post	405	D
3f2		405	D
3f3		405	D
3f4		405	D
4.1a	14 days pre	10	A
4.1b		10	A
4.1c		10	A
4.1d		10	A
4.1e		10	A
4.1f		10	A
4.2a	7 days pre	10	A
4.2b		10	A
4.2c		10	A
4.2d		10	A
4.2e		10	A

4.2f		10	A
4a1	7 days post	2732	NG
4a2		2732	NG
4a3		2732	NG
4a4		2732	NG
4a5		2732	NG
4a6		69	D
4b1	30 days post	69	D
4b2		69	D
4b3		69	D
4b4		69	D
4c1	49 days post	69	D
4c2		69*	D
4c3		69*	D
4c4		69	D
4d1	91 days post	648	B1
4d2		648	B1
4d3		648	B1
4d4		648	B1
4.e.1	152 days post	69	D
4.e.2		69	D
4.e.3		69*	D
4.e.4		69	D
5.1a	7 days Pre-Uzbekistan	452	B2
5.1b		452	B2
5.1c		452	B2
5.1d		452	B2
5.1e		452	B2
5.1f		452	B2
5.2a	4 days post Uzbek.	38	D
5.2b		38	D
5.2c		38	D
5.2d		405	D
5.2e		38	D
5.2f		38	D
5.3a	29 days post Uzbek.	405	D
5.3b		405	D
5.3c		405	D

5.3d		131	B2
5.3e		131	B2
5.3f		131	B2
5a1	3 days post India	48	A
5a2		48	A
5a3		48	A
5a4		48	A
5b1	8 days post India	48	A
5b2		48*	A
5b3		48	A
5b4		48	A
5c1	28 days post India	10	A
5c2		10	A
5c3		10	A
5c4		48	A
5d1	60 days post India	10	A
5d2		10	A
5d3		10	A
5d4		10	A
5.e.1	91 days post India	6338	NG
5.e.2		6338	NG
5.e.3		38	D
5.e.4		38	D
5f1	183 days post India	88	C
5f2		48*	A
5f3		48	A
5f4		88	C
5g1	244 days post India	3478	A
5g2		3478	A
5g3		3478	A
5g4		3478	A
5H1	364 days post India	10	A
5H2		10*	A
5H3		10	A
5H4		NS	
6.1a	42 days pre	69	D
6.1b		69	D
6.1c		73	B2

6.1d		69	D
6.1e		73	B2
6.1f		69	D
6.2a	28 days pre	73	B2
6.2b		73	B2
6.2c		73	B2
6.2d		73	B2
6.3a	1 day pre	73	B2
6.3b		73	B2
6.3c		73	B2
6.3d		73	B2
6.3e		73	B2
6.3f		73	B2
6a1	4 days post	450	NG
6a2		450	NG
6a3		450	NG
6a4		450	NG
6b1	21 days post	8095	D
6b2		8095	D
6b3		8095	D
6b4		8095	D
6c1	42 days post	2221	B1
6c2		73	B2
6c3		450	NG
6c4		73	B2
6d1	180 days post	73	B2
6d2		73	B2
6d3		73	B2
6d4		73	B2
7.1a	60 days pre	10	A
7.1b		10	A
7.1c		10	A
7.1d		10	A
7.1e		10	A
7.1f		10	A
7.2b	29 days pre	69	D
7.3a	7 days pre	69	D
7.3b		10	A

7.3c		10	A
7.3d		10	A
7.3e		10	A
7.3f		10	A
7a1	5 days post	10	A
7a2		6438	NG
7a3		6438	NG
7a4		6438	NG
7b1	10 days post	10	A
7b2		10	A
7b3		10*	A
7b4		43	A
7c2	28 days post	200	B1
7c3		200	B1
7c4		200	B1
7d1	60 days post	200	B1
7d2		200	B1
7d3		200	B1
7d4		200	B1
7.e.1	122 days post	450	NG
7f2	245 days post	3036	NG
7f4		69	D
8.1a	7 days pre	69	D
8.1b		1163	NG
8.1c		69	D
8.1f		2712	D
8.2a	1 day pre	10	A
8.2b		10	A
8.2c		FS	
8.2d		10	A
8.2e		10	A
8.2f		10	A
8a1	10 days post	448	B1
8a2		448	B1
8a3		448	B1
8a4		448	B1
8b1	16 days post	648	B1
8b2		10	A

8b3		10	A
8b4		10	A
8c1	40 days post	10	A
8c2		10	A
8c3		10	A
8c4		10	A
8d1	58 days post	10	A
8d2		10	A
8d3		648	B1
8d4		10	A
8.e.1	120 days post	648	B1
8.e.2		648	B1
8.e.3		648	B1
8.e.4		648	B1
8f1	244 days post	648	B1
8f2		648	B1
8f3		648	B1
8f4		648	B1
8g1	330 days post	131	B2
8g2		131	B2
8g3		38	D
8g4		131	B2
9.1a	28 days pre	NT	Clade V^
9.1b		NT	Clade V^
9.1c		NT	Clade V^
9.1d		NT	Clade V^
9.1e		NT	Clade V^
9.1f		NT	Clade V^
9.2a	30 days pre	NT	Clade V^
9.2b		69	D
9.2c		69	D
9.2d		69	D
9.2e		69	D
9.2f		69	D
9.3a	7 days pre	3727	NG
9.3b		3727	NG
9.3c		3727	NG
9.3d		3727	NG

9.3e		3727	NG
9.3f		3727	NG
9a1	5 days post	4	A
9a2		200	B1
9a3		4	A
9a4		4	A
9b1	14 days post	2617	F
9b2		2617	F
9c1	35 days post	2617	F
9c2		2617	F
9c3		2617	F
9c4		2617	F
9d1	91 days post	2617	F
9d2		2617*	F
9d3		2617	F
9d4		2617	F
9.e.1	152 days post	2617	F
9.e.2		2617	F
9.e.3		2617	F
9.e.4		2617	F
9f1	210 days post	2617	F
9f2		2617	F
9f3		2617	F
9f4		2617	F
10.1a	61 days pre	354	F
10.1b		354	F
10.1c		354	F
10.1d		354	F
10.1e		354	F
10.1f		354	F
10.2a	30 days pre	354	F
10.2b		354	F
10.2c		354	F
10.2d		354	F
10.2e		354	F
10.2f		354	F
10.3a	7 days pre	642	B1
10.3b		642	B1

10.3c		642	B1
10.3d		642	B1
10.3e		642	B1
10.3f		642	B1
10a1	5 days post	200	B1
10a2		34	A
10b1	14 days post	200	B1
10b2		200	B1
10c1	35 days post	10*	A
10e.1	152 days post	10*	A
10e.2		10*	A
12.1a	14 days pre	1314	A
12.1b		1314	A
12.1c		10	A
12.1d		10	A
12.1e		1314	A
12.1f		10	A
12.2a	7 days pre	10	A
12.2b		10	A
12.2c		752	A
12.2d		10	A
12.2e		770	NG
12.2f		10	A
12a1	3 days post	43	A
12a2		43	A
12a3		43	A
12a4		43	A
12b1	21 days post	43	A
12b2		43	A
12b3		43	A
12b4		43	A
12c1	29 days post	43	A
12c2		43	A
12c3		43	A
12c4		43	A
12d1	56 days post India	43	A
12d2		43	A

12d3		43	A
12d4		43	A
12.e.1	115 days post India	43	A
12.e.2		43	A
12.e.3		43	A
12.e.4		43	A
12fs	213 days post India	1276	F
12g1	305 days post India	189	A
12g2		189	A
12g3		189	A
12g4		189	A
12h1	360 days post India	5919	NG
12h2		5919	NG
12h3		216	A
12h4		5919	NG
15.1a	28 days pre	210	NG
15.1b		210	NG
15.1c		10	A
15.1d		10	A
15.1e		210	NG
15.1f		210	NG
15.2c	14 days pre	10	A
15.2d		10	A
15.2e		210	NG
15.2f		210	NG
15a1	1 day post	167	A
15a2		167	A
15a3		167	A
15a4		167	A
15b	122 days post	52	A
15c1	150 days post	10	A
15c2		10	A
15c3		10	A
15c4		10	A
16.1a	42 days	349	D
16.1b		349	D

16.1c		349	D
16.1d		349	D
16.1e		349	D
16.1f		349	D
16.2a	14 days	349	D
16.2b		349	D
16.2c		349	D
16.2d		349	D
16.2e		349	D
16.2f		349	D
16a1	7 days post	38	D
16a2		38	D
16a3		38	D
16a4		38	D
16b1	40 days post	227	A
16b2		131	B2
16b3		38	D
16b4		227	A
16c1	91 days post	131	B2
16c2		131	B2
16c3		227	A
16c4		131	B2
16d1	121 days post	131	B2
16d2		131	B2
16d3		131	B2
16d4		131	B2
16 e1	179 days post	131	B2
16 e2		131	B2
16 e3		131	B2
16 e4		131	B2
17.1a	30 days pre	NT	clade V^
17.1b		NT	clade V^
17.1c		NT	clade V^
17.1d		NT	clade V^
17.1e		NT	clade V^
17.1f		NT	clade V^
17.2a	3 days pre	131	B2
17.2b		131	B2

17.2c		131	B2
17.2d		131	B2
17.2e		131	B2
17.2f		131	B2
17a1	21 days post	450	NG
17a2		450	NG
17a3		450	NG
17a4		450	NG
17b1	42 days post	43	A
17b2		43	A
17b3		43	A
17b4		43	A
17c1	84 days post	131	B2
17c2		131	B2
17c3		131	B2
17c4		131	B2
18.1a	54 days pre	NS	
18.1b		NS	
18.1c		NS	
18.1d		NS	
18.1e		NS	
18.1f		NS	
18.2a	36 days pre	NS	
18.2b		NS	
18.2c		NS	
18.2d		NS	
18.2e		NS	
18.2f		NS	
18a1	11 days post	NS	
18a2		NS	
18a3		NS	
18a4		NS	
18b	27 days post	NS	
19.1a	42 days pre	10	A
19.1b		10	A
19.1c		10	A
19.1d		10	A
19.1e		10	A

19.1f		10	A
19.2a	30 days pre	10	A
19.2b		10	A
19.2c		10	A
19.2d		10	A
19.2e		10	A
19.2f		10	A
19a1	14 days post	43	A
19a2		43	A
19a3		43	A
19a4		43	A
20.1a	10 days pre	10	A
20.1b		10	A
20.1c		10	A
20.1d		10	A
20.1e		452	NG
20.1f		NT	B2
20.2a	4 days pre	10	A
20.2b		10	A
20.2c		10	A
20.2d		10	A
20.2e		10	A
20.2f		10	A
20a1	7 days post	6438	NG
20a2		6438	NG
20a3		162	NG
20a4		162	NG
20b1	42 days post	162	NG
20b2		162	NG
20b3		162	NG
20b4		162	NG
20c1	56 days post	131	B2
20c2		226	NG
20c3		131	B2
20c4		226	NG
20.e.3	91 days post	405	NG
20.e.4		405	NG
20f1	120 days post	10	A

20g1	183 days post	10	A
20g2		10	A
20g3		10	A
20g4		10	A
21.1b	16 days	NS	
21.1c		NS	
21.1d		NS	
21.1e		NS	
21.1f		NS	
21a1	15 days	NS	
21a2		NS	
21a3		NS	
21a4		NS	
21b1	36 days	NS	
21b2		NS	
21b3		NS	
21b4		NS	
21c1	96 days	NS	
21c2		NS	
21c3		NS	
21c4		NS	
22.1a	52 days	2619	B2
22.1b		2619	B2
22.1c		2619	B2
22.1d		2619	B2
22.1e		2619	B2
22.1f		2619	B2
22.2a	11 days	2619	B2
22.2b		2619	B2
22.2c		2619	B2
22.2d		2619	B2
22.2e		2619	B2
22.2f		2619	B2
22a1	6	226	A
22b1	14	NS	
22c1	36	607	A
22c2		2967	A
22c3		7174	NG

22c4		7174	NG

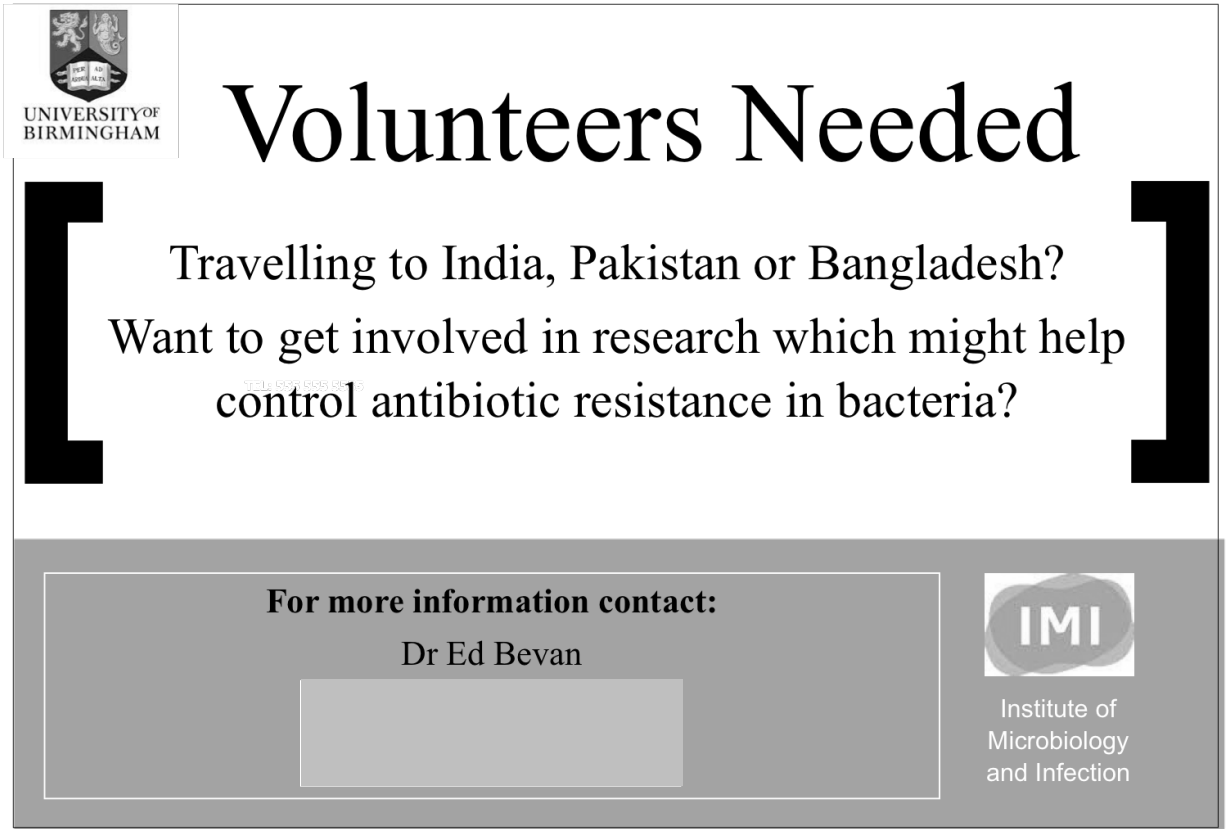
NT: no sequence type based on Warwick MLST scheme;
NG: no phylogenetic group assigned to the Warwick MLST;
NS: not sequenced.
No data shown for sample 10d as no gram-negative bacteria grew from faecal cultures (only Enterococcus spp.).
FS: failed assembly post short read sequencing

*MLST typing with WGS data failed on a single locus. Core phylogenetic analysis using SNPs with parsnp confirmed clustering with this ST.

^MLST confirmed E. coli but no ST type determined using Enterobase (http://enterobase.warwick.ac.uk). However all possible matches were to E. coli cryptic clade V.

APPENDIX 2

STUDY MATERIALS



CONSENT FORM

STUDY TITLE: **The molecular epidemiology of CTX-M antibiotic resistance genes in the faecal microbiome of humans acquiring ESBL-producing Enterobacteriaceae**

NAME OF PI: **Prof Peter Hawkey**

1. I confirm that I have read and understood the participant information sheet (version 5, 10/12/2015) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without any medical care or legal rights being affected. I understand that my personal data and samples will continue to be used for the duration of the study unless I specifically ask for them to be withdrawn

3. I understand that my identifiable data, contact details, medical history, and samples in relation to this study will be retained securely by the researchers on secure password protected computers at University of Birmingham for a maximum 4 years, and then destroyed.

4. In the unlikely event that I should suffer loss of mental capacity during the study, I understand that my personal data and samples submitted will continue to be used for the duration of the study.

5. I agree that stool samples I provide may be used for the duration of the study (maximum 4 years). I also agree that any bacterial isolates cultured from my samples may be utilised for future Research Ethics Committee-approved research projects

6. I agree to be contacted for further ethically-approved research

7. I understand that relevant sections of medical data collected during the study may be looked at by individuals from the University of Birmingham, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records

8. I agree to take part in this study.

PARTICIPANT
SIGNITURE…….
PRINT NAME…….
DATE…….

PERSON TAKING CONSENT
SIGNITURE…….
PRINT NAME……..
DATE…….

PARTICIPANT INFORMATION & CONSENT FORM

Do visitors travelling to the Indian subcontinent acquire local antibiotic resistant *E.coli* and carry those on return, or do resistance genes transfer via a plasmid to their resident *E.coli*?

We would like to invite you to take part in a research project investigating the mechanisms of antibiotic resistance in returning travellers.
It is entirely up to you whether to take part in the research. This information sheet explains why we are undertaking this research and what to expect if you decide to participate

WHY IS THIS RESEARCH BEING DONE?

Infections caused by antibiotic resistant bacteria are an increasing international problem. The bacterium *Escherichia coli* (*E.coli*) normally inhabits the bowel in healthy individuals, but is also the commonest cause of urinary tract infections and septicaemia in adults. Unfortunately, *E.coli* is often resistant to the most effective antibiotics available, and this is a particular problem in countries such as India, Pakistan, Bangladesh and Sri Lanka.

The spread of resistant *E.coli* through international travel presents a significant challenge, as these bacteria may spread within hospitals. Travellers from western countries who visit India, Pakistan, Bangladesh or Sri Lanka often become asymptomatic carriers, sometimes for several months after their return home. For example, in a recent study, 88% of those travelling to India acquired antibiotic-resistant *E.coli*[1]. The mechanism of acquisition of resistant bacteria in these cases is not fully understood.

DNA is the genetic material carried within all bacterial cells, and is the blueprint for the production of proteins which are essential for bacterial survival. Genes are long segments of DNA which encode specific proteins, e.g. proteins capable of degrading antibiotics which would otherwise be lethal to the bacteria. Genes for antibiotic resistance are often carried on circular rings of bacterial DNA called plasmids which can move between different bacterial cells of the same or different species. Resistant *E.coli* are acquired from environmental sources, such as contaminated food or drinking water, which leads to asymptomatic colonisation of the bowel. It is possible that in returning travellers, these new *E.coli* either:

1. Remain as colonisers of the bowel
   or
2. Transfer their antibiotic resistance genes to existing ‘normal’ host *E.coli* in the bowel via plasmid transfer

Using a group of volunteers who plan to travel to India, Pakistan, Bangladesh or Sri Lanka, this study will determine the mechanism by which antibiotic resistant genes are acquired. This study is important because it will support the development of

techniques to prevent the acquisition of antibiotic-resistant bacteria, and the removal of resistant bacteria using new technologies. Written informed consent will be obtained from all volunteers.

This research is also part of a PhD being carried out by Edward Bevan, who is funded through Public Health England (PHE).

PARTICIPANT INSTRUCTIONS

1. We will ask you to attend an initial 15 minute meeting with an investigator to complete a pre-travel questionnaire, and to collect specimen containers.
2. You will also be asked to complete a travel questionnaire about your trip on return to the UK. This will include questions on the specific places you visited, and whether you became unwell or took medication at any point.
3. We will ask you to submit stool samples on several occasions before and after travel:
    a. 8 weeks pre-departure
    b. 4 weeks pre-departure
    c. 2 weeks pre-departure
    d. As soon as possible on return to the UK
    e. 1 week after return
    f. 4 weeks after return
    g. 8 weeks after return
    h. 16 weeks after return
    i. If you still carry resistant E.coli at 16 weeks, we will ask you for 2-3 further samples up to a year after you return.

    Stool samples should be collected using the spatula and container provided, then sealed in the transparent plastic bag.

    Samples can be submitted in person to an investigator (Dr Ed Bevan) at the Biosciences Building, University of Birmingham, or to the Microbiology Department at Heartlands Hospital.

IMPORTANT INFORMATION

- Please note that your individual results cannot be made available to you
- Stool samples will be frozen and retained for the duration of the research period (4 years). Bacterial cultures may be retained indefinitely for further research.
- Your contact details will be needed for the purposes of follow-up. Your details will be stored securely on a University password protected computer for the duration of the research period (4 years). Any paper-based documents will be stored securely at the university.
- You may also be contacted relating to different ethically-approved research
- You may withdraw your consent for participation at any time.
- Fully anonymised results of the study will be published in international literature, and will form part of a PhD thesis, however no identifiable information will be

published or be disclosed to any third parties who are not directly involved in the research.
- Please do not hesitate to contact the lead investigator with any additional questions about the study: .

WHAT ARE THE RISKS OF TAKING PART?

- There are no risks to your health
- The study involves devoting a small amount of time in completing questionnaires and delivering samples to the Biosciences Building on campus.
- Your contact details and completed questionnaires will be treated as highly confidential and will not be transferred beyond the University. Should a lapse in data protection occur, you will be informed immediately.
- For ethical and legal purposes, the study is sponsored and insured by University of Birmingham.

WHAT IF SOMETHING GOES WRONG?

- If you have any concerns or complaints about this research, please contact one of the researchers, Dr Ed Bevan, or Prof Peter Hawkey, .

1. Tangden T, Cars O, Melhus A et al. Foreign travel is a major risk factor for colonization with Escherichia coli producing CTX-M-type extended-spectrum beta-lactamases: a prospective study with Swedish volunteers. *Antimicrobial agents and chemotherapy* 2010; **54**: 3564-8.