Beta-lactam resistant urinary tract infections: prevalence, the development of rapid diagnostics and novel treatments

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List of Abbreviations

AC: adenylyl cyclase

AMP: adenosine monophosphate

BCAM: basal cell adhesion molecule

CAUTI: catheter-associated urinary tract infection

cfu: colony forming unit

CNF1: cytotoxic necrotising factor

DNA: deoxyribonucleic acid

E.coli: Escherichia coli

ECDC: european centre for disease prevention and control

EDTA: Ethylenediaminetetraacetic acid

ESBL: extended-spectrum beta-lactamase

EUCAST: european committee on antimicrobial susceptibility

HlyA: alpha haemolysin

HPA: health protection agency

IBC: intracellular bacterial communities

Inc: incompatibility

IS: insertion sequence

LPS: lipopolysaccharide

LRI: Leicester Royal Infirmary

MDR: multidrug resistance

MIC: minimum inhibitory concentration

NTC: no template control

ORI: origin of replication

PAC: proanthocyanin

PBP: penicillin-binding proteins

PBRT: polymerase chain reaction based replicon typing

PBS: phosphate buffered saline

QIR: quiescent intracellular reservoir

qRT-PCR: quantitative reverse transcriptase PCR

REP: replication initiator protein encoding

RNA: ribonucleic acid

TLR4: toll-like-receptor

UPEC: uropathogenic *Escherichia coli*

UTI: urinary tract infection

WHO: world health organisation

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Abstract

Background

The 21st century has seen antibiotic resistance rise to be a major public health concern. The O'Neill report in 2016 reiterated the importance of antibiotic resistance and proposed many actions, including strengthening surveillance systems, the development of rapid diagnostics, and investment in new classes of antibiotics and alternatives.

Urinary tract infections (UTIs) are one of the most common infections diagnosed in the United Kingdom with uropathogenic *Escherichia coli* (UPEC) the most common cause. It is thought that the main reason why UPEC is so successful at causing UTIs, is its expression of a wide range of virulence factors including adhesins, capsules, toxins and iron-acquisition systems. Overuse of beta-lactam antibiotics to treat UTIs has led to selection for extended-spectrum β-lactamase (ESBL) producing *Enterobacteriaceae,* a major cause of antibiotic resistant urinary tract infections (UTIs). The prevalence of ESBL producing UTIs has dramatically risen, limiting treatment options. The most common ESBL types seen today are CTX-M, TEM, OXA and SHV. The emergence and spread of these types of ESBLs is thought to be through the horizontal transmission of antibiotic resistance plasmids IncL/M, IncF, IncN and IncI1. These conjugative plasmids have been directly linked to major outbreaks of antibiotic resistance.

Standard detection methods for ESBL-producing UTIs are time-consuming, with disputed accuracy. Patients are often treated empirically with broad-spectrum antibiotics. Reducing the time spent on broad-spectrum antibiotics by prescribing a more appropriate treatment, increases the favorability of the outcome of the patient and shortens the stay in hospital.

Aims

This study had three aims: to investigate the prevalence of ESBLs and the relationship between plasmids and ESBLs in Leicestershire; to develop a rapid, accurate method to detect ESBLs (TEM, SHV, OXA, and CTX-M) using real-time PCR and to investigate the effect of cranberry (Cysticlean®) on the expression of 10 virulence genes.

Methods

A total of 236 uropathogenic *E. coli* ESBL-producing isolates were collected from the Leicester Royal Infirmary. This study identified ESBL genes (CTX-M, SHV, TEM and OXA) and multiple CTX-M gene subtypes by multiplex PCR. A multiplex PCR-based replicon typing assay identified IncFIA, IncI1, IncL/M, IncN and IncFII. A real-time PCR assay was designed using amplicon melting analysis and the Plexor system to detect the ESBL family.

A CTX-M-producing *E.coli* isolate was treated with the cranberry extract Cysticlean®. Taqman qRT-PCR was used to detect the relative expression of the virulence genes SAT, USP, ChuA, SoxS, KPSM, TraT, RecA, IdfB and HcaT and the antibiotic resistance gene CTX-M.

Results

ESBL genes were identified as follows: CTX-M (71.6%), OXA (7.6%), TEM (3.8%) and SHV (3.8%) with multiple genes detected in 10.2% of isolates. CTX-M-1 (84.1%) was the most frequently detected CTX-M subtype. Replicon typing results were as follows: IncL/M (29.2%), IncN (14.4%), IncI1 (5.1%), IncFII (27.5%) and IncFIA (23.3%). A combination

of IncL/M, IncFII and IncFIA was the most common at 9.8%. A positive correlation between CTX-M and all plasmids except IncI1 was found.

The qualitative real-time PCR assay correctly identified 97.7% isolates tested, with a sensitivity and specificity of 98.7% and 83.3% respectively. The positive predictive value was 97.5% and the negative predictive value was 90.9%.

Results from the qRT-PCR assay showed that Cysticlean® was able to significantly reduce the expression of all the genes investigated.

Conclusions

This is the first study to analyse the prevalence of uropathogenic ESBLs in Leicestershire. The ability to rapidly and accurately detect ESBL genes is an important step in improving antimicrobial stewardship and reducing morbidity and mortality as a result of ESBL-producing pathogenic infections. The ability to reduce the expression of critical virulence factors, could lead to the development of alternatives to antibiotics.

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Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

No portion of the work referred to in this thesis has been submitted in support of an application for another degree.

Publications

Reid R, Samarasinghe S (2018) The development and evaluation of a multiplex real-time PCR assay for the detection of ESBL genes in urinary tract infections. *International Journal of Clinical Microbiology* 1: 1.

The contents of this paper can be found in chapter 3 and futher dicussed in chapter 5.

Baho S, **Reid R**, Samarasinghe S (2018) Adaptability to Various Growth Conditions of Biofilm Associated Extended-Spectrum-Beta- Lactamases Producing Bacteria. *Journal of Infectious Diseases and Diagnosis* 3: 121. DOI: 10.4172/2576-389X.1000121

This paper is not discussed within the thesis.

Reid R, Al-Bayati M & Samarasinghe S (2018) Genotypic Identification of Extended-Spectrum Beta-Lactamase (ESBL) – Producing *Enterobacteriaceae* from Urinary Tract Infections in the Leicestershire Area, United Kingdom: A One Health Prospective. *Journal of Infectious Disease and Diagnosis* 3: 122. DOI:10.4172/2576-389X.1000122

The contents of this paper can be found in chapter 2 and futher dicussed in chapter 5.

In preparation:

Reid R & Samarasinghe S. The effect of cranberry extract Cysticlean on the expression of virulence genes in CTX-M-producing *Escherichia coli.*

The contents of this paper can be found in chapter 4 and futher dicussed in chapter 5.

Chapter 1

General Introduction

1 Introduction

1.1 Urinary Tract Infection

Escherichia coli (*E.coli*) is often part of the normal flora of the gastrointestinal flora of both humans and animals. In most circumstances, the presence of *E.coli* in the gastrointestinal tract is beneficial to the host, by promoting homeostasis and outcompeting harmful bacteria. When freed from the confines of the gastrointestinal tract, where *E.coli* rarely causes infection, even non-pathogenic *E.coli* strains can cause infection and strains can take on a more pathogenic nature. These strains are still able to colonise the gastrointestinal tract, but also have the ability to disseminate into other areas of the host such as the blood, central nervous system and the urinary tract (Bien, et al., 2012).

Urinary tract infections (UTIs) effect approximately 150 million people annually worldwide, thus becoming one of the most common infections seen in man (Dash, et al., 2018). UTIs are a substantial cause of morbidity, especially in females, elderly men and infants (Foxman, 2003). The complications of UTI can be serious, involving recurrent infections, pyelonephritis with sepsis, pre-term birth and renal damage in children. Other consequences include the potential for antibiotic resistance due to frequent antibiotic usage and *Clostridium difficile* colitis (Foxman, 2003). Whether or not a person develops a UTI depends on anatomical factors, host defence mechanism integrity and the level of virulence of the infecting pathogen (Nicolle, 2002).

1.1.1 UTI Classification

UTIs can be classified as either uncomplicated or complicated. Uncomplicated infections generally effect patients that are otherwise healthy, including no urinary tract abnormalities (Svanborg, 1997). Complicated UTIs are associated with aspects that compromise either the urinary tract, or host defences, or both. This includes urinary obstruction, neurological disease leading to urinary retention, immunosuppression and renal failure (Svanborg, 1997). Urinary tract infections are commonly related with catheters. Catheter-associated UTIs (CAUTIs) have been related to increased morbidity and mortality and frequently cause secondary bloodstream infections. CAUTI risk factors include female gender, older age, diabetes and prolonged catheterisation (Svanborg, 1997). UTIs are also categorised depending on the site of infection. Infection of the bladder is termed cystitis, infection of the kidney is termed pyelonephritis, and infection of the urine is bacteriuria (Bien, et al., 2012).

Bacteriuria can be asymptomatic, and this does not normally require treatment. Additionally, in some cases colonisation of the pathogen can prevent the infection of a more virulent pathogen (Bien, et al., 2012).

More than 80% of UTIs, including asymptomatic bacteriuria, are caused by uropathogenic *E. coli* (UPEC) strains (Trautner, 2003) (Darouiche, 2001) (Hull, 2000).
The majority of community-acquired and a large portion of nosocomial UTIs, are caused by these UPEC strains. UPEC infections cause substantial medical costs and increase morbidity and mortality worldwide (Bien, et al., 2012).

1.1.2 Infection and Colonisation Mechanisms

In order for pathogens to successfully infect a host, they must adhere to host cells and colonise tissues whilst overcoming host defences (Foxman, 2003). The bladder epithelium comprises three main cell types: umbrella cells, intermediate cells and basal cells (Wiles, et al., 2008). UPEC bind to umbrella cells via uroplakins, that protect the tissue from damaging chemicals in urine by forming a crystalline array in the apical membrane (see figure 1-I) (Wiles, et al., 2008). Uroplakins are not the only place that UPEC can bind, α3β1 integrins, expressed at the surface of uroepithelial cells, can also function as receptors for UPEC (Wiles, et al., 2008). Colonisation of the urinary tract by UPEC, may lead to ascension towards the bladder, leading to cystitis, causing painful, frequent and urgent urination. From the bladder, UPEC can travel via the ureters to the kidney, causing pyelonephritis which can result in irreversible kidney damage and even death (Scholes, 2005).

1.1.3 UPEC Virulence Factors

Virulence factors increase the capacity of bacteria to cause an infection. UPEC strains can acquire a number of virulence factors, which aid them in colonizing and persisting in the urinary tract. Virulence genes are commonly located on mobile genetic elements such as pathogenicity islands, transposons, bacteriophages or plasmids and are generally acquired by DNA horizontal transfer.

In general, the more important virulence factors implicated in the successful infection and persistence in the urinary tract are those that are associated with the surface of the bacterial cell, and those that are secreted and exported to the site of action (Bien, et al., 2012). More

specifically, the main virulence factors associated with UPEC are adhesins, capsules, toxins and iron-acquisition systems (Muller, 2016). Research suggests that adhesions, or fimbriae, are the most important UPEC virulence factors and determine the pathogenicity of the bacterial strain. These adhesive organelles promote the attachment of bacterial cells to host tissues within the urinary tract. Fimbriae contribute to the virulence of the bacterial strain by activating cell signaling pathways in both the bacteria and the host, enabling bacterial product delivery to host tissues and potentially most importantly, by promoting bacterial invasion of host tissues.

1.1.4 Adhesins

1.1.4.1 Type 1 Fimbriae

The virulence determinant of type 1 fimbriae is poorly understood in human pathology, however in animal models, it has been shown to enhance bacterial survival, stimulate inflammation of the mucosal lining and to promote biofilm growth. The difficulty in determining the role of type 1 fimbriae in humans is due to the fact that they are expressed in both pathogenic and non-pathogenic strains at the same frequency. In both pathogenic and non-pathogenic strains, type 1 fimbriae bind to uroplakin Ia and IIIa, located at the top of the fimbriae, via a FimH adhesion subunit (Bien, et al., 2012). The interactions between the FimH- α3β1 integrin results in the activation of the RHO-family GTPases, via actin arrangement, leading to the invasion of bacteria into the cell (see figure 1-I) (Flores-Mireles, 2015). This then leads to the activation of signaling pathways, leading to molecular phosphorylation events involved in invasion and apoptosis (see figure 1-I). In addition, Tamm-Horsfall Protein is released into the urine by kidney cells, acting as a FimH

receptor. This can lead to the obstruction of UPEC's capability to interact with the receptor, reducing the ability to colonise the urinary tract. Once UPEC has navigated inside the host cell, it can then undermine host defenses and withstand antibiotic treatment (Bien, et al., 2012). However UPEC releases lipopolysaccharide (LPS), which can then be detected by Toll-like receptor 4 (TLR4) (see figure 1-I). This stimulates the production of cyclic AMP (cAMP) caused be adenylyl cyclase 3 (AC3) activation, which can cause the subsequent exocytosis of UPEC (see figure 1-I) (Bien, et al., 2012). This mechanism of innate immunity is disrupted by UPEC through evasion into the cytoplasm. When in the cytoplasm, UPEC proliferates into intracellular bacterial communities (IBCs) (see figure 1-I) (Flores-Mireles, 2015). These IBCs mature, causing the dispersal of bacteria, leading to the invasion of additional host cells, spreading the infection and beginning the IBC cycle once again (see figure 1-I) (Flores-Mireles, 2015). During IBC formation, UPEC develops into filamentous cells that upon emerging from their dormant state, are resistant to killing by neutrophils, leading to the colonization of other cells (Flores-Mireles, 2015).

An alternative mechanism of evading the immune response is the formation of quiescent intracellular reservoirs (QIRs) (see figure 1-I) comprising of 4-10 bacteria encased in F-actin. In this state, QIRs do not replicate and can persist dormant for several weeks, eventually becoming active and progressing into the bladder lumen as transitional cells differentiate to umbrella cells (Flores-Mireles, 2015).

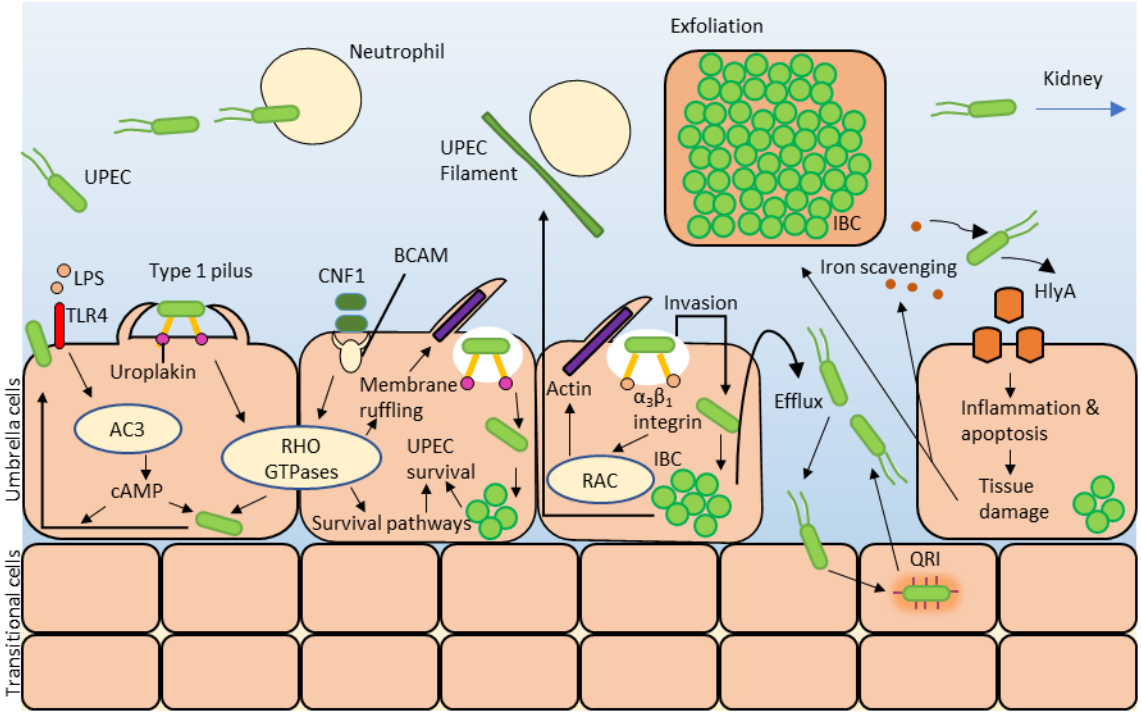

Figure 1-I Virulence factors of uropathogenic *Escherichia coli* that contribute to urinary tract infections. Various virulence factors contribute to the successful colonization, invasion and persistence of uropathogenic *Escherichia coli* (UPEC) in the bladder. The role of these virulence factors is described in the text. lipopolysaccharide (LPS) Toll-like receptor 4 (TLR4), cyclic AMP (cAMP), adenylyl cyclase 3 (AC3), intracellular bacterial communities (IBCs), quiescent intracellular reservoirs (QIRs), α-haemolysin toxin (HlyA), Cytotoxic necrotizing factor 1 (CNF1), basal cell adhesion molecule (BCAM) Figure adapted from (Flores-Mireles, 2015).

1.1.4.2 Capsules

Other virulence factors situated on the surface of the bacterial cell include the capsule and the lipopolysaccharide, an integral component of the bacterial cell wall. The main function of the capsule is to protect the bacterium from host defenses such as engulfment by phagocytes and the bactericidal effect produced by the complement system, via a polysaccharide structure. One of the ways that capsules do this is through molecular mimicry of tissue components, thus preventing an immune response. The lipopolysaccharide activates the inflammatory portion of the host response by inducing nitric oxide and cytokine production.

1.1.4.3 Iron Acquisition Systems

The urinary tract is naturally limited in iron (Bien, et al., 2012). Therefore, in order to be successful in persisting in the urinary tract, UPEC uses iron scavenging or siderophore systems to be able to grow in urine. These systems are composed of two parts: a siderophore for iron binding and a membrane receptor that transports the iron into the cell. It is thought that one of the reasons that UPEC is so successful in the urinary tract is the production of several siderophores, such as aerobactin and yersinabactin (Flores-Mireles, 2015).

1.1.4.4 Flagella

Flagella are organelles that are responsible for the motility of bacteria and the mediation of the interaction between UPEC and epithelial cells. It has been suggested that the flagella can be associated with pyelonephritis, participating in the ascension of UPEC from the bladder to the kidneys.

1.1.4.5 Toxins

1.1.4.5.1 HlyA

Toxins are another virulence factor that are important in UTIs and may also cause the activation of the inflammatory response, leading to UTI symptoms. α haemolysin (HlyA) is a lipoprotein that is believed to be one of the most significant virulence factors associated with UTI. These toxins are thought to be widespread in the gram-negative pathogenic community and approximately 50% of renal complications due to pyelonephritis are caused by HlyA. It is thought that this is due to induction of endothelial damage and renal vasoconstriction and permanent renal scarring is commonly associated with HlyA. When

HlyA is at a high concentration, it is capable of lysing erythrocytes and host cells, leading to the passage of UPEC across mucosal barriers, damaging the host cells, allowing the acquisition of iron and other host nutrients (see figure 1-I). At low concentrations, HlyA still has many abilities, particularly the apoptosis of host immune cells, renal cells and also the exfoliation of urinary tract epithelial cells (Bien, et al., 2012).

1.1.4.5.2 CNF1

Another important secreted toxin is cytotoxic necrotizing factor 1 (CNF1) and its main purpose is to bind to the receptor basal cell adhesion molecule (BCAM) on host cells (see figure 1-I). This binding stimulates the activation of RHO GTPases, leading to membrane ruffling (see figure 1-I). Activation of RHO GTPases also activates the pro-survival pathway, leading to the prevention of apoptosis of infected cells and the expansion of UPEC cells (see figure 1-I) (Bien, et al., 2012).

1.2 Host Response

As the urinary tract is normally a sterile environment, the presence of bacteria such as UPEC will trigger the host innate immune response. This response includes the production of inflammatory mediators such as cytokines which results in the recruitment of neutrophils into the infected area to ingest bacteria and to release enzymes that destroy bacteria. The host inflammatory response also causes the exfoliation of epithelial cells infected by bacteria and the generation of antimicrobial compounds such as reactive nitrogen and oxygen species (Bien, et al., 2012).

1.2.1 The Urinary Tract

In order to keep the urine sterile, the urinary tract has a number of defences. The main way the urinary tract clears bacteria is through the bulk flow of urine in the bladder. Further defence is provided by the secretion of glucosamine by transitional cells, this forms a mucin layer that prevents bacterial adherence. The urinary tract environment has a low pH and contains salts, urea and organic acids that naturally limit bacterial survival. The urinary tract also produces defensins, in response to the presence of bacteria. Defensins are peptides that have the ability to cause cell death in bacteria by increasing cell permeability via disrupting cell membrane function.

1.2.1.1 Exfoliation of Infected Cells

One of the most important parts of the inflammation process is the exfoliation of infected cells via a disruption of urothelial integrity. Infected host cells are then excreted. This process is FimH dependent and occurs via an apoptosis-like pathway.

1.2.1.2 Inflammation

If UPEC is able to evade the exfoliation process and adhere to the uroepithelium, the presence of bacteria activates the innate and adaptive immune responses including the production of cytokines, neutrophil recruitment and local tissue damage.

1.3 Antimicrobial Agents

1.3.1 Definition

Traditional antibiotics are natural metabolic products fungi and bacteria, that are able to inhibit or kill bacteria. Today most of the natural antibiotics in clinical use are then modified, to produce semi-synthetic antibiotics, improving their antibacterial or pharmacologic properties. Others are completely synthetic, such as sulphonamides and quinolones.

1.3.2 Discovery and Development

During the first decade of 20th century, Paul Ehrlich was the first to propose the relationship between microbial pathogens and drugs, as his experiments led to the arsphenamines for the treatment of syphilis. The present era of antimicrobial chemotherapy began in 1935, with the discovery of the sulphonamides by Gerhard Domagk. In 1929, penicillin was discovered by Alexander Fleming and he proposed that penicillin could be an effective chemotherapeutic agent. However, Fleming was unable to exploit this clinically and it was left to Florey and Chain to purify penicillin and prepare it for clinical use in 1940 (Aminov, 2010). The years of the 1950s and 1970s are considered the golden era of antibiotic discovery, with several novel classes identified. However, with no new classes discovered since then underlines the need to tackle antibiotic resistance to the drugs currently available.

1.4 Modes of Action of Antibiotics

Antimicrobial drugs have several mechanisms that lead them to inhibit or destroy bacteria. These include:

1. The interference of bacterial cell wall synthesis. For example, β-lactam antibiotics such as Cephalosporins and Carbapenams
2. The interference of protein synthesis: For example, Aminoglycosides, Macrolides and Tetracyclines
3. Interference with DNA synthesis. For example, Quinolones and Metronidazole.
4. Inhibition of RNA synthesis. For example, Rifamycins.
5. Inhibition of metabolic pathways. For example, Trimethoprim and Sulfamethoxazole.
6. Disruption of membrane structure. For example, Polymyxins (Etebu & Arikekpar, 2016).

1.5 Beta-Lactam Antibiotics

1.5.1 Structure

β-Lactams are one of the three largest classes of antibiotics and includes penicillins, cephalosporins, carbapenems, and monobactams (Fisher, et al., 2005). β-Lactams have been amongst the most successful drugs for the treatment of bacterial infections caused by numerous species for the past 70 years (Coleman, 2011). Clearly, this class of antibiotics has played a vital role to the preservation of human health, as stated by Fisher et al (2005). Their name comes from the presence of a β-lactam ring in their structure (Figure 1-II); this ring is vital for antimicrobial activity (Worthington & Melander, 2013). β-Lactams exert

their antibiotic effects by disrupting cell wall synthesis by inhibiting the normal function of the family of enzymes known as penicillin-binding proteins (PBP). Usually PBP would form cross-links between adjacent glycan chains in the bacterial cell wall (Figure 1-III). However, β-Lactam antibiotics are able to mimic the natural D-Ala-D-Ala substrate of the PBP, thus preventing the formation of cross-links between adjacent glycan chains (Figure 1-III) (Tipper & Strominger, 1965).

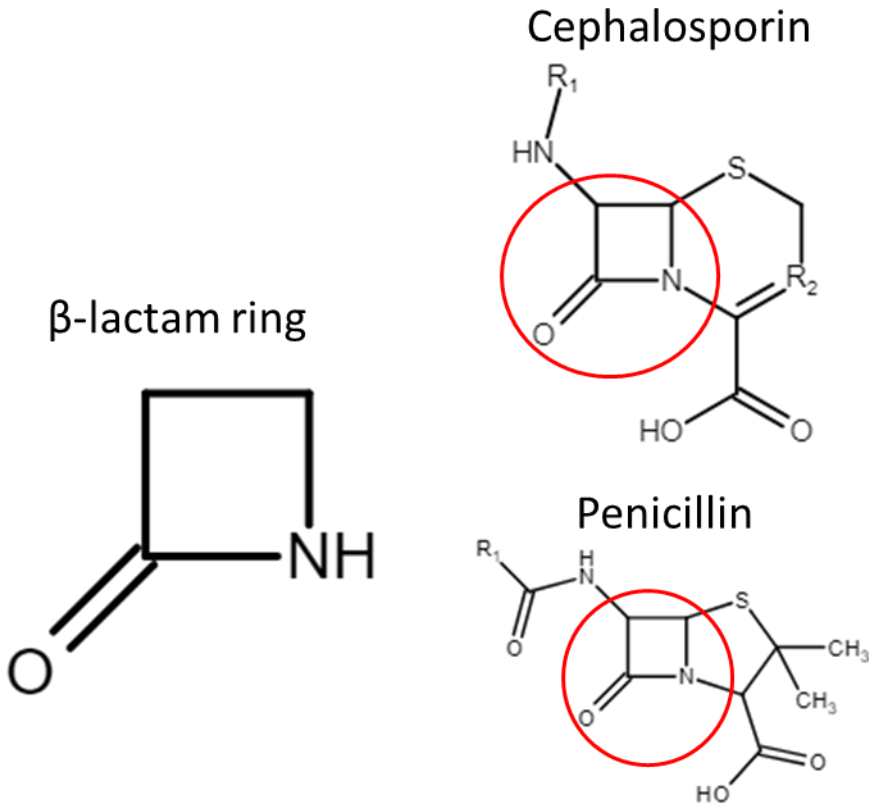

Figure 1-II beta-lactam antibiotic structure. All beta-lactam antibiotics contain the same core 4-member "beta-lactam" ring (Left). The beta-lactam ring structure is similar to the shape of the terminal D-Ala-D-Ala peptide substrate for penicillin binding proteins or cell wall transpeptidases that form covalent bonds between different peptidoglycan chains during cell growth. The 4-ring structure and associated side groups result in tight binding to the active site of Penicillin Binding Proteins, inhibiting enzyme activity, and consequently preventing cell wall formation (See figure 1-III). Modification of the structure of the naturally occurring penicillins resulted in the development of both synthetic penicillin analogs, as well as new families of beta-lactams such as cephalosporins (Right). Chemical structures were drawn using chem-space.com. Figure adapted from (Clarkson, 2015).

1.5.2 Mechanism of action

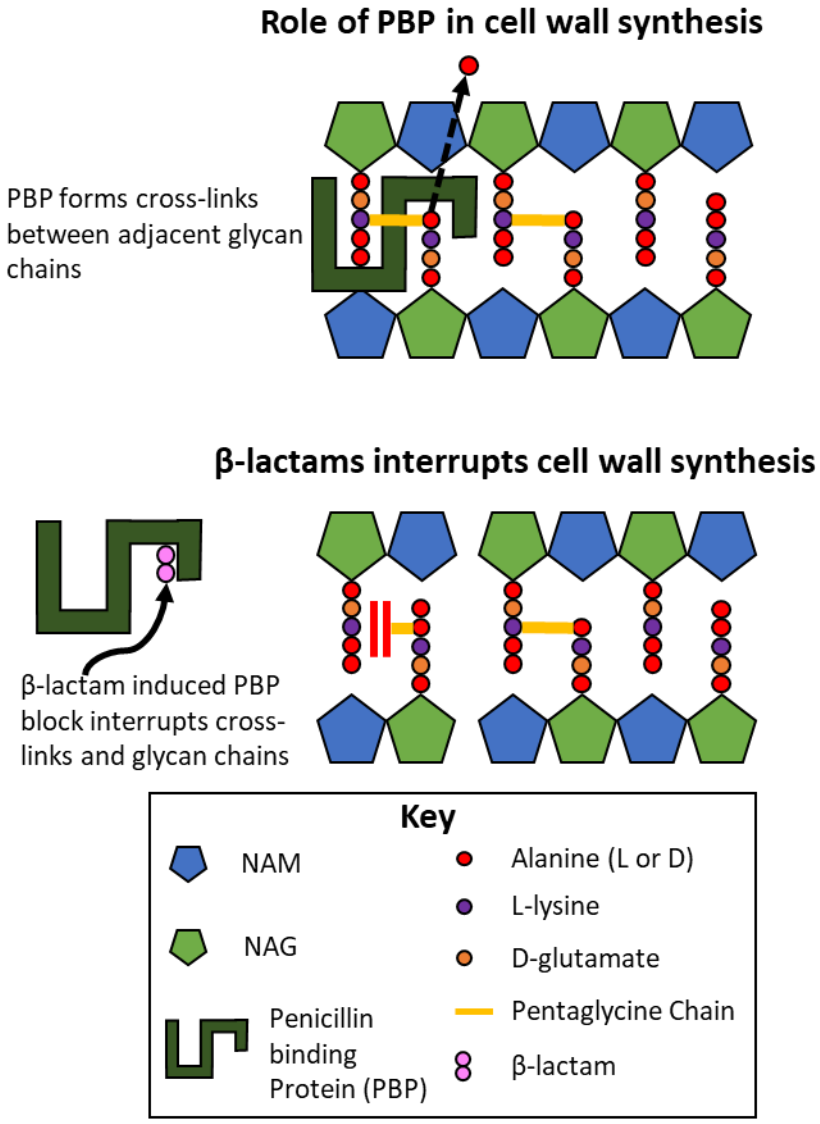

Figure 1-III **Mechanism of action of beta-lactam antibiotics**. Penicillin Binding Proteins (PBP) play a key role in forming a rigid cell wall that protects the bacterial cell from osmotic forces. Penicillin Binding Proteins (PBP) form Pentaglycine Chains between adjacent glycan chains, this catalytic reaction also results in the removal of a terminal D-alanine residue (Black arrow) from one of the peptidoglycan precursors. The structural resemblance of the Beta-lactam ring, in Beta-lactam antibiotics like penicillin, to the D-Ala-D-Ala substrate for the PBP, allows the Beta-lactam ring to bind tightly to the PBP. The binding of a Beta-lactam antibiotic to PBP prevents the formation of Pentaglycine Chains (vertical red lines), this results in a cell wall vulnerable to osmotic forces that may cause cell rupture. Figure adapted from (Clarkson, 2015).

1.6 Resistance to Antimicrobials

Whenever a new antibiotic is introduced, antibiotic resistance typically occurs within a few years. As most antibiotics are derived from microbial or fungal products, this is to be expected. The overuse and misuse of antibiotics creates selective pressure for those bacteria that are intrinsically (naturally) resistant or those that have acquired resistance, usually through horizontal gene transfer.

Resistance to antibiotics generally occurs via one of four mechanisms. 1) Bacteria may possess genes that encode enzymes that inhibit antibiotics, before they can take effect e.g. β-lactamases. 2) Bacteria may possess efflux pumps that prevent the antibiotic from binding to the target site by eliminating the antibiotic from the cell. 3) Bacteria may have the ability to alter the targets used by antibiotics. 4) Bacteria may possess mutation in genes that regulate porins, limiting the access of antibiotics (Kapoor, et al., 2017).

1.6.1 Intrinsic Resistance

Resistance of a bacteria to an antibiotic may be as a result of a general natural evolution, that is not related to any particular antibiotic class. An example of this is the outer membrane of Gram-negative bacteria, gives a natural resistance to some antibiotics (Kapoor, et al., 2017).

1.6.2 Acquired Resistance

Acquired resistance causes the most concern. Rather than the development of naturally occurring resistance to antibiotics, bacteria can develop resistance through the acquisition of resistance determinants via conjugation, transduction and transformation. This can occur not only between strains of the same species, but also between different species and genera. This process combined with mutations and selection via antibiotic pressure, enable bacteria to rapidly adapt to the introduction of a new antibiotic (Kapoor, et al., 2017).

1.7 Extended-Spectrum Beta Lactamases

Beta-lactam antibiotics are the most frequently used antibiotic worldwide, therefore bacterial resistance has continued to rise since their introduction due to a permanent selective force for resistance mechanisms (Pitout, et al., 1997; Bush & Macielag, 2010). This is an example of accelerated evolution following the Darwinian paradigm, whereby the most adapted to the environment will survive and pass on their genes to the next generation (Canton, 2012). Extended-spectrum β-lactamases (ESBLs) are mainly plasmid-encoded enzymes that are able to inactivate a variety of β-lactam antibiotics, including penicillins, 2nd-, 3rd- and 4th-generation cephalosporins and monobactams (e.g. aztreonam) (Zurfluh, 2015). However, ESBLs are generally susceptible to clavulanic acid (a beta-lactamase inhibitor), sulbactam and tazobactam (Shaikh, et al., 2015).

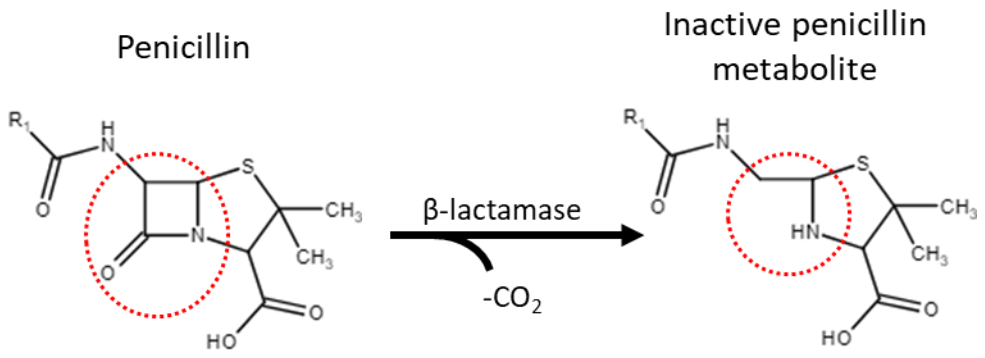

Figure 1-IV **β-lactamases confer resistance against β-lactam antibiotics**. The synthesis of β-lactamases is the most common mechanism that imparts drug resistance to β-lactam antibiotics (Hall et al, 2003). β-lactamases hydrolyse the beta-lactam ring, this prevents the antibiotic from inhibiting cell wall synthesis. Extended-Spectrum Beta-Lactamases (ESBLs) are enzymes produced by several types of gram-negative bacteria (*E coli*, *Klebsiella*, *Enterobacter*, *Proteus*) that enable bacteria to have resistance to all penicillins, cephalosporins, and monobactams, but do not affect sensitivity to carbapenems. Chemical structures were drawn using chem-space.com. Figure adapted from (Clarkson, 2015).

1.7.1 ESBL Classification

There are two distinct classification systems for beta-lactamases: the Ambler molecular classification and the Bush–Jacoby–Medeiros functional classification. The protein homology of the beta-lactamases is used to determine the Ambler scheme of four classes (Shaikh, et al., 2015). The four classes can then be differentiated further, classes A, C and D are serine beta-lactamases and class B are metallo-beta-lactamases. In contrast, the Bush–Jacoby–Medeiros functional scheme takes advantage of the functional properties of the beta-lactamase enzymes to classify them, for example their substrate and inhibitor profiles (Shaikh, et al., 2015). The Ambler Scheme shall be used from here onwards.

1.7.2 Important ESBLs

Clinically, the most important class A ESBLs include: TEM, SHV and CTX-M (Rahman, et al., 2018). The highest diversification and quickest evolution is within the CTX-M family of class A. The most important class D beta-lactamase is the OXA-type. The TEM, SHV and OXA type ESBL enzymes originate from point mutations within the plasmid-mediated TEM-1, TEM-2, SHV-1 and OXA-10 enzymes. ESBLs of the TEM and SHV-type were most frequently found in the 80's and 90's and are mainly associated with hospital outbreaks of *Klebsiella pneumoniae* (Canton, 2012). To this date (September 2018), there have been over 172 different CXT-M enzymes discovered, classified into five groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25, based on their amino acid composition (Rahman, et al., 2018). Being part of the class A ESBLS, it would be assumed that CTX-M producing enzymes would be related to TEM and SHV groups. However, this is not the case. Each of the CTX-M groups presents a 94% amino acid homology within the group itself and 90% homology between the other CTX-M groups (Shin, 2015). The CTX-M family is unlike other ESBLs in that it includes a complex and non-homologous group of enzymes. It has been suggested by phylogenetic analysis that the CTX-M enzymes did not originate by mutations from plasmid mediated enzymes but through chromosomal mobilization of bla genes from the *Kluyvera* species through mobile genetic elements. Like the other ESBLs, after these genes had been incorporated, the CTX-M enzymes evolved through punctual mutations due to antibiotic selective pressure. This brought about the variants of CTX-Ms that can be seen today (Canton, 2012). Within the CTX-M family of ESBLs, CTX-M-15 and CTX-M-14 are the most important, as they can

be found almost everywhere all over the world. In the UK the CTX-M-15 type is the most prominent (Canton, 2012). The first report of a CTX-M positive isolate was from Japan in 1986. The ESBL was originally called TOHO-1 but later changed to CTX-M due to its ability to resist cefotaxime. During the first decade or so after it was discovered, general dissemination and infrequent nosocomial outbreak was reported, mainly from Argentina. Over the last 15 years, CTX-M producing *E.coli* has become a major cause of community-onset UTI all over the world, creating the so-called "CTX-M pandemic" (Shin, 2015). Within the CTX-M family, CTX-M-15 has become the most important and widely distributed CTX-M enzyme. It was first discovered in India in 2001. The CTX-M-15 enzyme belongs to the CTX-M-1 cluster of ESBLs, thought to originate from the CTX-M-3 family of genes, differing by one amino acid substitution – an Asp to Gly change at position 240. This results in an increased resistance to Ceftazidime, due to increased catalytic activity (Peirano, 2010).

1.7.3 Discovery and Evolution of ESBL Groups

1.7.3.1 CTX-M

Phylogenetic trees of the CTX-M family suggest that the family can be differentiated into five clusters (Naas, et al., 2007; Bonnet, 2004). Each of these clusters has been linked to the chromosomal bla genes found in the *kluyvera* species of bacteria. The *kluyvera* species is a normal part of the intestinal flora of humans, found in low amounts and is associated with urinary tract, skin and soft tissue opportunistic infections. It is also found in water, soil, sewage and animal food products as free-living organisms. The ancestor of the CTX-M-1 cluster has been suggested to be the chromosomal bla gene kluC, from the *kluyvera*

cryocrescens. The origin of the CTX-M-2 cluster is said to be from the *kluyvera ascorbata* species. CTX-M-8, CTX-M-9 and CTX-M25 originated from three chromosomal bla genes from *kluyvera georgiana*, kluG, kluY and blaCTX-M-78. The CTX-M-74 and CTX-M-75 have been found to only have a single amino acid change from CTX-M-2 but may be considered as a new cluster, showing that the ESBLs are still evolving and diverging (Decousser, et al., 2001; Humeniuk, et al., 2002).

This being said, several questions about the lineage of the CTX-M family remain unanswered. When comparing the phylogenetic trees of CTX-M and 16s rDNA sequences of *Kluyvera* species, it is revealed that the evolutionary relationships do not correlate consistently (Canton, 2012).

1.7.3.2 OXA

The OXA-type are ESBLs and are so named because of their oxacillin-hydrolyzing abilities (Shaikh, et al., 2015). OXAs are beta-lactamases that facilitate resistance to ampicillin, cephalothin, oxacillin and cloxacillin, however are resistant to the beta-lactamase inhibitor clavulanic acid and are generally encoded by plasmids. The first OXA ESBL is believed to have been discovered in Turkey, from *P. aeruginosa* isolates in a single hospital (Shaikh, et al., 2015).

1.7.3.3 TEM

When first discovered in 1965, the TEM beta-lactamases substrate and inhibition profiles were comparable to SHV-1, for example TEM-1 is only capable of hydrolyzing penicillins and first generation cephalosporins. Since 1965, the TEM beta-lactamases have evolved and diverged, variants now have increased activity against extended spectrum cephalosporins, for example TEM-3 (Shaikh, et al., 2015).

1.7.3.4 SHV

The SHV beta- lactamases have been suggested to have originated from *Klebsiella spp.* Like the TEM beta lactamases, the SHV family has evolved from resistance to broad-spectrum penicillins such as ampicillin, tigecycline and piperacillin but not to the oxyimino substituted cephalosporins to conferring an extended spectrum resistance today (Shaikh, et al., 2015).

1.8 ESBL-Producing Urinary Tract Infections

It has recently become apparent that ESBL producing bacteria can be found not only in symptom producing patients, but also in asymptomatic human carriers. Increased risk of ESBL producing community-onset urinary tract infections can be due to many risk factors including multiple previous urinary tract infections, renal pathology, extended antibiotic use (especially cephalosporins), hospilisation/nursing home care, liver pathology, diabetes and international travel (Fournier, 2013; Zurfluh, 2015). Serious infection can be linked to length of hospital stay, admittance to an intensive care unit and catheterization. These infections in particular correlate with increased mortality, morbidity and healthcare cost (Wintermans, 2013; Shin, 2015). Those patients that suffer from a symptomatic UTI are

usually treated with antibiotics. Antibiotic treatment of UTI has been associated with long-term alteration of normal micro-biota of the vagina. Long-term antibiotic treatment for recurrent UTI is related to the development of antibiotic resistance (Al-Mayahie, 2013).

1.9 Epidemiology

1.9.1 Difficulties in Acquiring Epidemiology Data

ESBL epidemiology is very complicated and there are a number of issues to consider. The geographical area (country), the reservoir (humans, environment, companion animals, livestock etc.) and location of acquired infection (hospital or community) should all be taken into account when conducting prevalence studies (Shaikh, et al., 2015).

1.9.2 Global Epidemiology

1.9.2.1 Europe

There are still reports in Europe of new TEM and SHV types emerging. With regards to CTX-M, different subtypes are common in different countries. CTX-M-9 is common in Spain, whilst CTX-M-3 is common in Eastern Europe. Overall in Europe, the CTX-M-1 group (including CTX-M-15) is the most widespread and has been associated with isolates found in the community (Shaikh, et al., 2015).

In the UK, the Surveillance Atlas of Infectious Diseases reports the level of *E.coli* resistant to third generation cephalosporins in 2016 to be 9.2%. This is actually a decline in incidence since 2013. In comparison, the incidence level in Bulgaria in 2016 was 41.6%. Other problematic areas in Europe include Cyprus (30.2%), Italy (29.8%) and Slovakia (29.7%) as can be seen in figure I-V.

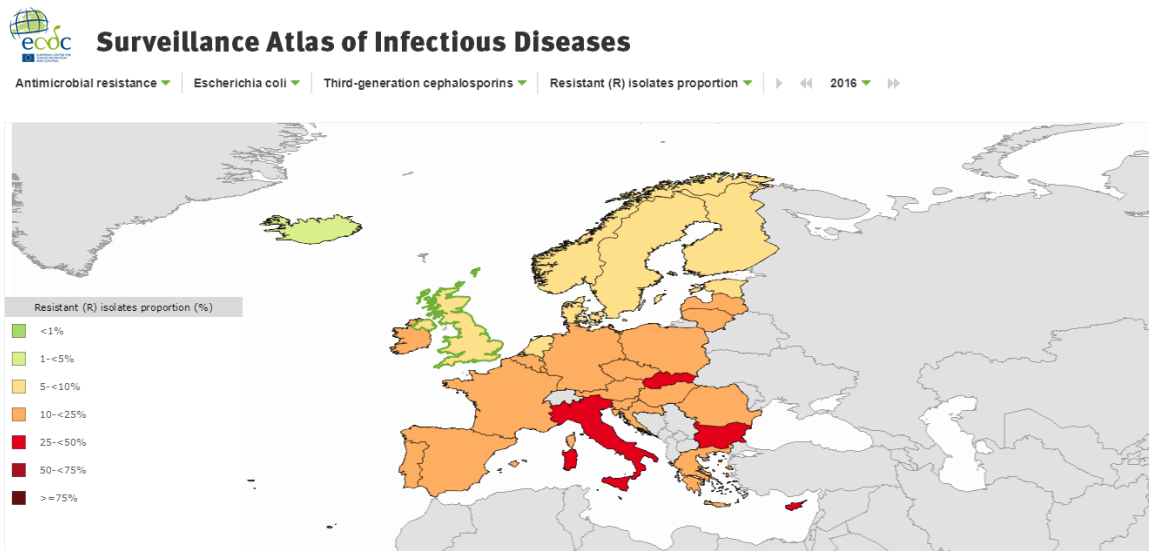

Figure 1-V Map of surveillance information for third-generation Cephalosporin resistance in *E. coli*. Dataset provided by ECDC based on data provided by WHO and Ministries of Health from the affected countries. Surveillance information contains percentages of *E. coli* isolates that are resistant to third-generation Cephalosporins in Europe, in 2016 (European Centre for Disease Prevention and Control, 2017).

1.9.2.2 Asia and the Indian Subcontinent

It has only been recently that we have begun to understand the degree to which ESBL-producing *Enterobacteriaceae* is effecting parts of Asia and the Indian subcontinent, and the incidence of ESBLs is continuing to rise (Shaikh, et al., 2015). In India specifically, three medical centers reported that 66% of third generation cephalosporin resistant *Enterobacteriaceae* harbored CTX-M-15. Ten other centers in India reported ESBL rates of 70% within *Enterobacteriaceae* (Shaikh, et al., 2015).

It has been suggested that several of the most successful clones that harbor ESBLs have originated from Asia (Shaikh, et al., 2015). The main factors in the development of resistance include inadequate sewage practices, insufficient quality of drinking water and a lack of regulation with regards to the sale of antibiotics over the counter and the over prescription of antibiotics be doctors (Shaikh, et al., 2015).

1.9.2.3 The Middle Eastern Countries

Incidences of 26% for ESBL-producing *Klebsiella pneumoniae* isolates have been seen in Saudi Arabia, whereas a prevalence of 26.5% for *E. coli* and 43% for *Klebsiella* has been reported in Iran (Tawfik, et al., 2011). A different study in Iran reported that 42.1% of the *E. coli* isolates derived from patients with clinical symptoms of UTIs were of ESBL-producing (Ebrahim-Saraie, et al., 2018).

1.9.2.4 Africa

In Tanzania, hospitals have described rates of ESBL-producing isolates in *Klebsiella pneumoniae* as high as 64%. An orphanage in Mali reported that 100% of the children carried ESBL-producing *Enterobacteriaceae* (Shaikh, et al., 2015). Likewise, in Madagascar, it was observed that 10% of non-hospitalised patients carried ESBLs. It has been suggested that poverty is an important risk factor for carriage of ESBLs (Shaikh, et al., 2015). A systematic search review that used Twenty-six studies (409215 isolates) from 13 African countries ESBL-producing isolates from a urinary source varied from 1.5% to 22.8% (Tansarli, et al., 2014). Combined, these reports may indicate that the prevalence in Africa may be increasing.

1.9.2.5 America

It was estimated in 2001 that 5.3% of *E.coli* in America was ESBL-producing. A more recent study in 2009 in a cancer center revealed 9% of *E.coli* isolates were ESBL-producers.

As can be seen here, there is substantial global variation in the incidence of antibiotic resistance. This variation has been linked to the usage of antibiotics. Some countries have been working hard to reduce their antibiotic consumption, whereas others have seen a rapid rise in usage. Despite the variation in antibiotic consumption and resistance rates, it is clear that antibiotic resistance is a concern worldwide.

1.10 The One Health Approach

The One Health Approach to tackling antibiotic resistance outlines the importance of understanding the relationship between humans, animals and the environment when it comes to the spread of antibiotic resistance genes. The main aim of the One Health Approach is to ensure that antimicrobial agents continue to be effective by developing policies that promote the responsible use of antimicrobial agents. This involves investigating the possible transmission between these three vectors and creating suitable antibiotic stewardship and other policies for reducing resistance rates in isolates found in humans, animals and what is inevitably released into the environment (Conrad, et al., 2013).

1.11 Spread of ESBLs

The rise and ease of international travel of both humans and animals has increased the opportunity for antibiotic resistance determinants to spread worldwide. As previously mentioned, bacteria can share and acquire genes that increase their antibiotic resistance. This can create new antibiotic resistant strains at a much faster rate than before (Sleeman, et al., 2017). A summary of this mechanism can be seen in figure I-VIII.

1.11.1 Mobile Genetic Elements

Mobile genetic elements are sequences of genetic material that can change places on a chromosome, and be exchanged between chromosomes, between bacteria, and even between species (Frost, et al., 2005). Plasmids are an example of this. Tracking mobile genetic elements between bacterial species and locations allows for the study and prediction of evolution.

1.11.2 Insertion Sequences

Insertion sequences (IS) are similar to mobile genetic elements, in that they can move from one location to another, however they are limited to the bacterial chromosome. Insertion sequences often appear in the middle of a gene, and this can disable the expression of that gene by interrupting the coding sequence. They can also inactivate the expression of genes downstream of the insertion sequence in the same operon, owning to the presence of termination signals of transcription and translation (Griffiths, et al., 2000).

It has been found that there is a link between CTX-M genes and the surrounding genetic elements. As mentioned previously, the theory is that CTX-M genes originated from the *Kluyvera* species. It is thought that this occurred due to mobilisation of CTX-M genes by insertion sequences and possibly bacteriophages. Insertion sequences have many functions, but with relation to CTX-M, they are responsible for the over-expression of CTX-M genes, leading to a high resistance phenotype. Some insertion sequences are adjacent to integron structures, which in turn are integrated into transposition units, called supra-structures. Successful or high-risk clones often contain these supra-structures, which are incorporated within conjugative plasmids. In addition to this, supra-structures can also perform as

evolutionary units and units of selection (Canton, 2012). Integrons which contain multiple gene cassettes are called super-intergrons. Many gene cassettes contain genes which code for antibiotic resistance, therefore benefitting their host (Ravi, 2014). Mobilisation of CTX-M genes has been demonstrated experimentally due to insertion sequences located upstream. The promoter P_{out} increases the expression of CTX-M by effecting upstream insertion sequences. It is thought that this had a role in the selection and dissemination of CTX-M. Upstream sequences that have been identified include ISEcp1, ISCR1, IS10 and IS26. The most common insertion sequence by far is ISEcp1, originally found upstream of CTX-M-15 in 1999. It has also been found in all other clusters of ESBLs, apart from CTX-M-8 (Canton, 2012).

1.11.3 Spacer Sequences

A spacer sequence is a region of non-coding DNA between genes. Spacer sequences, in relation to the genetic distance between ISEcp1 and CTX-M, is mentioned in multiple studies (see figure 1-VI). It is well documented that the spacer sequence between ISEcp1 and CTX-M has an influence on Cephalosporin MIC. The distance of spacer sequence ranges between CTX-M clusters. In CTX-M-1, the spacer sequence is between 48 and 127bp in length. There is a strong homology between spacer sequences in the CTX-M-1 cluster (Canton, 2012; Ma, et al., 2011).

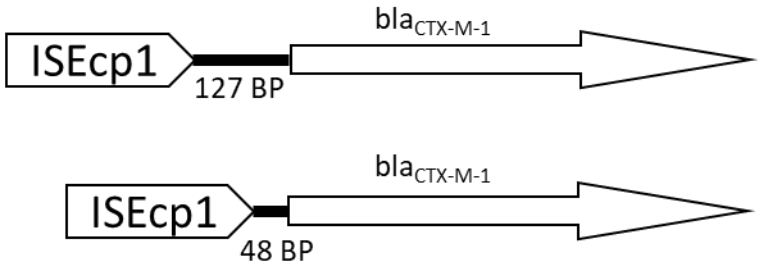

Figure 1-VI **The relationship between spacer sequence and antibiotic susceptibility**. The length of the spacer sequence between the insertion sequence ISEcp1 and the gene cassete CTX-M-1 is variable. This example shows spacer sequences of 127 bp (top) and 48bp (bottom). The larger the spacer sequence, the higher the susceptibility of the isolate to Beta-lactam antibiotics. Figure adapted from (Zong, 2010).

1.11.4 Plasmids

Plasmids are a type of mobile genetic element that are able to integrate directly into the chromosome or persist in the bacterial cytoplasm (see figure 1-VII). Plasmids are key agents of change in bacterial populations. All bacteria, including *E.coli*, transport plasmids. A plasmid is a self-replicating double stranded, circular or linear genetic element and vary in size from a few to more than several hundred kb. Plasmids usually regulate their rate of replication to control their concentration within the cell, maintaining their characteristic copy number throughout the cell line. There is a minimal part of the plasmid that must replicate with the characteristic copy number of the parent plasmid, called the basic replicon. These replicons incorporate the ori, but also contain specific replication initiator protein (REP) encoding genes. REPs bind to the ori and their regulating factors. Plasmids spread through the promotion of conjugation between bacteria of different genera and kingdoms. Conjugation involves transferring plasmids from a donor to a recipient cell. This is a contact–dependent, energy-driven system. There are plasmids that are unable to transfer themselves via conjugation, these can be transferred with the use of a helper plasmid.

Plasmids are selfish genetic elements, as they are able to facilitate their own replication. This being said, most plasmids also carry genes that are beneficial to their bacterial host. It is quite often beneficial to carry certain plasmids as they carry a selectable phenotype, for instance virulence of some kind, or antibiotic resistance, along with insertion sequences, transposons and class 1 integrons. Plasmids can carry more than one gene for selectable advantage. For example, resistance plasmids can also carry virulence factors such as bacteriocins and cytotoxins. Likewise, virulence plasmids have also been known to carry resistance genes such as those that code for ESBLs. Under antibiotic and infectious pressure, these traits may facilitate the successful spread of certain plasmid types between different bacteria and geographical locations (Moran, 2015). It is this spread that has made the tracking of plasmids so important. Tracking plasmids helps to understand how plasmids are disseminated so widely and quickly. Tracking is not as easy as it seems, as there are many different types of plasmid, and each type of plasmid has many variations that need to be taken into account (Moran, 2015). A plasmid classification scheme was designed by Datta and Hedges in 1971 based plasmid incompatibility (Inc). Plasmid Inc is determined by the stability of the plasmid during conjugation. Two plasmids of the same Inc group cannot be conjugated into the same cell stably. There are currently 27 recognised Inc groups within *Enterobacteriaceae*. Plasmid typing on a molecular scale was first proposed in 1988 by Couturier, involving replicons of each Inc types and southern blot hybridisations. It wasn't until 2005 that a PCR-Based Replicon Typing (PBRT) scheme was introduced. This uses multiplex PCR to detect the Inc groups of the major plasmid types occurring in *Enterobacteriaceae* (Carattoli, 2005). Many papers have discussed the

importance of plasmids in the spread of ESBL genes, especially CTX-M (Carattoli, 2011). It appears that CTX-M is primarily associated with plasmids of Inc group FII and have been termed as “epidemic resistance plasmids”. FII plasmids are narrow host-range and are characterised by a low-copy number in the host bacteria. They are able to easily acquire resistance genes and transfer amongst bacteria, making them a major concern. It is thought that FII plasmids contain replicon types that help speed up the rate at which evolution and diversification of plasmids occurs. It has also been suggested that these plasmids were already incorporated into many *Enterobacteriaceae*, meaning that they were already well suited to these bacteria before acquisition of resistance genes occurred. This explains the rapid dissemination of CTX-M genes within *Enterobacteriaceae*. Also, these plasmids may have already contained genes coding for resistance other than beta-lactamases, contributing to the co-selection process during their evolution. It is thought that these genes will persist and evolve over many generations, giving rise to new CTX-M variations and yet wider dissemination (Canton, 2012).

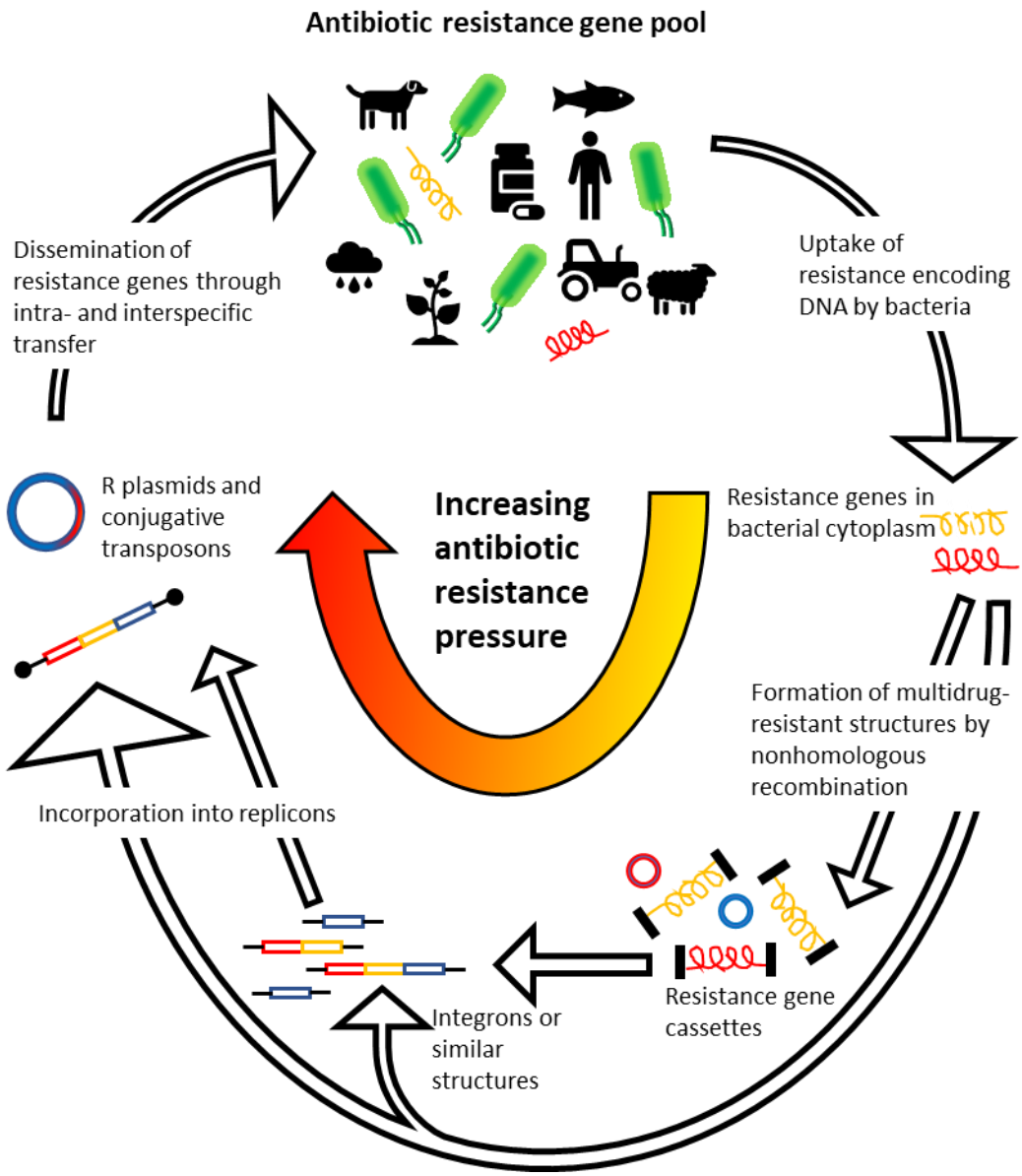

Figure 1-VII **Dissemination of antibiotic resistance genes and production of resistant bacteria**. The diagram summarises how bacteria can acquire, incorporate, express and spread genes from their environment. This process is exacerbated by antibiotic misuse which results in bacteria utilising increasing antibiotic pressure as a method of survival. Over time this can result in the development of multi-drug resistant bacteria which may spread throughout the environment. Figure adapted from (Davies, 1994).

1.11.5 Addiction Systems

Plasmids produce addiction systems (or toxin-antitoxin) to facilitate the maintenance of plasmids during replication. Addiction systems are small genetic elements that, when not present, can cause bacterial cell death and/or cell arrest. The plasmid produces both a toxin and an anti-toxin. The anti-toxin prevents the toxin's deleterious activity. Therefore, when the plasmid is removed from the cell, the toxin produced previously is able to cause replication or segregation errors. Therefore, it is in the cell's best interest to maintain the presence of these plasmids (Hayes, 2003). It is thought that CTX-M producing plasmids use many addiction systems to ensure that the plasmids are maintained in multiple strains of bacteria, facilitating their dissemination (Tamang, 2014). On the other hand, plasmid addiction systems represent potential antibacterial targets, resulting in the development of novel drugs to counteract plasmids contains genes coding for multi-drug resistance (Tamang, 2014).

1.12 ESBL Detection in Clinical Practice

In order to detect a UTI, a midstream, clean-catch urine sample is taken from a patient suspected of having cystitis (Schmiemann, et al., 2010). Cystitis is then determined as the presence of $\geq 10^3$ bacteria/ml. Clinicians can also screen samples by colorimetric dipstick testing for nitrites and leukocyte esterase, however, there have been many reports of false-negative results. Therefore, further microscopic urinalysis and culture is performed (Schmiemann, et al., 2010). Once in the clinical microbiology laboratory, a sample is then cultured on agar plates and those that grow $\geqslant$104 cfu/ml of a single or predominant species of uropathogenic bacteria are considered culture positive (Davenport, et al., 2017).

Chromogenic agar can help to distinguish between *E.coli, Staphylococcus saprophyticus and Enterococcus* species (D'Souza, et al., 2004). The time taken from urine collection to identification of the causative pathogen is normally 18-30 hours.

1.12.1 Defining Antibiotic Resistance

One of the significant roles of the clinical microbiology laboratory is the determination of antibiotic susceptibility, taking an additional 24-48 hours depending on the pathogen and the method used. In the UK, the Health Protection Agency (HPA) provides guidelines for the detection of ESBLs in *Enterobacteriaceae* and recommend antibiotic susceptibility testing with either 1 mg/L of cefpodoxime, 1 mg/L each of cefotaxime, ceftazidime, ceftriaxone, or aztreonam, followed by confirmatory tests with both cefotaxime and ceftazidime in combination with clavulanate at a concentration of 4 lg/mL. This method shows a high sensitivity and specificity, 94% and 98% respectively (Shaikh, et al., 2015). Some automation has been carried out in some laboratories, with high-throughput instruments such as the Microscan Walkaway (Beckman Coulter), Phoenix Automated Microbiology System (BD), and Vitek 2 (BioMérieux). These instruments can improve turnaround time to around 10-16 hours (Eigner, et al., 2005; Thomson, et al., 2007).

1.12.2 Genotypic Detection

Genotypic detection of ESBLs generally involves PCR amplification, and in the case of TEM and SHV genes, subsequent sequencing to distinguish between ESBL and non-ESBL genes. Recently, several studies have depicted various new approaches to rapidly detect ESBLs (Davenport, et al., 2017).

1.13 Treatment

Antibiotics are currently the recommended treatment for symptomatic UTI including trimethoprim sulfamethoxazole, ciprofloxacin and ampicillin (Hooton, 1997). However, increasing rates of resistance has led to the use of carbapenems instead. The use of carbapenems as an alternative treatment has led, as expected, to an increased prevalence of carbapenems producing multi-drug resistant *Enterobacteriaceae*. (Vigil et al., 2010 and Gqunta. 2015).

1.13.1 Alternative Treatments

1.13.1.1 Treatment with Cranberry

Cranberry fruit has been suggested as an alternative for the treatment and prevention of UTI (Fu, et al., 2017). Cranberries are mostly composed of water, but also contain organic acids, fructose, a high level of vitamin C, flavonoids, anthocyanidins, catechins and triterpenoids (Guay, 2009). There are many species of cranberry, however the American cranberry (*Vaccinium macrocarpon*) is the only one that has been shown to have a role in UTI. It is thought that this is due to a reduction in bacterial adhesion to the urinary tract wall. Cranberries are most commonly taken as whole berries, juices (usually only 10-25% cranberry) and capsules. Juices are the most common (Vostalova, et al., 2015). Pure cranberry juice is unpalatable due to its acidity. It is recommended that people consume cranberry either before a meal, or two hours after and drinking excess water is generally necessary, particularly after drinking dehydrated juices. Inhibition of adherence by cranberry has also been seen in multidrug resistant UPEC strains. The combination of cranberries and antibiotics has also been tested. In a combination of β-lactams and

cranberry, a modest delay in absorption was seen, however overall absorption was not affected. The biosafety of cranberries has been tested, with no biochemical or haematological alterations found and it has been suggested that cranberry has up to an 8-hour window that it decreases UPEC adherence after consumption (Hisano, et al., 2012).

1.14 Summary

UTIs are one of the most common bacterial infections. Both the numerous uropathogens, which encode a wide range of virulence factors, and the spread of antimicrobial resistance threaten the only effective treatment option available — antibiotics. Moreover, high rates of recurrent UTIs suggest that antibiotics are not an effective therapy for all UTIs.

The CTX-M family of genes has become an important area of study in recent years. It is thought that the CTX-M genes originated from the *Kluyvera* spp, however there is some contradictory research with regards to this. Since its origination, CTX-M has spread considerably, creating the so-called “CTX-M epidemic”. Successful dissemination has been associated with the co-existence of CTX-M and other resistance determinants, leading to multi-drug resistance. CTX-M genes are closely linked to IncFII plasmids, thought to be present in multiple *Enterobacteriaceae* before the evolution of CTX-M genes, one explanation as to how this particular type of ESBL was able to spread so rapidly. However, research is conflicted as to the correlation between the presence of CTX-M genes and the level of virulence factors. It is clear that current recommended methods for the detection of CTX-M positive isolates are not sufficient in current circumstances. Rapid methods such as Real Time-PCR have been suggested due to their greater sensitivity and the fact that identification is at the genetic level. Whichever method is used, it is clear that the CTX-M

family of genes will remain an important issue for some time, and their evolution and further dissemination needs to be closely monitored.

1.15 Aims

1. Complete genotypic study to establish the prevalence of ESBLs and the CTX-M family within samples collected from the Leicester Royal Infirmary (LRI).
2. Complete plasmid replicon typing of samples from LRI.
3. Develop a rapid detection method for ESBLs in urinary tract infection.
4. Analyse the effect of a natural product on the expression levels of virulence genes produced by *E. coli* using quantitative reverse transcriptase-PCR (RT-qPCR).

1.16 Thesis Layout

This thesis is manuscript based and has been organized into five chapters. Chapter 1 introduces the topic and outlines the rationale and research objectives. Chapter 2 is a manuscript describing the prevalence of ESBLs in the Leicestershire area. Chapter 3 describes the development of a rapid qualitative real-time PCR based method to detect ESBLs. Chapter 4 is a manuscript showing results of an analysis of the effect of cranberry on the expression of virulence genes in *E.coli*. Chapter 5 is the overall discussion and conclusion of the thesis. Appendix A outlines the experimental development and rationale.

Chapter 2:

Genotypic Identification of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Enterobacteriaceae* from Urinary Tract Infections in the Leicestershire Area, United Kingdom: A One Health Prospective

Statement of Contributions of Joint Authorship

Reid, R: (Candidate)

Writing and compilation of manuscript, carrying out of experiments, data analysis, preparation of tables and figures

Samarasinghe, S: (Supervisor)

Assisted with manuscript compilation and editing.

The journal paper referred to below has been adapted for use within a thesis.

Genotypic Identification of Extended-Spectrum Beta-Lactamase (ESBL) - Producing *Enterobacteriaceae* from Urinary Tract Infections in the Leicestershire Area, United Kingdom: A One Health Prospective

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Running title: Genotypic Identification of ESBLs in Leicestershire, UK

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Abstract

Aims

Urinary tract infections (UTIs) are one of the most common infections diagnosed in the United Kingdom (UK). The prevalence of extended-spectrum-beta-lactamase (ESBL) producing UTIs has dramatically risen, limiting treatment options. The emergence and spread of ESBLs is thought to be through the horizontal transmission of antibiotic resistance plasmids IncL/M, IncF, IncN and IncI1. These conjugative plasmids have been described as important vectors and directly linked to major outbreaks of antibiotic resistance. This study aimed to investigate the prevalence of ESBLs in Leicestershire, UK and their relationship with antibiotic resistance plasmids.

Methods

A total of 236 ESBL producing uropathogenic *Enterobacteriaceae* isolates were obtained from the Leicester Royal Infirmary (Leicestershire, UK). ESBL production was confirmed phenotypically via the MAST ID double disc synergy test. ESBL-producing genes (CTX-M, SHV, TEM and OXA) were identified by multiplex PCR. The CTX-M family was then further characterised into (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) by multiplex PCR. The relationship between ESBL-producing genes and plasmid type was then investigated by multiplex PCR-based replicon typing to detect IncFIA, IncI1, IncL/M, IncN and IncFII.

Results

ESBL genes were identified as follows: CTX-M (71.6%), OXA (7.6%), TEM (3.8%) and SHV (3.8%). Multiple resistance genes were detected in 16% of isolates. CTX-M genes were identified as follows: CTX-M-1 (84.1%), CTX-M-9 (12.5%), CTX-M-25 (1.7%), CTX-M-8 (1.1%) and CTX-M-2 (0.6%). Replicon typing results were as follows: IncL/M (29.2%), IncN (14.4%), IncI1 (5.1%), IncFII (27.5%) and IncFIA (23.3%). A combination of IncL/M, IncFII and IncFIA was the most common at 9.8%. A positive correlation between CTX-M and all plasmids except IncI1 was found.

Conclusion

CTX-M producing *Enterobacteriaceae* are associated with multiple plasmids, which can be linked to its rapid spread across the world. Prevalence studies help to inform policy about antibiotic stewardship and resistance evolution, aiming to reduce resistance levels in the future.

Keywords: Extended-spectrum beta-lactamases, multiplex PCR, antibiotic resistance, CTX-M, urinary tract infection, replicon typing, One Health Approach.

2 Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infectious diseases diagnosed in outpatients in the UK. Of the causes of UTIs, uropathogenic *Escherichia coli* (UPEC) is the most prevalent (Al-Assil., 2013). Risk factors for acquiring a UTI include previous exposure to 3rd and 4th generation cephalosporins and fluroquinolones; hospitalisation; old age; female gender; recurrent UTI infection, diabetes and catheterisation (Al-Assil., 2013).

The most common type of resistance in UTIs is beta-lactamase production, resulting in extended-spectrum beta-lactamase (ESBL) producing bacteria (Lupo, 2013). These bacteria produce enzymes that can hydrolyse oxyimino-cephalosporins (ceftriaxone, cefotaxime, ceftazidime and cefepime) and monobactams (aztreonam) but not cephamycins and carbapenems. Based on their amino acid sequence, these enzymes have been classed into four groups – A – D (Lupo, 2013). Class A enzymes have been shown to be the most clinically important, due to their link to treatment failure, increased morbidity, mortality and healthcare costs. The most important class D beta-lactamase is the OXA-type. There are 3 main families of class A: TEM, SHV and CTX-M. While TEM, SHV and OXA ESBLs arise via substitutions in strategically positioned amino acids from the natural narrow-spectrum TEM-1/-2, SHV-1 and OXA-10 β-lactamases, all CTX-M variants demonstrate an ESBL phenotype (Lupo, 2013). Previously, most ESBLs detected were of the TEM/SHV group. From the 1990's the CTX-M family became increasingly more common, and now it is reported to be the most prevalent type of ESBL detected at present

worldwide (Lupo, 2013). The worldwide dissemination and dramatically increasing prevalence of the CTX-M family of ESBLs is due to the selective pressure caused by the over-use, and more importantly, mis-use of the beta lactam antibiotics (Lupo, 2013).

Risk factors for an ESBL-producing UTI are: recent hospilisation; treatment with 3rd generation Cephalosporins; old age (over 65); diabetes; recurrent UTI; indwelling catheters and female gender (Dash, et al., 2018). It has been reported that ESBL-producing isolates are more likely to be associated with significant pyuria, suggesting that ESBLs are more likely to cause a clinically significant UTI (Qiao, et al., 2017; Wragg, et al., 2017). Although most ESBL-producing UTIs are acquired in the community, a hospitalised patient is approximately 4 times more likely to be diagnosed with an ESBL-producing UTI (Dash, et al., 2018).

The significant increase in movement of livestock and agricultural produce and human travel has facilitated the rapid amalgamation and dissemination of antibiotic resistance genes (Bengtsson-Palme, et al., 2018). The emergence and spread of antibiotic resistance determinants is often through horizontal transmission of mobile genetic elements such as plasmids (Roshani, et al., 2017).

It is the large, low-copy, self-transmitting resistance conjugative plasmids that are increasingly threatening the efficacy of antibiotics for Gram-negative infections; and have been directly linked to antibiotic resistance outbreaks. Conjugative plasmids can carry more than one gene for a selectable advantage such as antibiotic resistance genes and virulence factors such as bacteriocins and cytotoxins. Under antibiotic and infectious pressure, these

traits may facilitate the successful spread of certain plasmid types between different bacterial hosts and geographical locations (Moran, 2015). This results in ESBL-producing isolates that can show resistance to other types of antibiotics, leading to multidrug resistance (MDR). MDR limits treatment options, resulting in less favourable outcomes for patients (Shaikh, et al., 2015). Surveillance of the spread of these plasmids can help track antibiotic resistance determinants and phylogenetic and comparative analysis can identify the origin and evolution of these genes (Bennett, 2008).

Many papers have discussed the importance of plasmids in the spread of blaCTX-M genes, especially CTX-M-1 (Carattoli, 2011; Agyekum, et al., 2016; Bevan, et al., 2017; Bonnet, 2004; Canton, 2012; Zurfluh, et al., 2015). Studies have suggested that CTX-M is primarily associated with plasmids of IncF, IncL/M, IncN and IncI1 and these have been termed as "epidemic resistance plasmids" in *Enterobacteriaceae* (Wang, et al., 2013). It is suggested that these plasmids are successful as they likely provide virulence and antimicrobial resistance determinants, such as ESBL-production, that contributes to the fitness of the bacterial host. They also guarantee their survival and stability in the host through the production of several addiction systems, such as toxin-antitoxin systems. Therefore, they are maintained without the need for selective pressure of antibiotic treatment. This could explain the rapid dissemination of CTX-M genes within *Enterobacteriaceae* (Carattoli, 2009).

IncF plasmids are narrow host-range and are characterised by a low-copy number in the host bacteria. They are able to easily acquire resistance genes and transfer amongst bacteria, making them a major concern. It is thought that IncF plasmids contain replicon

types that help speed up the rate at which evolution and diversification of plasmids occurs (Mathers, et al., 2015). It has also been suggested that these plasmids were already incorporated into many *Enterobacteriaceae*, meaning that they were already well suited to these bacteria before acquisition of resistance genes occurred (Carattoli, 2009). All IncI1 plasmids contain genes for the type IV pili in their plasmid backbone structure. This has been suggested to contribute to the adhesive and invasive properties of shiga-toxigenic *E. coli* (Zurfluh, et al., 2015). IncL/M are broad-host range plasmids and reports have suggested that the IncL/M family are mostly responsible for the dissemination of CTX-M-3 in Eastern Europe, France, Belgium and Korea (Carattoli, 2009). It has been observed that the IncL/M plasmid has a higher plasmid copy number than IncF co-purified in the same host, suggesting that IncL/M could induce a higher resistance level in its host due to increased gene dosage (Adamczuk, et al., 2015).

IncN are also broad-host range plasmids and some studies suggest that IncN is specific to the food chain and it has been confirmed that these plasmids are circulating among food producing animals (Zurfluh, et al., 2014). CTX−M−1 has frequently been reported on the broad host-range replicon plasmids IncI and IncN, which appear to have a reservoir in animals. In particular, a high prevalence among *E. coli* of the avian faecal flora has been found (Zurfluh, et al., 2014).

Realising the major health concern that antibiotic resistance presents, the world health organisation (WHO) developed a global strategy for the containment of antimicrobial resistance in 2001. This involved monitoring the prevalence of ESBLs in the UK, and other countries all over the world. Prevalence studies help to inform policy on infection

prevention and antibiotic stewardship. They allow monitoring of evolution of antibiotic resistance genes and prediction of possible future evolution (World Health Organization, 2001). The importance of surveillance was reiterated in the O'Neill report in 2016 where it was suggested that all antibiotic prescriptions should be informed by up-to-date surveillance information (O'Neill, 2016).

The One Health Approach to tackling antibiotic resistance outlines the importance of understanding the relationship between humans, animals and the environment when it comes to the spread of antibiotic resistance genes. This involves investigating the possible transmission between these three vectors and creating suitable antibiotic stewardship and other policies for reducing resistance rates in isolates found in humans, animals and what is inevitably released into the environment (Robinson, et al., 2016). Prevalence studies are an important way of monitoring the transmission of resistance determinants between vectors, and whether stewardship and other measures are working effectively (O'Neill, 2016).

It has been known for some time that companion animals can act as a reservoir for antibiotic resistance determinants. Pets can not only be a source of contamination, but due to close contact with their owners, could be contaminated by human bacteria (Melo, et al., 2018). Contributing factors include the growing population of both humans and companion animals and urban development, leading to increased interactions among people and animals (Sleeman, et al., 2017).

The contribution of food production to the global antibiotic resistance problem has been questioned, as there are not that many reports of animal-associated infections in humans. Due to the way that antibiotics are used in food production, at sub-therapeutic

concentrations and with long exposure, this creates the ideal conditions for antibiotic resistance determinants to be acquired and subsequently transmitted to humans via human-adapted pathogens through farmers, contaminated food or the environment. Therefore, the misuse of antibiotics in food production can be a driver of antibiotic resistance that needs to be monitored and rectified (Prestinaci, et al., 2015).

The environmental element of the One Health Approach is the least well understood. Antibiotics are used in plant protection and environmental bacteria serve as a source for antibiotic resistance in a similar manner to that of animals, which can be incorporated into pathogens that infect animals and humans. The problem is exacerbated by the discarding of waste into the environment that contains antibiotic resistance genes and antibiotic residues from the pharmaceutical industry, livestock and hospitals. They are therefore providing a resistome, as a mixture of resistant and non-resistant, pathogenic and non-pathogenic bacteria that can exchange plasmids and therefore antibiotic resistance genes such as ESBLs between one another (Zurfluh, et al., 2015). This is more likely to be a significant problem in developing countries, due to issues with environmental legislation and enforcement.

The aim of this study is to identify the prevalence of the different ESBL types in Leicestershire by multiplex PCR. PCR-based replicon typing will be used to investigate whether prevalence is associated with antibiotic resistance plasmids. It is hypothesised that the prevalence of ESBLs in this area is related to the conjugative resistance plasmids IncL/M, IncF, IncN and IncI1.

2.1 Materials and Methods

2.1.1 Isolate Collection and Phenotypic Detection

Bacterial isolates (n=236) of *Enterobacteriaceae* isolated from urinary tract infections were obtained from the Leicester Royal Infirmary hospital (Leicester, England). More description of isolate collection and storage can be found in 6.1 of Appendix. The Leicester Royal Infirmary was chosen as a collection site as a large number of samples are received from all over the Midlands, and the Midlands has a large population of a wide variety of ethnic groups. Informed consent from patients was not required, as waste material (bacterial cultures) was collected only, therefore there is no link to patient data. ESBL production was confirmed phenotypically via the MAST ESBL ID double disc synergy method, conforming to the European Committee on Antimicrobial Susceptibility (EUCAST) standards. Discs contained Cefotaxime, Ceftazidime and Cefpodoxime with an ESBL inhibitor (clavulanic acid) counterpart. Plates were incubated for 18 hours at 37°C. A zone of inhibition difference of 5mm or more between the beta-lactam and the ESBL inhibitor clavulanic acid, indicated a positive result for ESBL production. Four control isolates were obtained from Public Health England: NCTC 13353 (CTX-M-15), NCTC 13351 (TEM-3), NCTC 13368 (SHV-18) and NCTC 13442 (OXA-48) for use as controls in phenotypic and genotypic tests (see table 6-1).

2.1.2 DNA extraction and Genotypic Detection by Multiplex PCR.

The method used for DNA extraction has previously been outlined (Reid & Samarasinghe, 2018). Genotypic identification was by means of a multiplex PCR method for the detection of the ESBL-producing genes (CTX-M, SHV, TEM and OXA). These were then further characterised into the CTX-M family (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) by another multiplex PCR assay. The GoTaq G2 Flexi DNA Polymerase kit was chosen for all PCR assays (Promega, Southampton, UK). PCR amplification reactions were performed using the PikoReal® 96 well Real Time-PCR platform (ThermoFisher, Loughborough, UK) in a volume of 25µl containing 5µl of 5X Flexi Buffer, 2µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.125 µl of 5U/µl GoTaq G2 Flexi DNA polymerase, 400 nM of each primer, 2.5µl of DNA template and made up to 25µl with water.

Cycling parameters for the first multiplex assay were as follows: an initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, annealing at 65°C for 1 min (gradual temperature decrements of 0.5°C per cycle, final annealing temperature 48°C), and 72°C for 1 min; and with a final extension at 72°C for 10 min.

Cycling parameter for the second multiplex assay were as follows: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 25 s; 52°C for 40s and 72°C for 50 s; and a final extension at 72°C for 6 min. Primers can be found in table 2-1. More details of primer design can be found in 6.4.1.

Table 2-1 Primers used in this study. Primers for the first multiplex PCR assay were TEM, SHV, CTX-M and OXA. The primers for the second multiplex PCR assay were CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Primers were designed using the OligoAnalyzer 3.1 (Integrated DNA Technologies Inc, Illinois, USA) Primers were then checked for specificity by the BLAST software (National Center for Biotechnology Information, Bethesda, USA). All PCR products were ran on a 2% agarose gel (UltraPure™ Agarose, ThermoFisher Scientific, Paisley, UK) for 90 mins at 60 Volts. A GeneRuler 50 bp DNA Ladder, ready-to-use (ThermoFisher Scientific, Paisley, UK) was used for comparison.

Primer	Forward Primer	Reverse Primer	Product Size	Reference
TEM-1	CGG ATG GCA TGA CAG TAA GAG	AGG ACC ACT TCT GCG CTC G	782	(Reid & Samarasinghe, 2018)
SHV-18	CTCAAGGATGTATTGTGGTTATGC	CTA CGA GCC GGA TAA CGC G	914	(Reid & Samarasinghe, 2018)
CTX-M	CGTCATCTATGTTCGCCGAC	GCATCTCAGTCGGATCGAGC	551	(Reid & Samarasinghe, 2018)
OXA-48	CGGAATGCCTGCGGTAGCAAAG	CAGCCCTAAACCATCCGATG	700	(Reid & Samarasinghe, 2018)
CTX-M-1	AAA AAT CAC TGC GCC AGT TC	AGC TTA TTC ATC GCC ACG TT	415	(Al-Mayahie, 2013)
CTX-M-2	AGC TTA TTC ATC GCC ACG TT	CCA GCG TCA GAT TTT TCA GG	552	(Al-Mayahie, 2013)
CTX-M-8	TCG CGT TAA GCG GAT GAT GC	CAA AGA GAG TGC AAC GGA TG	666	(Al-Mayahie, 2013)
CTX-M-9	CAA AGA GAG TGC AAC GGA TG	ATT GGA AAG CGT TCA TCA CC	205	(Al-Mayahie, 2013)
CTX-M-25	GCA CGA TGA CAT TCG GG	AAC CCA CGA TGT GGG TAG C	327	(Al-Mayahie, 2013)

2.1.3 Multiplex PCR-based Replicon Typing

Each isolate was sub-cultured in Luria Betani broth overnight at 37°C (recipe can be found in table 6-2 of Appendix). Plasmids were extracted using the Illustra PlasmidPrep Mini Spin Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). More detail of the plasmid extraction method can be found in 6.3.2 of Appendix. Detection of plasmids was by means of adapting the replicon typing assay designed by (Carattoli, 2005). PCR amplifications

were performed in a volume of 25ul containing 5 µl of 5X Flexi Buffer, 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.125 µl of 5U/µl GoTaq G2 Flexi DNA polymerase, 400 nM of each primer and 2.5 µl of plasmid DNA. Cycling parameters were as follows: initial denaturation at 94°C for 5 min 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 min. Primers can be found below in table 2-2. More details of primer design can be found in 6.4.1.

Table 2-2 Primers used in multiplex replicon-typing. Primers for IncFII, IncI1, IncFIA, IncN and IncL/M were designed using the OligoAnalyzer 3.1 (Integrated DNA Technologies Inc, Illinois, USA) Primers were then checked for specificity by the BLAST software (National Center for Biotechnology Information, Bethesda, USA). All PCR products were ran on a 2% agarose gel (UltraPure™ Agarose, ThermoFisher Scientific, Paisley, UK) for 90 mins at 60 Volts. A GeneRuler 50 bp DNA Ladder, ready-to-use (ThermoFisher Scientific, Paisley, UK) was used for comparison.

Primer	Forward Primer	Reverse Primer	Product Size	Reference
IncFII	TGTTTCCACTATGACCCGCT	TGATACATCGAGGGCAGCAA	246	This study
IncI1	CGGGAATGTCTGTTGTTGCA	ATCGGCTTCATCCTGGTGAA	155	This study
IncFIA	CCA TGC TGG TTC TAG AGAAGGTG	GTATATCCTTACTGGCTTCCGCAG	462	(Carattoli, 2005)
IncN	GTCTAACGAGCTTACCGAAG	GTTTCAACTCTGCCAAGTTC	559	(Carattoli, 2005)
IncL/M	GGATGAAAACTATCAGCATCTGAAG	CTGCAGGGGCGATTCTTTAGG	785	(Carattoli, 2005)

2.1.4 Statistical Analysis

The Chi Square test was used for statistical comparison and the Spearman Rank Correlation test was used to analyse the relationship between plasmids and ESBLs. P values < 0.05 were regarded as significant.

2.2 Results

236 *Enterobacteriaceae* were tested by multiplex PCR for the ESBL genes CTX-M, TEM, SHV and OXA; and the CTX-M sub-groups CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Multiplex PCR-based replicon typing tested for IncFII, IncFIA, IncL/M, IncN and IncI1. Results were ran on agarose gels and can be found in figure 5-VI and 5VII. The PCR assay assigned the four controls to their correct phylogenetic groups.

ESBL genes were identified as follows: CTX-M = 169 (71.6%) OXA = 18 (7.6%), TEM = 9 (3.8%) and SHV = 9 (3.8%). Multiple resistance genes were detected in 16% of isolates, specifically CTX-M + OXA = 16 (6.8%), CTX-M + TEM = 12 (5.1%), CTX-M + OXA + SHV = 5 (2.1%) and CTX-M + SHV = 5 (2.1%). This can be seen in figure 2-I. A total of 31 isolates did not contain any of the four genes tested.

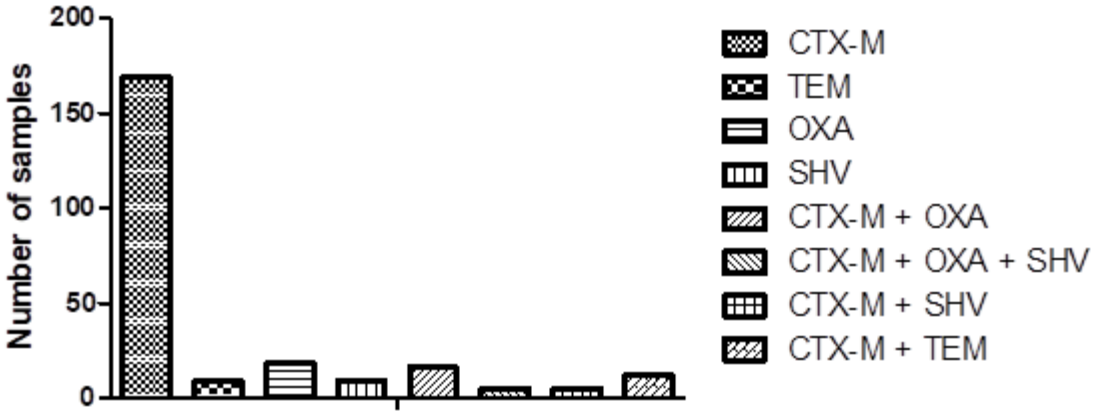

Figure 2-I Frequency of ESBL producing UTI isolates in the Leicestershire area. 236 isolates were tested for the presence of CTX-M, TEM, OXA and SHV genes by multiplex PCR analysis. All genes were detected; however, CTX-M was the most frequent.

CTX-M genes were identified as follows: CTX-M-1 = 148 (84.1%), CTX-M-9 = 22 (12.5%), CTX-M-25 = 3 (1.7%), CTX-M-8 = 2 (1.1%) and CTX-M-2 = 1 (0.6%). This can be seen in figure 2-II. All isolates identified as containing CTX-M in the previous assay, contained at least one of the CTX-M sub-groups tested. One isolate contained both CTX-M-1 and CTX-M-8.

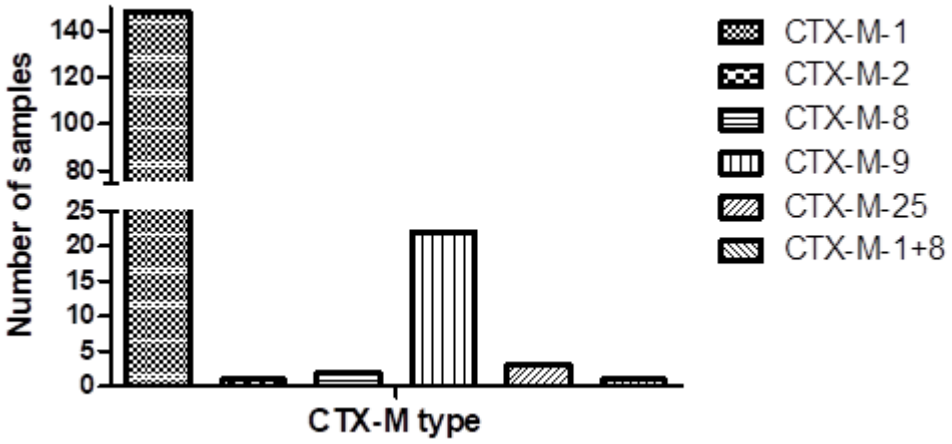

Figure 2-II Frequency of CTX-M sub-groups in UTI isolates in the Leicestershire area. 169 isolates previously identified as containing the CTX-M gene, were tested for the presence of CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 genes by multiplex PCR analysis. All genes were detected, however CTX-M-1 was the most frequent. A combination of CTX-M-1 and CTX-M-8 was found.

Plasmids identified were as follows: IncL/M was detected in 69 (29.2%) of isolates, IncFII was detected in 65 (27.5%) of isolates, IncN was detected in 34 (14.4%) of isolates, IncFIA was detected in 55 (23.3%) of isolates and IncI1 was detected in 12 (5.1%) of isolates. This can be found in figure 2-III. 235 of the 236 isolates contained at least one plasmid.

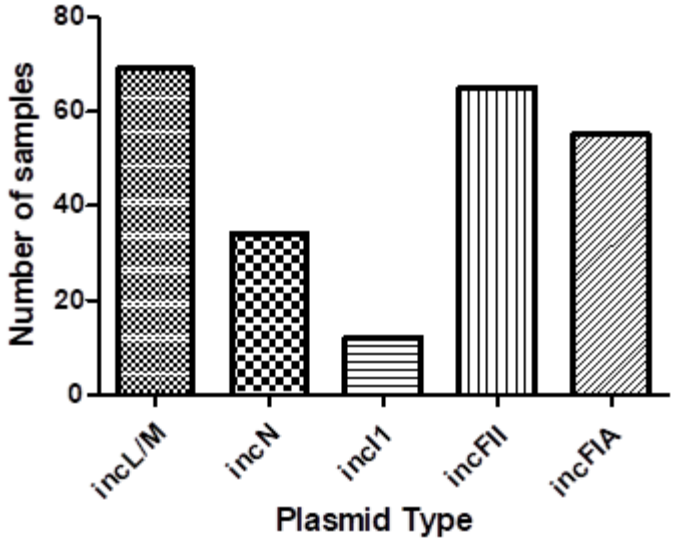

Figure 2-III Frequency of replicon types in ESBL producing UTI isolates in the Leicestershire area. 236 isolates were tested for the presence of IncL/M, IncN, IncI1, IncFII and IncFIA genes by multiplex replicon-typing PCR analysis. All genes were detected; however, IncL/M was the most frequent, followed closely by IncFII.

Multiple plasmids were found in 37% of isolates. IncL/M, IncFII + IncFIA was detected in 23 (9.8%) isolates. IncN + IncFII was detected in 13 (5.5%) isolates. IncL/M + IncN was detected in 12 (5.1%) isolates. IncFII + IncFIA was detected in 12 (5.1%) isolates. IncL/M + IncFIA was detected in 11 (4.7%) isolates. IncL/M, IncI1 + IncFIA was detected in 3 (1.3%) isolates IncL/M, IncN and IncFII was detected in 3 (1.3%) isolates. IncI1 and IncFII was detected in 3 (1.3%) isolates. IncN, IncI1, IncFII + IncFIA was detected in 2 (0.9%) isolates. IncL/M + IncFII was detected in 2 (0.9%) isolates. IncL/M + IncI1 was detected in 1 (0.4%) isolate. IncN, IncI1 + IncFII was detected in 1 (0.4%) isolate. IncI1 + IncFIA was detected in 1 (0.4%) isolate. This can be found in figure 2-IV.

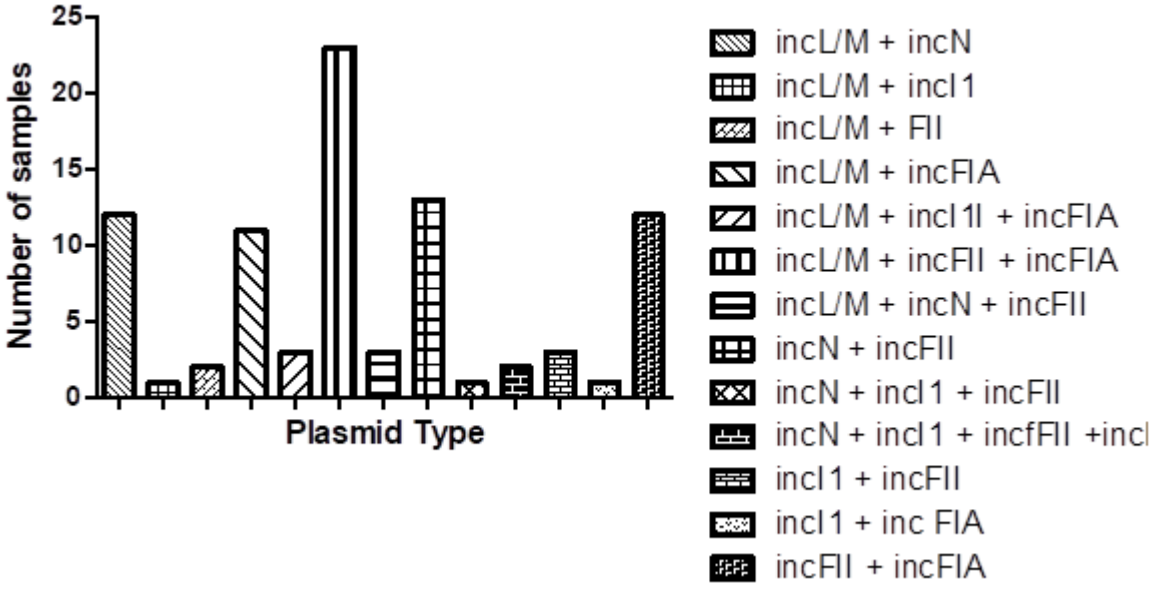

Figure 2-IV Frequency of dual, triple and quadruple plasmids in ESBL producing UTI isolates in the Leicestershire area. 236 isolates were tested for the presence of IncL/M, IncN, IncI1, IncFII and IncFIA genes by multiplex PCR analysis. All genes were detected; however, CTX-M was the most frequent.

A spearman rank was carried out to access the correlation between ESBL genes and plasmid type. A significant positive correlation between the CTX-M gene and IncL/M, N, FII and FIA plasmids was found. Also found was a significant positive correlation between the IncFII plasmid and the CTX-M-8 gene. No significant correlation was found for the TEM, SHV and OXA genes and any of the plasmids. None of the other CTX-M sub-types had a significant correlation with any of the plasmids.

2.3 Discussion

The major cause of antibiotic resistance in *Enterobacteriaceae* is ESBL-production. ESBL-producing UTIs are a major problem in the UK, due to their increasing prevalence and limited treatment options (Harwalkar, 2013). Given how common ESBL-producing infections are worldwide, prevalence studies are important to ascertain where resistance is a major problem.

In this paper, the prevalence of ESBL genes was investigated in UTIs in the Leicestershire area of the UK. In short, the CTX-M gene was found in 71.6% of samples, in comparison genes TEM, SHV and OXA were detected in ≤16% of samples. The most prevalent CTX-M was CTX-M-1 at 84.1%. The most common plasmid found was IncL/M at 29.2%, with IncFII following closely at 27.5%. Multiple plasmids were found in 37% of isolates, with the IncL/M, IncFII and IncFIA combination the most common. A significant correlation between CTX-M and all plasmids except IncI1 was found, however no other gene family significantly correlated with any of the plasmids.

Here, the relevance of findings of this study in relation to other studies will be evaluated within the context of the One Health Approach. The One Health Approach describes a holistic and multisectoral approach to antimicrobial resistance, as resistant organisms exist in humans, animals, food and the environment (Goutard, et al., 2017). The main aim of the One Health Approach is to ensure that antimicrobial agents continue to be effective by developing policies that promote the responsible use of antimicrobial agents. The development of policy decisions depends on economic and scientific evidence. Prevalence studies are an important part of this (Aidara-Kane, et al., 2016).

This is the first study to investigate the prevalence of ESBL-producing *Enterobacteriaceae* and their relationship to plasmids, in the Leicestershire area of the UK.

In this study, it has been found that CTX-M was the most common ESBL and the most common sub-group was CTX-M-1. This is comparable with other European countries, and is consistent with the worldwide dissemination of the CTX-M ESBL (Shaikh, et al., 2015; Canton, 2012; Celik, 2010; McNulty, et al., 2018). CTX-M-1 has been found to be the most prevalent gene in humans, animals and food in the UK, frequently found in poultry and cattle isolates (Day, et al., 2016). It has been established that the prevalence of ESBLs differs significantly between countries. Within Europe, a high prevalence has been seen in Southern Europe, while a generally lower prevalence is seen in Northern Europe. Outside of Europe, Turkey and India have reported high levels of ESBLs (Bevan, et al., 2017). The high number of CTX-M isolates found in this area may be due to the multicultural nature of the Leicestershire community. A study in a hospital in Birmingham, UK also found a high level of ESBL and suggested that this could be due to a high immigrant

population (Wragg, et al., 2017). One possible cause of elevated levels of ESBLs in high immigrant populations could be faecal colonisation. Transmission of faeces to the urinary tract is frequently a cause of UTI. As *E.coli* is a common inhabitant of the gastrointestinal tract, it is possible that CTX-M-producing *E.coli* could be a commensal bacteria that can become pathogenic upon colonization of the urinary tract (Bien, et al., 2012). To further this, a direct link between conjugative resistance plasmids in the microflora and increased treatment failure has been found (Kamruzzaman, et al., 2017). In another study in Birmingham, they found a direct link between place of birth and CTX-M-1 colonisation in stool samples. Those that were originally born in Afghanistan saw the highest colonisation at 60%, followed by those born on the Indian subcontinent (India, Pakistan, Bangladesh or Sri Lanka) with a 25% colonisation. Those that travel to certain areas were also associated with CTX-M colonisation. Travellers to South Asia (India, Pakistan, Bangladesh, Sri Lanka or Nepal) in the last year had a 38.5% colonisation, suggesting a geographical, rather than ethnic susceptibility (McNulty, et al., 2018).

In India, a prevalence rate of 53% for CTX-M was seen in a study detecting ESBLs in UTIs. Antibiotic usage in India is far higher than in the UK, due to issues with lack of restriction in antibiotic prescribing which could explain why they saw a higher incidence of CTX-M (Rath, 2011).

Prevalence rates of CTX-M have been seen as high as 94.4% in Iran, 73% in Mexico, 98.7% in Japan and 100% in Ghana (McNulty, et al., 2018). These studies show that the problem of ESBL producing infections is not limited to the UK. As bacteria do not

recognise geographical borders, so too does the approach to tackling AMR need to be borderless.

This study found that 16% of isolates contained more than one ESBL type. Interestingly, in all of the multi-ESBL isolates CTX-M was always present, this suggests that CTX-M may play a key role in the production of MDR bacteria. Of these, CTX-M and OXA were the most common multi-ESBL, detected in 16 isolates. 2% of isolates contained three genes, specifically, CTX-M, OXA and TEM. It has been reported that CTX-M-15 combined with an OXA-30 gene can survive β-lactamase inhibitors, since OXA-30 is poorly inhibited and also confers resistance to cefepime (Agyekum, et al., 2016). Although this study did not specifically type the ESBL genes in these isolates, it is possible that it could be this combination present. The coexistence of CTX-M and other antibiotic resistance genes could be one of the reasons why CTX-M has been so successful. This has been found elsewhere, with incidences of combinations of ESBLs as high as 95.4%, with regards to the CTX-M and SHV combination (Barrios, et al., 2017; Moghanni, et al., 2018). The combination of three genes found in this study has also been found elsewhere (Moghanni, et al., 2018). Furthermore, other Multi ESBL combinations have also been found in animals and the environment, suggesting that multiple ESBL combinations are not limited to humans. This data combined shows that multi ESBLs are widespread and could pose a significant threat (Qiao, et al., 2017; Zarfel, et al., 2013).

A similar incidence of ESBLs have been reported in livestock and companion animals globally (Qiao, et al., 2017; Tippelskirch, et al., 2018; Schill, et al., 2017; Melo, et al., 2018; Zogg, et al., 2018). In a study looking at canine UPEC in Switzerland, CTX-M-1

was found in 28.6% of ESBL-producers suggesting that dogs, a common companion animal worldwide, may be a reservoir for CTX-M-1 spread into humans (Zogg, et al., 2018). In addition to this, blaCTX-M-14 IncK pCT plasmids, previously only found in cattle in the UK, have been detected in turkey and human isolates. This suggests a possible dissemination of CTX-M genes amongst other animals and humans. (Stokes, et al., 2012). Both livestock and companion animals could be considered a major vector of ESBL-producing bacteria (Day, et al., 2016). With raw meat a speciality in some countries and the rise of raw food diets in both humans and companion animals, a high level of ESBLs in livestock is a cause for concern. As it has been suggested that the majority of ESBL infections are community-acquired, this could suggest a relationship between companion animals, livestock and humans.

This study focused on the prevalence of IncL/M, IncFII, IncI1, IncFIA and IncN. These conjugative plasmids have previously been associated with the dissemination of antibiotic resistance determinants such as ESBLs, in *Enterobacteriaceae* and other bacteria.

Whilst IncL/M was the most common plasmid found in the isolates tested, when IncFII and IncFIA (both members of the IncF group) are combined, the frequency is far higher at 50.8%. The IncF group is one of the most common plasmids found in UTI isolates and is frequently associated with CTX-M (Agyekum, et al., 2016). Studies show that IncL/M is also highly common, and both have been termed an “epidemic” plasmid (Carattoli, 2011). Both of these plasmids can persist for months without the need for selective pressure by

antibiotic usage (Kamruzzaman, et al., 2017). This suggests that simply reducing antibiotics by antibiotic stewardship alone will not reduce the prevalence of these plasmids.

The distribution of plasmids harbouring ESBL genes in this population differs from the distribution described in other studies. Studies have agreed that CTX-M is associated with IncN, IncFII and IncL/M, however reports have shown that CTX-M is also associated with IncI1. IncI1 plasmids harbouring CTX-M have been described in the UK, US, Belgium, Netherlands, France and Australia in isolates from horse, cattle and human isolates (Zurfluh, et al., 2015). Studies have also found that other ESBL types are associated with particular plasmids. It has been found that SHV can be associated with IncFII, IncL/M and IncI1. Likewise, TEM can be associated with the same plasmids, and additionally IncI1 (Carattoli, 2009). A reason for the differing results could be due to different antibiotic and environmental selection pressures in this population, leading to selection of CTX-M harbouring IncL/M, IncF, and IncN plasmids, but not IncI1 plasmids.

In this study, no significant correlation between CTX-M-1 (the most common CTX-M subtype) and any other plasmid was found. There are conflicting reports within the literature with regards to the most significant association between CTX-M-1 and plasmid types. Some reports suggest a correlation between CTX-M-1 and IncFII, whilst others, mainly in Europe, suggest a correlation between CTX-M-1 and IncI1. Other reports suggest no overall correlation at all (Madec, et al., 2015; Day, et al., 2016). It appears that the association between CTX-M-1 and plasmids differs between geographical locations and sample type. These differences in reports could be attributed to socioeconomic factors,

animals and number of samples. It could be suggested that CTX-M-1 is equally adapted to most plasmid types, therefore a significant correlation between just one plasmid was not seen. It could also be suggested that the high level of CTX-M-1 in this population is also due to another mechanism other than conjugative plasmids, such as chromosomally mediated resistance.

A significant correlation between CTX-M-8 and IncFII was found. Whilst CTX-M-8 is not the highest prevailing gene found in this study, it is a significant finding, as IncFII was very common. A link has been found between CTX-M-8 harbouring IncI1 plasmids and companion animals. This could further strengthen the argument that CTX-M genes could be passed, via plasmids, from companion animals to humans (Melo, et al., 2018).

Though studies suggest that IncN does not contribute to the prevalence of CTX-M in humans to the same extent as incI1, this was not the case in this study, as IncN was seen to be more prevalent than IncI1 and a significant correlation was found (Zurfluh, et al., 2014). This theory has also been contradicted by another study, that found that IncN was in fact associated with humans and 95.5% of isolates containing IncN harboured CTX-M-1 (Day, et al., 2016). In a Danish study, IncN plasmids carrying CTX-M-1 were seen in both pigs and farm workers, and it was demonstrated that these plasmids were transmitted within the farm, showing animal to human transmission of these plasmids harbouring CTX-M (Carattoli, 2011).

Multi-replicon plasmids were found in 37% of isolates. Multiple plasmids frequently occur in the same bacterial cell, however, cross-interference between plasmid replicons

guarantees that the most closely related plasmids are incompatible and cannot stably persist together (Kamruzzaman, et al., 2017). When multi-replicon plasmids occur, generally one replicon is usually highly conserved, and the other is free to diverge. This gives the bacterial host the benefit of being able to change antibiotic resistance determinants and virulence factors, depending on antibiotic pressure and environment (Zurfluh, et al., 2015). Multi-replicons have been found in other UTI isolates, and multi-replicons containing multiple IncF types appear to be common (Agyekum, et al., 2016). Multi replicons are also common in the environment. A study found that the diversity of plasmids found in the environment was higher than in UTIs in the same area. CTX-M-1's in the environment have been shown to be associated with IncI1 and IncN (Zarfel, et al., 2013). A report has suggested a direct link between wastewater treatment plants and ESBL-producing UTIs. Sewage sludge used for agriculture may be one of the ways that ESBL-producing isolates enter the food chain (Zarfel, et al., 2013). It has been established that CTX-M-1 producing *E.coli* in manure spread on fields can survive in the soil for at least a year. This demonstrates the capability of antibiotic resistant bacteria to survive under environmental conditions in the absence of antibiotic selection pressure (Zurfluh, et al., 2014).

A limitation of this study is that isolates were not categorised into source of isolate (community/hospital acquired). However, other studies suggest that the CTX-M family and ESBLs in general are associated with community-acquired infection (Hayakawa, et al., 2017; Qiao, et al., 2017). In this study, only ESBL-positive isolates were collected. From this, the level of ESBL resistance as a whole cannot be determined. This study should be followed up regularly, to study the trends of antibiotic resistance in this area. More

surveillance studies on animal and environmental isolates are needed, to fully understand the relationship between these and human infections.

In this study, similar results to what has been found in the rest of the UK and worldwide have been found. CTX-M-producing *Enterobacteriaceae* is a major threat to patient care and healthcare costs. Genotypic prevalence studies are an important contribution to the understanding and prediction of antibiotic resistance evolution and antibiotic stewardship. Knowledge of plasmid types circulating in bacterial populations is vital to advancing a new prospective to control these plasmids, such as replicon-targeting compounds. A strategy to prevent the further dissemination of these plasmids needs to be implemented.
Combined, these findings highlight the importance of restricting antibiotics sales to only those with medical prescriptions and appropriate use, as many low and middle-income countries have not yet enforced policies that prevent widespread self-medication with antibiotics.

This study reinforces the One Health approach and underpins the importance of antibiotic stewardship and infection prevention schemes in humans, animals, food production and the environment in order to limit the spread of ESBL-producing *Enterobacteriaceae*, not only in the UK, but worldwide.

2.4 Acknowledgements

The authors would like to acknowledge Christopher Holmes of University Hospitals of Leicester NHS Trust, for providing bacterial isolates used in this study, Adrian Slater of De Montfort University and Avninder Bhambra of De Montfort University for providing guidance in the development of this study.

2.5 Conflict of Interest

None declared.

2.6 Funding

This work was supported by the De Montfort University fees only scholarship awarded to Ruth Reid.

Chapter 3:

The Development and Evaluation of a Multiplex Real-Time PCR Assay for the Detection of ESBL Genes in Urinary Tract Infections

Statement of Contributions of Joint Authorship

Reid, R: (Candidate)

Writing and compilation of manuscript, carrying out of experiments, data analysis, preparation of tables and figures

Samarasinghe, S: (Supervisor)

Assisted with manuscript compilation and editing.

The journal paper referred to below has been adapted for use within a thesis.

The development and evaluation of a multiplex real-time PCR assay for the detection of ESBL genes in urinary tract infections

Running title: Development of assay to detect ESBLs in UTI.

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Abstract

Background

Overuse of beta-lactam antibiotics has led to selection for extended-spectrum β-lactamase (ESBL) producing *Enterobacteriaceae,* a major cause of antibiotic resistant urinary tract infections (UTIs). Standard detection methods are time-consuming, with disputed accuracy. This study describes a novel real-time PCR method to detect CTX-M, SHV, OXA and TEM.

Methods

A total of 181 *Enterobacteriaceae* isolates from UTIs were collected from the Leicester Royal Infirmary, UK. A multiplex Plexor®-based real-time PCR assay detected ESBLs using their specific amplicon melting temperature, during each cycle, removing the need for a melt-curve analysis. Validation was achieved by end-point PCR and disk diffusion.

Results

The method was able to produce rapid and accurate results, achieving a sensitivity and specificity of 94.9% and 72% respectively, and the assay can differentiate between the different ESBL genes, with ease.

Conclusions

With further investigation, a Plexor®-based assay could form the basis of a high-throughput kit that health services could use to detect ESBLs or other antibiotic resistance genes.

Keywords: Extended-spectrum beta-lactamases, urinary tract infections, Real-Time PCR, rapid diagnostics, antibiotic resistance.

3 Introduction

Antibiotic resistance is a natural process, whereby bacteria exchange genes via horizontal transfer, however the overuse of antibiotics has caused a permanent selective force for resistance mechanisms (Canton, et al., 2012). In the O'Neill report in 2016, it was estimated that every year at least 700,000 people die as a result of antibiotic resistant infections (O'Neill, 2016).

Extended-spectrum β-lactamases (ESBLs) are one of the most common types of antibiotic resistance found in *Enterobacteriaceae* and are a major public health concern, both in hospital settings and in the community (Fournier, et al., 2013; Esteve-Palau, et al., 2015). First discovered in the 1980's, ESBLs have since increased massively, becoming a major public concern (Canton, et al., 2012).

ESBLs are able to hydrolyze extended – spectrum cephalosporins (cefotaxime, ceftriaxone, ceftazidime and cefepime) and monobactams (aztreonam) (Esteve-Palau, et al., 2015) (Roschanski, et al., 2014). The most common families of ESBLs found are CTX-M, SHV, OXA and TEM (Canton, et al., 2012).

Urinary tract infections (UTIs) are the most common infection found in humans (Yang, et al., 2010). The majority of both community-acquired and nosocomial UTIs are caused by uropathogenic *Escherichia coli* (UPEC) strains (Yang, et al., 2010). Serious infection by ESBL-producing UPEC can be linked to increased length of hospital stay, admittance to an intensive care unit, catheterization, increased mortality, morbidity and healthcare cost (Birkett, et al., 2007) (Esteve-Palau, et al., 2015) (Shin & Ko, 2015) (Wintermans, et al.,

2013). Therefore, early detection would benefit both the patient and the health care provider. Screening for ESBL-producing uropathogens is commonly achieved by means of selective agar plates, such as the commercially available ESBL agar. Isolates are also tested for antibiotic sensitivity by disk diffusion or broth microdilution (Roschanski, et al., 2014). These procedures can take 4-6 days to obtain an antibiotic resistance profile after the culture, isolation and characterization of pathogens from a patient sample.

In the O'Neill report, it was suggested that rapid diagnosis could change the way antibiotics are used. Reducing the time spent on broad-spectrum antibiotics by prescribing a more appropriate treatment, increases the favorability of the outcome of the patient and shortens the stay in hospital. Rapid detection of ESBL isolates also has other benefits, including infection control and prevention of outbreaks through the use of hospital hygiene precaution measures (Birkett, et al., 2007) (Lupo, et al., 2013) (Swayne, et al., 2013). Therefore, rapid and sensitive detection of infections thought to be of the ESBL type is of the utmost importance for patient outcome, the economy and outbreak control.

In this study, a multiplex real-time assay was developed and validated to detect the most prevalent ESBL-producing genes in bacterial isolates found in UTIs, based on the amplicon melting temperature.

3.1 Methods

3.1.1 Bacterial Isolates and DNA Extraction

Bacterial isolates (n=181) of *Enterobacteriaceae* isolated from urinary tract infections were obtained from the Leicester Royal Infirmary hospital (Leicester, England). More description of isolate collection and storage can be found in 6.1 of Appendix. The Leicester Royal Infirmary was chosen as a collection site as a large number of samples are received from all over the Midlands, giving a wide population base. A total of 25 of the samples were non β-lactamase producing strains of *Enterobacteriaceae,* and 156 were β-lactamase producing strains. The presence or absence of ESBL production was previously identified by disk diffusion. Waste material (agar plates) was collected and therefore informed consent from patients was not required. Both catheter and non-catheter patients, symptomatic and non-symptomatic patients were included. Four control isolates were incorporated: NCTC 13353 (CTX-M-15), NCTC 13351 (TEM-3), NCTC 13368 (SHV-18) and NCTC 13442 (OXA-48), obtained from Public Health England (see table 6-1 in Appendix). Each isolate was sub-cultured on Müller-Hinton agar plates for 24 hr. at 37°C (recipe can be found in table 6-2 of Appendix). 2-3 colonies were picked and suspended in 50 µl sterile distilled water and then heated in a AccuBlock dry bath (Labnet International Inc, Windsor, Berkshire) for 10 min at 94°C.

3.1.2 Primers

Table 3-1 Primers used in this study

Oligo	Forward	Reverse	Product Size	Reference
OXA48	AGCAAAGGAATGGCAAGAAA	CGCCCTGTGATTTATGTTCA	65	Singh, et al., 2016)
SHV	GGTCAGCGAAAAACAYCTTG	GCCTCATTCAGTTCCGTTTC	195	(Singh, et al., 2016)
TEM	GATACGGGAGGGCTTACCAT	GGATGGAGGCGGATAAAGTT	146	(Singh, et al., 2016)
CTX-M	AATCTGACGCTGGGTAAAG	CCGCTGCCGGTTTTATC	304	(Singh, et al., 2016)

The allelic variants that these primers can detect have been previously shown and can detect 149 variants of ESBLs (Singh, et al., 2016). These variants include CTX-M-1, CTX-M-15 and CTX-M-9 that have been shown to be highly prevalent in UTIs in the UK (Canton, et al., 2012). These can be found in table 3-1. More details of primer design can be found in 6.4.2.

3.1.3 Qualitative Real-Time PCR

The Plexor® qPCR (Promega, Southampton, UK) mastermix kit was used for its suitability in multiplex real-time PCR and its previous use in diagnostic assays. The Plexor® qPCR system mechanism has been previously described (Promega Corporation, 2009). In typical real-time PCR assays, the accumulation of a product is accompanied by an increase in

fluorescence. However, the Plexor® qPCR system differs in that the accumulation of a product leads to a decrease in fluorescence. Primers are easy to design, and less expensive than using probes. Primer design requires the addition of an iso-dC label adjacent to a fluorescent reporter on one strand. The Plexor® mastermix contains a dabcyl-iso-dGTP label as a quencher. This can be seen in figure 3-I. Plexor® primers are less expensive than probes and have a higher specificity than SYBR green chemistry (Promega Corporation, 2009).

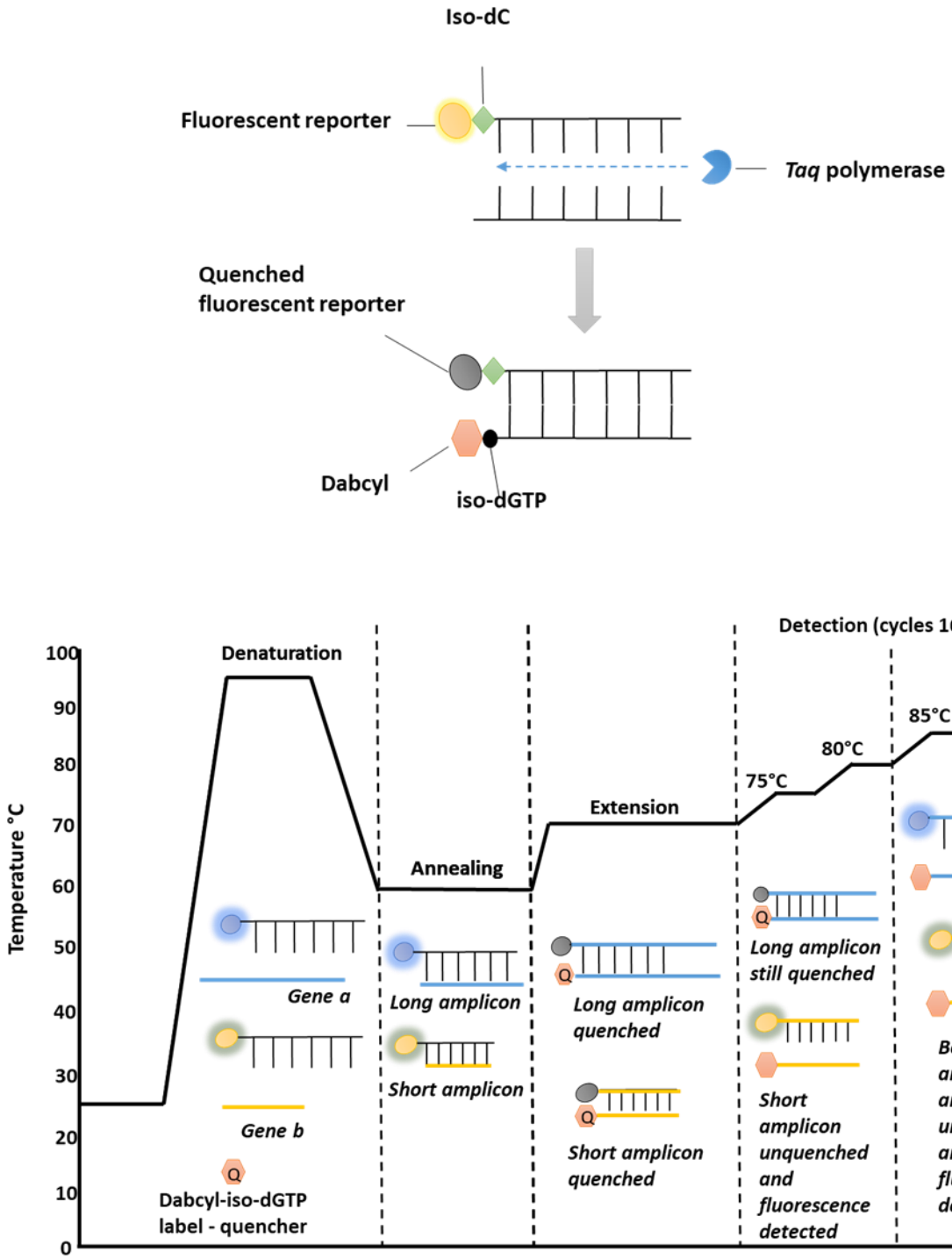

Figure 3-I The Plexor® qPCR system and the method of detection used in this study. The Plexor® qPCR system requires a florescent reporter, adjacent to an iso-dC label, to be attached to one of the primers in each pair. Florescence is then quenched by incorporation of a Dabcyl-iso-dGTP label contained in the mastermix, when bound to another DNA strand during the extension phase of the PCR cycle. Therefore, as product increases, florescence decreases. When the amplicon melts, the strands dissociate and the quencher is released. Shorter amplicons melt at lower temperatures to longer ones, allowing differentiation between the products.

Qualitative real-time PCR conditions such as annealing temperature and primer concentration were optimised in simplex, and once satisfied, in multiplex. The amplicons of the control isolates were initially tested using high resolution melting curve analysis, first in simplex and then multiplex to determine the melting point of the amplicon. An increase in temperature increments between 84°C and 86°C was implemented to aid resolution between the amplicon melting points of CTX-M and TEM. See 6.6 of Appendix for more details on optimization of the assay. An agarose gel image of the results of the optimization stage can be found in figure 6-X.

Assays were performed in 12.5 µl reactions consisting of 6.25 µl of 2X Plexor® Master Mix, 0.5 µl of each primer mix, (forward and reverse primer combined) at a final concentration of 200 nM and 2.5 µl template DNA. The five previously mentioned controls were included in the assay, along with a no template control (NTC).

The qualitative real-time PCR amplification protocol was performed in a PikoReal® 96 well Real Time-PCR platform (ThermoFisher, Loughborough, UK). The qualitative real-time PCR amplification program comprised of the following steps: initial denaturation for 10 min at 95 °C, and then 40 cycles of 95 °C for 10 s, annealing at 70°C for 40 s (gradual temperature decrements of 0.2°C per cycle, final annealing temperature 60°C), and fluorescence readings were taken from cycle 16 onwards at 72°C, 76°C , 78°C, 84°C, 84.2°C, 84.4°C, 84.6°C, 84.8°C, 85°C, 85.2°C, 85.4°C, 85.6°C, 85.8°C, 86°C, 88°C and 90°C for 10 s each.

3.1.4 Limit of Detection

For each of the ESBL types, the limit of detection was determined in triplicate with five 1:10 dilutions using DNA extracted by the method mentioned previously. DNA concentration was measured using a Qubit 3 fluorometer for the highest DNA concentration, and then concentrations were estimated by calculation from then on.

3.1.5 Validation

ESBL production was confirmed phenotypically via the MAST ID double disc synergy method outlined by the European Committee on Antimicrobial Susceptibility (EUCAST) Discs contained Cefotaxime, Ceftazidime and Cefpodoxime with their clavulanic acid counterpart. Plates were incubated for 18 hours at 37°C. A zone of inhibition difference of 5mm or more indicated a positive result for ESBL production.

A multiplex end-point PCR assay was used as a reference for validation. PCR amplification reactions were performed in a volume of 25µl 5 µl of 5X Flexi Buffer, 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.125 µl of 5U/µl GoTaq G2 Flexi DNA polymerase ,400 nM concentrations of each primer, and 2.5µl of DNA template. Cycling parameters for the multiplex assay were as follows: an initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, annealing at 65°C for 1 min (gradual temperature decrements of 0.5°C per cycle, final annealing temperature 48°C), and 72°C for 1 min; and with a final extension at 72°C for 10 min. Primers can be found in table 3-2.

Table 3-2. Primers used in the validation end-point PCR assay

Primer	Forward Primer	Reverse Primer	Product Size	References
TEM-1	CGGATGGCATGACAGTAAGAG	AGGACCACTTCTGCGCTCG	782	Own study
SHV-18	CTCAAGGATGTATTGTGGTTATGC	CTACGAGCCGGATAACGCG	914	Own study
CTX-M	CGTCATCTATGTTCGCCGAC	GCATCTCAGTCGGATCGAGC	551	Own study
OXA-48	CGGAATGCCTGCGGTAGCAAAG	CAGCCCTAAACCATCCGATG	700	Own study

3.1.6 Statistical Analysis

The sensitivity, specificity, positive likelihood ratio and negative likelihood ratio was calculated using SPSS version 22. 95% confidence intervals for the sensitivity and specificity were calculated using exact Clopper-Pearson confidence intervals (Newcombe, 1998). Whereas the Log method was used to calculate 95% confidence intervals for the likelihood ratios (Altman, et al., 2000).

3.2 Results

A Plexor®-based multiplex real-time PCR assay was designed to simultaneously detect the ESBL genes CTX-M, TEM, OXA and SHV, using amplicon melting temperatures, without the use of melting curve analysis. Figure 3-II shows the results of the development of the real-time PCR method.

A total of 179 samples were collected from the Leicester Royal Infirmary and tested by qualitative real-time PCR. The result of the multiplex assay was mostly in agreement with the results of the multiplex end-point PCR. The assay correctly identified 91.7% isolates tested. A summary of the statistical results can be found in Table 3. It is typical for a new

diagnostic to be compared to a gold standard in order to determine if the new method diagnoses patients correctly. End-point PCR was used as the gold standard for comparison against the new diagnostic method. Seven very major errors/discrepancies and seven major errors/discrepancies were identified. Very major errors were defined as samples that showed as negative for ESBL genes by the qualitative real-time PCR assay, but positive by end-point PCR. These would be patients that may not be given treatment with the new method, which may have needed it. Major errors were defined as samples that showed as positive for ESBL genes by the qualitative real-time PCR assay, but negative by end-point PCR. These would be patients that would be given treatment with the new method, which may have not needed it.

Interestingly, the real-time PCR assay picked up additional TEM genes in two of the isolates, which the end-point PCR had failed to. The NTC was assigned correctly as predicted.

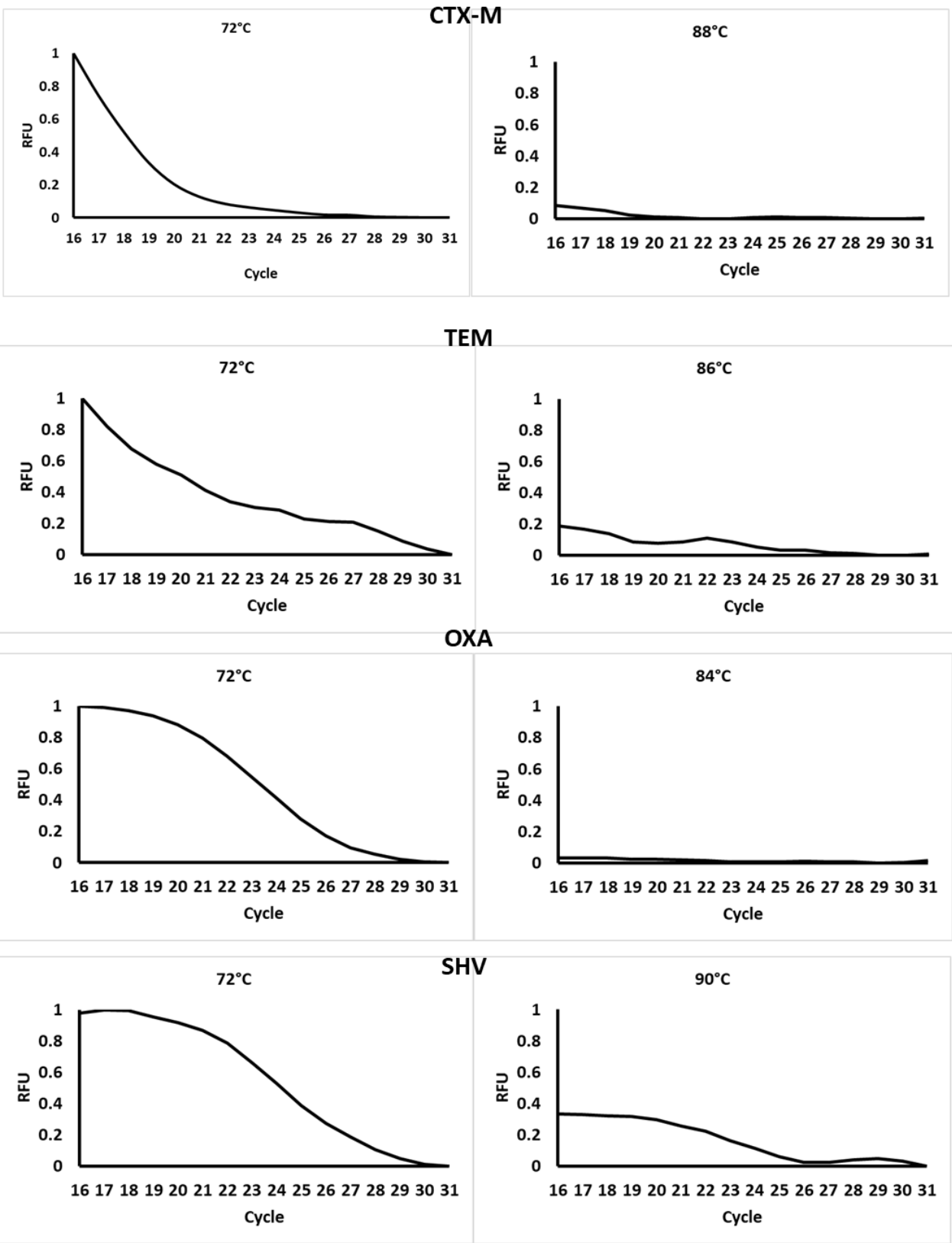

Figure 3-II Data showing the change in florescence at cycles 16-31 (when florescence was detected) at low temperatures and at the melting point of each amplicon Data was normalized to the highest relative florescent unit (RFU). At temperatures below the Tm, florescence deceases as product increases each cycle. This is due to the incorporation of the Dabcyl-iso-dGTP quencher contained within the mastermix. At higher temperatures, florescence is very low as the majority of florescent reporters have been quenched. This change in florescence occurs at different temperatures for each amplicon, therefore the different products can be distinguished based on temperature.

In comparison, the disk diffusion method correctly identified ESBL production in 87.36% of tested isolates. Whilst the sensitivity was slightly higher than the qualitative real-time PCR, the specificity was significantly lower (see Table 3). Eighteen very major errors and five major errors were identified. The results of the statistical analysis can be found in table 3-3.

Table 3-3. Summary of statistical analysis.

	Qualitative Real-Time PCR	Disk Diffusion
Accuracy	91.71% (86.70-95.29)	87.36% (81.64 – 91.82)
Sensitivity	94.87% (90.15-99.97)	96.82% (92.72 – 98.96)
Specificity	72% (50.61-87.93)	28.00% (12.07 – 49.39)
Positive likelihood ratio	3.39 (1.81-6.36)	1.34 (1.05 – 1.72)
Negative likelihood ratio	0.07 (0.03-0.15)	0.11 (0.04 – 0.33)

3.2.1 Limit of Detection

Measurement of DNA concentration and calculations are described in the methods. The limit of detection for each of the set of primers CTX-M, TEM, OXA and SHV was 0.0004125ng/µl, 0.0242ng/µl, 0.000404ng/µl, and 0.000362 respectively. As TEM was not detected below 0.0242ng/µl, it is recommended that at least this amount of DNA is included in the test.

3.3 Discussion

Although other methods have been described for the detection of ESBLs, this is the first study to accurately and rapidly describe a multiplex real-time PCR assay to detect ESBLs, based on amplicon melting temperatures, without the need for a high-resolution melting curve analysis. Whilst there are other chemistries applied in real-time PCR that do not

require high-resolution melt-curve analysis for detection, such as Taqman, these tend to be far more expensive and require a higher level of user experience (Promega Corporation, 2009). In this assay, the detection of products is based upon the melting temperature of amplicons, within each cycle. In the present study, 179 samples previously identified as ESBL-producers by disk diffusion methods, were tested using a Plexor®-based multiplex real-time PCR assay for the detection of 149 variants of the CTX-M, TEM, OXA and SHV genes in one amplification. The assay was able to correctly detect genes in 91.7% of the isolates in under 3 hours. In comparison, disk diffusion methods were able to correctly detect ESBL production in 87.4% of isolates. Whilst in this study, the disk diffusion method showed a slightly higher sensitivity than the qualitative real-time PCR method, this has not been seen in other studies (Yazdi, et al., 2012) (Tofteland, et al., 2006). It has also been reported that clinical failures may occur, even when an isolate is negative phenotypically (Gheldre, et al., 2003). This may have been seen in this study, as there were eighteen cases where ESBL genes were detected by end-point PCR, but the isolate was negative phenotypically. This further advocates the use of genotypic detection.

ESBL-producing *Enterobacteriaceae* are a global growing concern, especially when it comes to UTIs. Current susceptibility testing requires at least 24 hr.; therefore, it is common practice to treat with empirical antibiotic therapy, without full pathogen information. Treating in this way can lead to ineffective therapy, causing an increase in clinical symptoms, including the possibility of urosepsis and ascending infection. It is well known that empirical treatment with antibiotics increases the population of resistant pathogens (Van der Zee, et al., 2016). Therefore, it is crucial that reliable, accurate and

rapid detection methods of antibiotic resistance are available. In addition, prevalence studies are highly important for monitoring the spread and evolution of antibiotic resistance genes.

Not only can the assay detect ESBL production in isolates in under 3 hours, it can also differentiate between the major classes of ESBL, aiding in surveillance studies. In addition, this assay did not require any laborious primer modifications and the results are easy to interpret, with no further analysis required. The results from this assay could lead to treatment escalation much more quickly than current methods. However, due to the low number of negative samples tested in this study, it is difficult to determine if treatment de-escalation could be concluded from the results of this assay. Nevertheless, due to the increased expense of PCR-based methods compared to current susceptibility testing methods, it is likely that only those patients who are suspected of having an ESBL producing UTI, such as patients who suffer from recurrent UTIs or those that are at higher risk (pregnant women, children, immunocompromised) would be tested. Therefore, the patient population tested in this study, represents the samples that would most likely be tested in practice. Until the negative likelihood ratio of this test can be more reliably proven, it is suggested that this test be used as an add-on to current testing in any patients that do not fall into the above criteria.

In the future, more targets could be added to this multiplex assay, as long as they have a different amplicon melting temperature. This is achieved through primer design, as amplicon melting temperature is related to length and the ratio of CG/AT content. As technology improves, the melting temperature difference required between amplicons can

decrease, further increasing the number of targets possible in the assay. This study recommends that primers for other Carbapenamases and ampCs (other major antibiotic resistance genes) are added, to give this assay full breadth across the antibiotic resistance spectrum seen in UTIs.

A multiplex Plexor®-based real-time PCR assay could provide a rapid, more sensitive, easy to interpret detection method, thereby helping to prevent the inappropriate use of antibiotics. It was found that this method could aid antibiotic susceptibility testing, if ESBL production is suspected. With further investigation this assay could form the basis of a high throughput kit that health services could use to detect ESBLS or other antibiotic resistance genes.

3.4 Acknowledgements

The authors would like to acknowledge Christopher Holmes of University Hospitals of Leicester NHS Trust, Adrian Slater of De Montfort University and Avninder Bhambra of De Montfort University.

3.5 Funding

This work was funded by the De Montfort University Fees Only PhD Scholarship awarded to Ruth Reid.

3.6 Conflicts of Interest

None to declare.

Chapter 4

The Effect of Cranberry Extract Cysticlean® on the Expression of Virulence Genes in CTX-M Producing *Escherichia coli*

Statement of Contributions of Joint Authorship

Reid, R: (Candidate)

Writing and compilation of manuscript, carrying out of experiments, data analysis, preparation of tables and figures

Samarasinghe, S: (Supervisor)

Assisted with manuscript compilation and editing.

The journal paper referred to below has been adapted for use within a thesis.

The effect of cranberry extract Cysticlean® on the expression of virulence genes in CTX-M producing *Escherichia coli*

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Abstract

Background

Urinary tract infections (UTIs) are one of the most common infections found in humans, with uropathogenic *Escherichia coli* (UPEC) the most common cause. It is thought that the main reason why UPEC is so successful, is its expression of a wide range of virulence factors including adhesins, capsules, toxins and iron-acquisition systems. The American cranberry *Vaccinium macrocarpon* produces proanthocyanins (PACs) which are tannins or stable polyphenols, and are reported to confer protection against bacterial infection. Cysticlean® capsules contain 240 mg of PACs and have been shown to produce a significant inhibition of *E. coli* adherence, *in vitro* and *ex vivo*, to uroepithelial cells. In this study, the Cephalosporin resistant *E.coli* isolate NCTC 1553 was analysed by qRT-PCR for the expression of virulence factors, before and after treatment with the cranberry product Cysticlean®.

Methods

RNA lysate was extracted using the Cells-to-CT™ 1-Step TaqMan™ Kit (ThermoFisher Scientific, Loughborough, UK) and qRT-PCR was carried out to detect the toxins SAT, and USP, the iron acquisition system ChuA, the protectins SoxS, KPSM, TraT and RecA, the antibiotic resistance gene CTX-M (encoding beta-lactamase production) and the transporters, IdfB and HcaT.

Results

The results showed that Cysticlean® significantly reduces the expression of the ten genes tested responsible for virulence and antibiotic resistance. This will eventually weaken

bacterial coordination, decrease their survival abilities, and interfere with their virulence and antibiotic resistance mechanisms.

Conclusion

These results suggest that cranberry could be a suitable alternative to antibiotic prophylaxis for the prevention of recurrent UTI.

Keywords: urinary tract infections; cranberry, virulence, UPEC

4 Introduction

Urinary tract infections (UTIs) are one of the most common infections found in humans and uropathogenic *E.coli* (UPEC) accounts for approximately 80% of all UTIs (Bien, et al., 2012). Common symptoms include painful, frequent and urgent urination and can lead to irreversible kidney damage and even death (Flores-Mireles, 2015).

It is thought that the main reason why UPEC is so successful, is their expression of a wide range of virulence factors (Bien, et al., 2012). Virulence genes are commonly located on mobile genetic elements such as pathogenicity islands, transposons, bacteriophages or plasmids and are generally spread by DNA horizontal transfer (Johnson, 1991). By understanding the role of virulence factors and their expression enables research aiming to discover novel therapeutic mechanisms that can limit bacterial infection.

Virulence factors important to UPEC infection of the urinary tract include adhesins, capsules, toxins and iron-acquisition systems (Ranfaing, et al., 2018). These virulence factors contribute to the colonization and invasion of the urinary tract, leading to biofilm formation, tissue damage and ascension to the bladder and kidneys. Research suggests that adhesins, or fimbriae, are the most important virulence determinant in the infection of the urinary tract and have therefore been studied extensively (Bien, et al., 2012).

It has been demonstrated that cranberries can decrease the virulence of UPEC, however little work has been completed on virulence factors other than adhesins. There are many cranberry products on the market, particularly cranberry capsules. For this study, the

cranberry capsule Cysticlean® was chosen. Cysticlean® capsules contain 240 mg of PACs and *in vivo* and *in vitro* results have shown a significant inhibition of adherence of *E.coli* to uroepithelial cells. A great improvement of symptoms has been seen, including the reduction in painful, frequent and urgent urination (Rodriguez, et al., 2015).

This study focuses on the toxins SAT, and USP, the iron acquisition system ChuA, the protectins SoxS, KPSM, TraT and RecA, the antibiotic resistance gene CTX-M and the genes responsible for the normal function of the cell, IdfB and HcaT. In this study the CTX-M producing *E.coli* isolate NCTC 1553 was analysed by qRT-PCR for the expression of virulence factors, before and after treatment with the cranberry product Cysticlean®. The results reported herein show that cranberry treatment reduces the expression of multiple virulence factors. These data suggest that cranberry's mechanism may be multifaceted.

4.1 Methods

4.1.1 Bacterial Isolate and Cranberry Capsules

Escherichia coli isolate NCTC 13353 expressing the resistance gene CTX-M-15 was used in all parts of this study.

Cysticlean® capsules were purchased from VITA GREEN Europe S.A, which contained 240 mg of PACs per capsule.

4.1.2 Minimum Inhibitory Concentration

In order to determine the concentration of cranberry to use in the RT-qPCR experiment, the minimum inhibitory concentration was determined using the broth macrodilution method in duplicate. A 60mg/ml PAC stock solution in 20ml minimal medium (M9 Minimal Salts, 5X, Sigma Aldrich, Dorset, UK) was created (recipe for minimal medium can be found in table 6-2 of Appendix). Serial dilutions in duplicate of the following concentrations of Cysticlean®: 30, 15, 7.5, 3.75, 1.88, 0.94, 0.47, 0.23, 0.12, 0.059, 0.029 mg/ml were created using M9 Minimal Salts 5X (Sigma Aldrich, Dorset, UK). An overnight culture (10^8 CFU/ml) of *E.coli* NCTC 1553 was diluted to 0.5 McFarland standard in minimal medium and 100 µl of this diluted culture was added to each concentration. Cultures were incubated at 37°C with shaking overnight. The MIC was determined as the lowest concentration with no visible growth. A positive control, consisting of minimal medium and *E.coli* and a negative control consisting of minimal medium only, were included. See 6.7.1 of Appendix for more detail.

4.1.3 RNA Extraction

A 10ml volume of the MIC concentration was prepared in minimal medium. An overnight culture of *E.coli* NCTC 1553 was diluted to 0.5 McFarland standard and 100µl was added to the cranberry solution and incubated with shaking at 37°C for 4 hr. A second untreated culture was also incubated as an untreated control. A negative control consisting of minimal medium only was included. All cultures were then filtered with a Whatman 1 and 0.45 membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) before RNA lysate was extracted using the Cells-to-CT™ 1-Step TaqMan™ Kit (ThermoFisher Scientific,

Loughborough, UK) following manufacturer's instructions, except that the lysis + DNase time was doubled to 10 min. RNA concentration was measured using a Qubit 3 fluorometer (Thermofisher Scientific, Paisley, UK) (see figure 6-IV of Appendix). The quality of RNA and any presence of DNA was assessed using agarose gel electrophoresis (see figure 6-2 and 6.3.6 of Appendix). More detail of RNA extraction methods can be found in 6.3.4 of Appendix.

4.1.4 Reverse Transcriptase qPCR (RT-qPCR)

Primers and Taqman probes were designed using the PrimerQuest Tool (Integrated DNA Technologies) and can be found in table 5-1. More details of primer design can be found in 6.4.3 of Appendix. As it is not possible to run melting curve analysis on Taqman assays, primer specificity was confirmed before RT-qPCR using agarose gel electrophoresis and extracted DNA (see figure 6-XI and 6.7.3 in Appendix). The Cells-to-CT™ 1-Step TaqMan™ Kit was used for RT-qPCR. More details on the Cells-to-CT™ 1-Step TaqMan™ Kit can be found in 6.7.2.

PCR reactions were carried out using the PikoReal® 96 well Real Time-PCR platform (ThermoFisher, Loughborough, UK). PCR amplification reactions were performed using three technical replicates, in a volume of 10 µl containing 2.5 µl TaqMan® 1-Step qRT-PCR Mix (initial concentration not known), final concentrations of 500 nM primers, 250 nM probe and 1 µl of RNA lysate. Cycling parameters for the RT-qPCR assay were as follows: an initial reverse transcription step at 50 °C for 5 min, reverse transcriptase inactivation/initial denaturation at 95 °C for 20 s, followed by 40 cycles of 95 °C for 15 sec

and 60 °C for 1 min. A no-reverse transcriptase control was implemented in triplicate by adding the mastermix containing reverse transcriptase, after the reverse transcription step of the RT-qPCR assay. A no template control consisting of water instead of RNA, was also added in triplicate.

Table 4-1 Primers and probes used in this study. Primer and probe sets were designed using the PrimerQuest Tool (Integrated DNA Technologies). All probes contained a FAM fluorophore at the 5' end and a black hole quencher at the 3' end. Gene

	Forward	Reverse	Probe	Product Size	Reference
16S	GGAGACTGCCAGTGATAAA	GAGGTCGCTTCTCTTTGT	CCTTACGACCAGGGCTACACACGT	113	This study
KpsM	GTTATATGGGACCGTCATATT	GCCACACTCTTTCGTATC	CCCAGAGCTTGCGGATTATCAATC	125	This study
ChuA	CGATGGGCGAAATGTATAA	GTTAGTTTCCGGACGTAAG	TCTCGATTGGTCGCTTCTATACCA	98	This study
CTX-M	GAAAGCGAACCGAATCT	GACATCGTCCCATTGAC	ACCTTGTTAACTATAATCCGATTGCGG	101	This study
SAT	AGAAATATGGCATCTGTCACC	CAGACGATATAGTCGGTGTTTC	CAGCAACCATTACTCTGGGACAGC	97	This study
USP	TGAGTTCTGGTATGAGGAAGA	CCCGTATGAACACCATACAC	TACTGCCTGCTAGTGCTTTCTGCC	126	This study
SoxS	AGGGTTGATTACCAGTATCC	AACATATCGCAACACATCAC	ACAGCGGCAATCAGCGGCGATATA	70	This study
RecA	GAGACAATTTGGTACTCCATC	GATATCCAGTGAAAGCGAAC	CGCCTGGGTGAAGACCGTTCCAT	90	This study
TraT	AGATTGCAGAGCGTACTAAG	ACACGGGTCTGGTATTTATG	CAACGGATAATGTTGCCGCCCTGC	127	This study
IhfB	GACGGTTGAAGATGCAGTAA	CGGTAGTGCAAAGAGAAACT	AGCATATGGCCTCGACTCTTGCGC	108	This study
HcaT	GGCATTATGGGAGCAACTAC	CTGGCGAGCAGCAATATAAC	ACCACTATCAACCACGGCAACGCC	114	This study

4.1.5 Statistical Analysis

The 16SrRNA gene was selected as a reference gene and the stability of expression was analysed using a t-test (see 6.7.5 for more details). The relative expression levels of the genes of interest compared to untreated samples were determined and normalized to the expression of the reference gene using a modified ΔΔCt method to take into account primer efficiencies in Microsoft Excel. Primer efficiencies were calculated using serial dilutions of amplicons (diluted 1:1000) and this efficiency was used in ΔΔCt calculations (see 6.7.6 for more details.

All statistical analyses were carried out using the GraphPad Prism (version 5; GraphPad Software Inc.; La Jolla, CA, USA). To determine the significant differences between treated and untreated samples, the t-test was performed. A difference with a p-value of <0.05 was considered statistically significant.

4.2 Results

4.2.1 MIC

The MIC for *E.coli* strain NCTC 13353 was determined to be 15mg/ml and this was used in all further experiments.

4.2.2 RT-qPCR

In this study, RT-qPCR was used to evaluate the impact of exposure of *E.coli* NCTC 13353 cells to the commercial cranberry product Cysticlean® (at MIC; for 4h) on the expression of ten genes that have been shown to be associated with the virulence and fitness of UPEC. The selected genes included the toxins SAT, and USP, the iron acquisition system ChuA, the protectins SoxS, KPSM, TraT and RecA, the antibiotic resistance gene CTX-M and the

genes responsible for normal function of the cell, IdfB and HcaT. Figure 4-I shows the average Ct values, as a measure of expression, of these 10 genes in the absence or presence of cranberry pre-treatment. There was a significant decrease ($P < 0.05$) in the Ct value for each gene in the presence of cranberry, indicating a decrease in expression. Figure 4-II shows the decrease as a Double Delta Ct Value ($\Delta\Delta Ct$) -1. By presenting the data as $\Delta\Delta Ct$) -1 a value of 0 would indicate no change while a value between 0 and 1 indicates a downregulation in expression. The t-test showed that all the genes tested were significantly downregulated ($P < 0.05$) following treatment with cranberry.

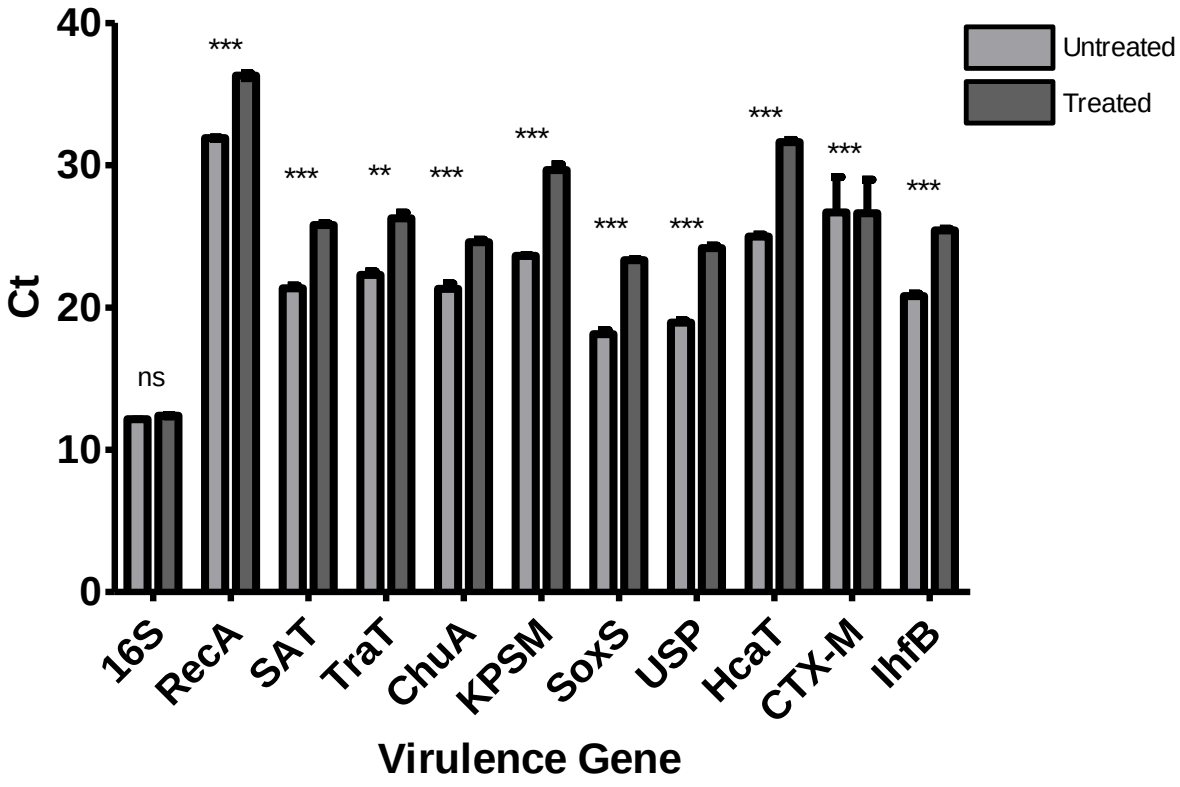

Figure 4-I Virulence genes tested. Genes for toxin production, iron acquisition, antibiotic resistance protectins and normal function of the cell were tested after exposure to Cysticlean® using RT-qPCR all genes were significantly downregulated when tested using a t-test. * p = 0.01 to 0.05 ** p = 0.001 to 0.01 *** p = < 0.001 ns = not significant.

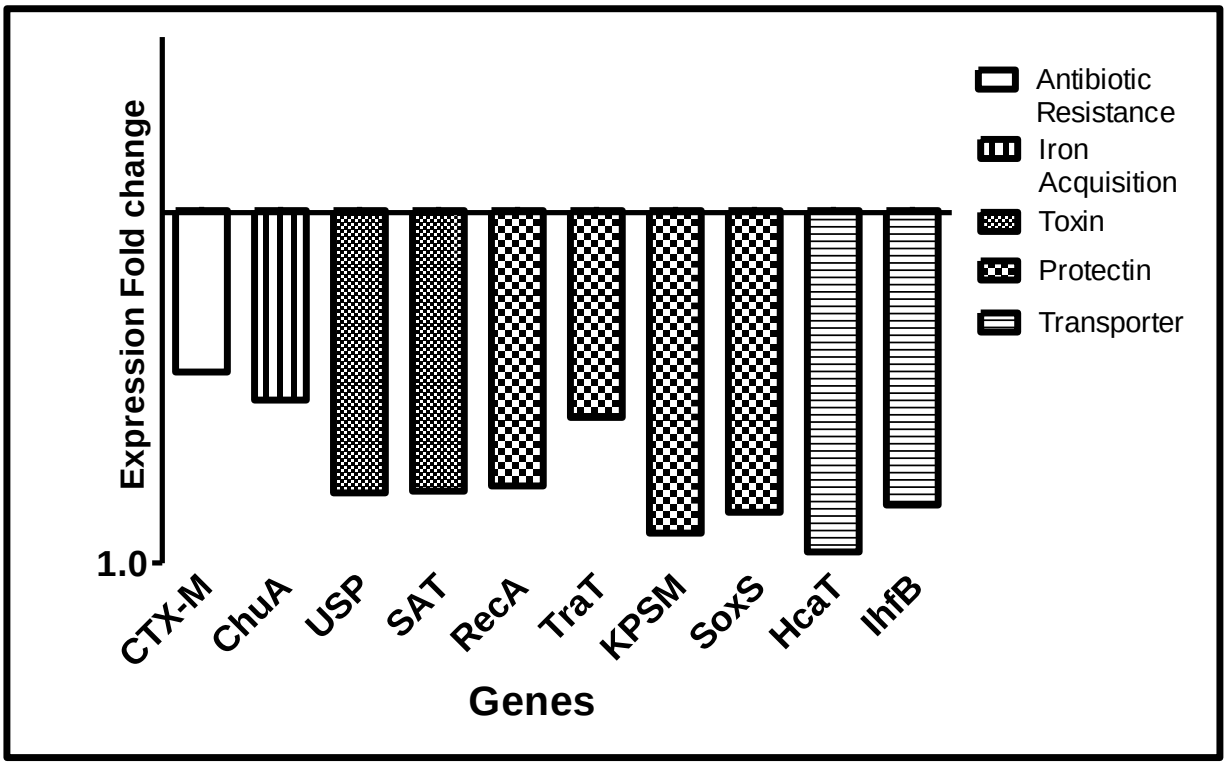

Figure 4-II The expression fold change of genes antibiotic resistance, iron acquisition, toxins, protectins and genes responsible for the normal function of the cell. The results of the expression fold change were normalized against the reference gene 16s and then were taken away from one to show the genes were downregulated.

It is not possible to run melting curve analysis on Taqman assays, due to the degenerate nature of Taqman probes. Non-specific products do not produce results in Taqman assays, therefore it is not necessary to run products on an agarose gel. However, non-specific products should be avoided as they reduce the efficiency of the reaction. Taqman assays should be checked for specificity before running qPCR (see figure 6-XI and 6.7.3 in Appendix).

4.3 Discussion

Reports have shown that numerous genes are implicated in the virulence and fitness of UPEC, therefore downregulating the expression of these genes could add to the efficiency of antibiotic treatment and could reduce the likelihood of treatment failure (Wasfi, et al., 2016). In this study, ten genes playing significant roles in the normal function of the cell, iron acquisition, toxin production and stress survival in *E. coli* were tested after exposure to the cranberry product Cysticlean ® and the relative gene expression was determined using qRT-PCR. Cysticlean® contains concentrated American cranberry extract, equivalent to 240mg of proanthocyanins (PAC).

The current results show that Cysticlean® significantly reduces the expression of a wide variety of genes responsible for fitness and virulence. This is the first study to access the effect of Cysticlean® on the expression of virulence and antibiotic resistance determinants. Even though it was the least downregulated gene, it could be stipulated that the most important downregulation was that of the CTX-M gene that causes extended-spectrum-β-lactamase (ESBL) production. ESBL-producing UTIs are a major problem in both the community and the hospital (Esteve-Palau, et al., 2015). The reduction in expression of this gene could lead to increased susceptibility of previously resistant isolates.

Reports have suggested that USP functions as a bacteriocin, targeting competing bacterial strains (see figure 4-III). It has also been shown to enhance infectivity in the urinary tract and play a role in UPEC pathogenesis (Crnigoj, et al., 2014; Paniagua-Contreras, et al., 2017; Tiba, et al., 2008). In a study by Bauer et al, results showed that the USP gene was found in 80% of cystitis isolates. (Bauer, et al., 2002). A reduction in the expression of USP

could lead to the outcompeting of this bacteria by other non-pathogenic bacteria and a reduction in its ability to cause pyelonephritis.

It is especially advantageous for UPEC to be able to utilize heme and/or hemoglobin, as the urinary tract is naturally low in iron. UPEC secrete cytotoxins that can access the heme reservoir within cells. These two capacities combined, could aid iron acquisition throughout the infection process (Subashchandrabose & Mobley, 2015). ChuA is an outer membrane receptor responsible for the transport of heme into the cell for utilization as iron (see figure 4-III) (Nagy, et al., 2001). Reduction in iron transport into the cell could lead to reduced growth of the bacteria or even cell death. Studies have shown that cranberry causes the over expression of iron-acquisition genes. In a study, cranberry treatment caused an up-regulation of genes encoding iron metabolism regulators (fur), transport protein (feoAB), genes involved in biogenesis pathway of Fe-S cluster (iscAS) and iron storage (ftnA, acnA, sodB) (Ranfaing, et al., 2018). This is the first study to investigate the expression of the ChuA gene after exposure to a cranberry product. Combining the literature with the results of this study, cranberry causes the over expression of iron-acquisition genes, but a downregulation of heme membrane receptors. It has been suggested that the expression of ChuA is influenced by RfaH in UPEC (Nagy, et al., 2001). Therefore, it may be that Cysticlean® has an effect on RfaH and this needs to be investigated further.

SAT is a serine protease, which is found predominantly in uropathogenic strains of *E.coli*, which has been shown to elicit a cytopathic effect on cultured epithelial cells (see figure 4-III). It has also been shown to be associated with pyelonephritis (Subashchandrabose &

Mobley, 2015). A reduction in the expression of this gene could lead to a reduction in the ability of UPEC to cause pyelonephritis.

KpsM is responsible for capsule production, protecting the bacterial cell from host defenses such as engulfment by phagocytes and the bactericidal effect produced by the complement system (see figure 4-III). KpsM is commonly associated with pyelonephritis (Paniagua-Contreras, et al., 2017). A downregulation in the expression of this gene could lead to cell death by phagocytes and the complement system.

SoxS increases tolerance to stress, activated by cytotoxic weapons produced by the human immune system such as nitric oxide and leads to direct activation of antibiotic resistance factors (see figure 4-III) (Warner & Levy, 2012). Reduction in the expression of soxS could lead to bacteria that are less tolerant to stress and generally less antibiotic resistant. In a study, cranberry treatment caused significant over-expression of genes involved in stress response (arcA, rpoS, cpxR), periplasmic stress (rseAC) and oxidative stress (oxyR). This being said, they did not specifically report results on the SoxS (Ranfaing, et al., 2018).
RecA plays a role in DNA recombination, by catalyzing the homologous pairing driven by ATP and strand exchange of DNA molecules (see figure 4-III) (Greene, 2016). A reduction in recombinational repair could lead to bacteria that are less able to survive.

TraT is an outer membrane lipoprotein that is responsible for preventing unproductive conjugation between bacteria carrying like plasmids (see figure 4-III). It has also been shown to interfere with complement-mediated killing, imparting a mild amount of serum

resistance (Johnson, 1991). A downregulation in the expression of this gene could lead to an increase in unproductive conjugation and a decrease in serum resistance.

The HcaT gene plays a role in the catabolism of 3-phenylpropionate and cinnamic acid, the products of which are fed into the mhp pathway (see figure 4-III). IhfB is responsible for the activation of transcription via sequence-specific DNA-binding and for site-specific recombination. It is uncommon to see changes in expression of these genes, in fact they have been recommended as suitable reference genes in transcriptional analysis of *E.coli* (Zhou, et al., 2011). This suggests that the effect of Cysticlean® is multifactorial, effecting many different pathways.

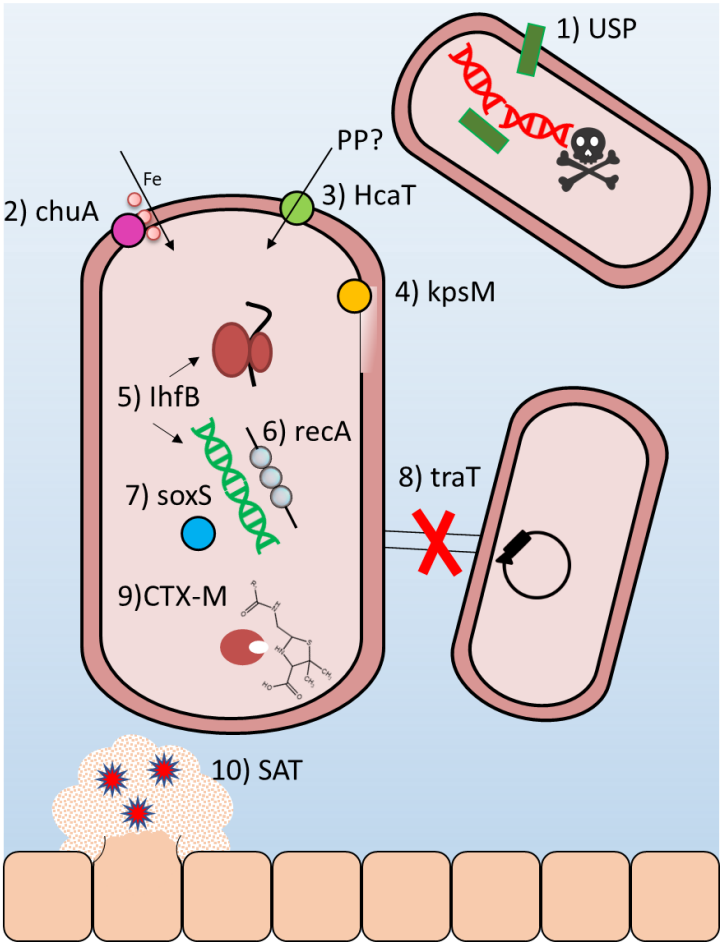

Figure 4-III **Reported mechanisms of action for 10 Virulence genes in *Escherichia coli* are downregulated by Cranberry treatment.** Various virulence factors are established to enhance the pathogenicity of UPEC. This study has shown that the following 10 genes, which all encode for virulence factors with different mechanisms, are all downregulated by cranberry treatment. These data suggest that cranberry's antimicrobial action is multifaceted. Below is a summary of the action of each gene downregulated by cranberry.1) Uropathogenic specific protein gene (USP) is a Bacteriocin that possesses DNase activity that can degrade plasmid DNA of competing uropathogens. 2) chuA is an outer membrane protein that plays a key role in heme uptake. 3) hcaT encodes a potential permease for the uptake of phenylpropionic acid (PP), an aromatic acid that can be utilised as a carbon and energy source. 4) kpsM protein plays a role in group 2 capsular polysaccharide synthesis. 5) IhfB is a specific DNA-binding protein that functions in genetic recombination as well as in transcriptional and translational control. 6) recA catalyses ATP-driven homologous pairing and strand exchange of DNA molecules necessary for DNA recombinational repair. 7) SoxS protein is a transcriptional activator of the superoxide response regulon of *E.coli*. 8) traT prevents unproductive conjugation between bacteria carrying like plasmids. 9) CTX-M produces beta-lactamases that can render beta-lactams inactive. 10) Sat is a secreted autotransporter toxin exhibits cytopathic activity and loosening of cellular junctions.

Other studies have shown that exposure of UPEC to cranberry PACs or cranberry powder results in the downregulation of the flagellin gene fliC which results in impeded motility via swimming and swarming. Honey has also been shown to have a similar effect of virulence genes to cranberry, however its ability to reach the urinary tract intact has been questioned (Wasfi, et al., 2016).

In vivo studies have previously been conducted on Cysticlean®. In experiments conducted with rats, urine samples taken after treatment with Cysticlean® capsules (118 mg PACs/ animal) showed a significant inhibition of *E. Coli* adherence. Many cranberry extracts available in the market, but, Cysticlean® sachets and capsules have shown the highest inhibition on bacterial adherence to urinary tract epithelium, and their use is related to a high tolerability, treatment compliance and effectiveness in preventing UTI. Cysticlean® has also been clinically proven to be highly effective in helping to reduce UTIs recurrence after 3-6months (Rodriguez, et al., 2015).

4.3.1 Mechanism of action

Two complementary mechanisms have been suggested to explain the ability of the American cranberry to reduce urinary tract infections. The first mechanism has been credited to its biocidal activity, through the production of several elements that can damage bacteria. The second mechanism is attributed to its anti-virulence activity, through the downregulation and interference of multiple virulence factors associated with tolerance to stress and survival in the urinary tract. The latter could ultimately result in the weakening of bacterial survival abilities and its ability to cause infection (Wasfi, et al., 2016).

Recurrent UTI is commonly treated with antibiotic prophylaxis, which has been shown to attribute to bacterial resistance (Othman, 2016). Antibiotic resistance is a major concern worldwide, therefore a re-evaluation of the treatment recommendations for UTI should be considered. An alternative to antibiotic prophylaxis could be treatment using cranberry products or a combination of cranberry and antibiotic treatment in an attempt to reduce resistance rates in UTIs.

Future studies should investigate the effect of Cysticlean® on other virulence and antibiotic resistance determinants and other bacterial species that cause UTI to combine with *in vivo* studies that have already been carried out. The effect of a cranberry and antibiotic combination should be considered further.

Chapter 5

Overall Discussion

5 Overall Discussion

5.1 Summary of Research

In this PhD project, I have used molecular microbiology methods to investigate four main areas of interest. Firstly, a study was carried out to investigate the prevalence of the ESBL family in the Leicestershire area and the relationship between plasmids and antibiotic resistance determinants. Given the high levels of ESBLs found in this study, a clear need was identified for a rapid test to detect ESBLs in the clinical laboratory. This lead to the development of a qualitative real-time PCR based method to detect 149 variants of the ESBL family in UTIs. It was also identified that alternatives to antibiotics are going to be required in an effort to counteract antibiotic resistance. In this study, the potential effect of the commercial product Cysticlean® on virulence factors associated with UTI was investigated. It was found that Cysticlean® is effective in reducing the expression of a wide variety of virulence factors and the CTX-M gene coding for beta-lactam resistance.

The results from these studies shall be discussed in the broader context of antibiotic resistance as a whole; and the challenges that face us in tackling it; including prevalence studies, rapid diagnostics, economics and treatment development.

5.2 Antibiotic Resistance

The 21st century has seen antibiotic resistance rise to be a major public health concern that threatens the effective prevention and treatment of a growing number of bacterial infections (Prestinaci, et al., 2015). The O'Neill report in 2016 reiterated the importance of the threat of antibiotic resistance and proposed many actions, including strengthening surveillance systems, the development of rapid diagnostics, and investment in new classes of antibiotics and alternatives (O'Neill, 2016).

5.3 Surveillance of Antibiotic Resistance

Surveillance of antibiotic resistance is vital in order to provide information on the scale and trends in resistance and to monitor the impact of interventions (Johnson, 2015; O'Neill, 2016; World Health Organization, 2001). The level at which data is collected (local, national or international) will determine the action required. Clinical management, treatment guidelines and infection control policies should be guided by frequent local prevalence data, as well as to educate prescribers (World Health Organization, 2001). Frequent prevalence information ensures that policy changes are made in a timely manner, as the speed of antibiotic resistance tends to be faster than policy change implementation (O'Neill, 2016). At a national level, collected data may also be used to inform policy, but also to update lists of essential antibiotics, treatment guidelines and the evaluation of cost-effectiveness interventions (World Health Organization, 2001). As antibiotic resistance is a global problem, international collaboration will always be needed in surveillance (O'Neill, 2016). There are gaps in knowledge of the extent of resistance in many parts of the world, due to the absence of suitable surveillance.

It is the hoped that the prevalence study completed as part of this PhD, can contribute to surveillance data in the UK and worldwide, and can help inform local antibiotic stewardship policy. It is also hoped that prevalence data on the levels of antibiotic resistance in farming, the environment and companion animals in the area will be combined with this study and others like it to give a true One Health perspective of the problem of antibiotic resistance.

As mentioned previously, Leicester has a high immigrant population and it was suggested that this may have contributed to the high level of CTX-M found. The findings of this study reinforce the need for a worldwide collaboration on antibiotic stewardship, as the actions of other countries can have a profound effect on antibiotic resistance levels on other countries through immigration.

Without adequate surveillance, the majority of attempts to prevent or reduce antibiotic resistance will become increasingly more difficult (World Health Organisation, 2014). Surveillance data can only be effective if it is acquired in an adequate amount of time. The development of rapid diagnostics is essential for not only patients, but to speed up the processing of surveillance isolates (O'Neill, 2016).

5.4 Rapid Diagnostics

The issue with current diagnostic methods is that they can give results conclusively, but not rapidly. Most methods are still based upon those developed by Louis Pasteur and Robert Koch in the nineteenth century (O'Neill, 2015). Current laboratory tests require at least 36 hours to give a result that can be used to guide antibiotic treatment. This is far too long and empirical treatment will have been started long before the result has been achieved, possibly with ineffective antibiotics due to resistance, or with unnecessarily broad-spectrum antibiotics, increasing the chance of resistance developing in the future (Lupo, et al., 2013; O'Neill, 2015).

It is well known that without rapid diagnostics, it is far more difficult to prescribe the right antibiotic to the right patient in a timely manner (Lupo, et al., 2013; O'Neill, 2015). This problem has largely been overcome in the treatment of long-term infections such as tuberculosis or HIV, where in higher income countries at least, patients are tested for resistance before treatment in started, not after (O'Neill, 2015). It is therefore surprising there is a lack of rapid diagnostics to achieve this with other bacterial infections.

5.5 Challenges in Developing Rapid Diagnostics for Bacterial Infections

There are many barriers to overcome in the development of bacterial diagnostics (Davenport, et al., 2017). Unfortunately, there is a lower level of public funding for the development of diagnostics for bacterial infections (O'Neill, 2015). Diagnostics for bacterial infections also have other challenges, when compared to other infections. The main problem is that many bacterial pathogens can be present without necessarily causing infection and so it is difficult for diagnostics to distinguish between harmless bacteria and

those that are causing infection. It is estimated that a third of people carry *Staphylococcus aureus* in their nose with it causing an infection, and therefore do not need antibiotics. On the other hand, if HIV is present in a patient, it is obvious that they require treatment. This makes it especially difficult to develop diagnostics for bacterial infections (O'Neill, 2015). The cost of rapid diagnostics is also considered another barrier, especially by prescribers (O'Neill, 2016; Ivanov, 2013). Most antibiotics are far less expensive that rapid diagnostic tests, therefore it is seen as an additional cost to diagnose infections before giving treatment (Ivanov, 2013). However, this way of thinking is very narrow minded, as it is evident that whilst the upfront cost is higher, both patients and healthcare systems benefit financially from rapid diagnostics. This opinion is not yet widespread, meaning that there are rapid diagnostics ready to come to market, that are not being bought and some that have been bought and not used.

Rapid diagnostics that allow the right antibiotic to be given to the right patient quickly, will likely help save money several ways. This includes reducing the length of stay in hospital for patients with bacterial infections and identifying those with antibiotic resistant infections so that infection control measures can be put in place to prevent spread. Infection control measures are expensive and rapid diagnostics could mean that precautionary measures put in place for high risk patients could be stepped down much more quickly (Bauer, et al., 2014). This was found to be the case in a study in the Netherlands, it was found that rapid diagnostics could reduce demand on intensive care isolation rooms by at least 40% (Wassenburg, et al., 2012).

On the other hand, it is common that diagnosis based on symptoms alone could miss out giving antibiotics to a patient that did in fact need them. Without antibiotics, the patient may deteriorate and end up in hospital, leading to much higher costs than using a rapid diagnostic test (Llor & Bjerrum, 2014).

Another benefit is the potential to alleviate some of the pressure on healthcare systems and save valuable doctor's time in surgeries by allowing screening to be completed in pharmacies or even at home. In some countries a diagnostic test for strep throat is available in some pharmacies. If the diagnostic test indicates that the infection is likely to be bacterial, the pharmacist can prescribe antibiotics for the patient, rather than the patient seeing a doctor (Thornley, et al., 2016).

There is clearly a need for rapid diagnostics that can be implemented throughout both high and low-income countries (Bauer, et al., 2014; Ivanov, 2013; World Health Organization, 2001). In the near future, rapid diagnostic tools should be point of care (at the bedside, in a doctor's office, pharmacy or at home), should be able to give meaningful information (bacterial or viral infection and resistance profiling) and should be combined with computer programming such as artificial intelligence that can access electronic medical records, combined with the symptoms that the patient is suffering, relevant and up to date literature and the latest local surveillance data, to assist doctors in recommending treatments. The information obtained by rapid diagnostics should immediately feed into real-time surveillance data for local, national and international infectious disease and antibiotic resistance, to monitor trends and enable the prediction of emerging problems so that interventions can be put in place rapidly.

Whilst the diagnostic method developed in this study does not fully fulfill these criteria, it is an important stepping stone in providing doctors with the most information currently available to aid them in suggesting treatment for a patient. With further optimization, this method could help antibiotic stewardship by reducing the time taken to get an antibiotic sensitivity result, helping to reduce resistance rates. It could also reduce the time patients spend in hospital, easing the bed crisis in the NHS and reduce the chance of infection spread within hospitals. Another benefit could be the opportunity to step down infection prevention measures earlier. If the method could be optimized for use in low-income countries, such as India, that have a huge problem with Cephalosporin resistance, this could improve healthcare dramatically. Infectious diseases are still a significant limitation in improving life expectancy in low income countries. In 22 countries, 70% or more of premature deaths are still caused by infectious diseases and related conditions. If methods such as this could be used in the developing world to detect infectious diseases earlier than current methods, this could go some way to improve life expectancy. All of this combined could not only save the NHS millions, but also give the world a fighting chance at tackling antibiotic resistance.

5.6 Alternatives to Antibiotics

There is clearly an awareness of the need for new antibiotics and alternatives to antibiotics, signified by the fact that there are antibiotics and vaccines in clinical trial at present (Flores-Mireles, 2015; O'Neill, 2016). However, antibiotic stewardship is understandably concerning to pharmaceutical companies, as this may have a negative impact on sales (O'Neill, 2016). This may lead to companies reducing their investment in antibiotic

research and development and may decrease the amount of antibiotics and vaccines that they donate, or reduce the cost of, to low income countries.

Cranberry is one alternative to antibiotics that was studied as part of this thesis and in light of the results, should be given more attention and moved from the “old wives tale” thinking into a potential therapeutic option. Cranberry, either as juices, powder or capsules, has been shown to be safe, effective and synergistic with antibiotics (Hisano, et al., 2012). This treatment could be most effective with antibiotic resistant UTIs, as this study has shown that it can reduce the expression of CTX-M in *E.coli.* If this is also true of other antibiotic resistance genes, cranberry could be a viable alternative to antibiotics for treatment of UTI, especially in those with recurrent infections. In this study, the effect of a cranberry capsule Cysticlean® was shown to decrease the expression of some key virulence genes linked to urinary tract infection caused by *E.coli.* It can be suggested that targeting some of these virulence factors or others, could be a potential treatment of UTI.

The development of vaccines is another possible alternative and there are vaccines currently undergoing clinical trial. Despite research that suggests that a prior UTI does not produce a protective immune response, animal model studies indicate that immunization with UPEC antigens can stimulate a mucosal immune response that may prevent UTI. However, the heterogenicity of uropathogens means that vaccine design is complicated and requires more research for an effective UPEC vaccine to be developed (Flores-Mireles, 2015). Vaccines currently under investigation include those that target adhesins, exotoxins, iron acquisition systems, flagella, capsule, and lipopolysaccharides.

Another alternative to antibiotics that may have merit is antimicrobial peptides. Antimicrobial peptides are natural components of the innate immunity of many plants and animals. Like cranberry, antimicrobial peptides kill bacteria by multiple mechanisms and this includes membrane pore formation. They are also synergistic with antibiotics (Flores-Mireles, 2015). However, resistance mechanisms have already been identified, and improvements in bioavailability, stability and reduced toxicity are required before these can be available for treatment (Fuente-Núñez, et al., 2012). Further research is needed; however, these provide promising alternatives to antibiotics.

5.7 Future Work

5.7.1 Prevalence

Prevalence studies are an important part of tackling antibiotic resistance and should be continued regularly. Other resistance mechanisms should be monitored in the Leicestershire area, such as carbapenamases, and should not be limited to humans; isolates from the environment, animals and livestock should always be included in surveillance data as well. As mentioned previously, in the future surveillance data should be real-time and combined with artificial intelligence in order to analyse it rapidly. Some work has gone into this, with the establishment of EARS-Net and GLASS, however results are not published fast enough. For example, using EARS-Net, results for third generation cephalosporin resistance in *E.coli* in the UK were only available up to 2016. The improvement of rapid diagnostics should go some way in speeding up this process.

5.7.2 Rapid Diagnostics

As mentioned previously, the optimization of the diagnostics method developed in this study could have great benefit. In order to optimize this method, other antibiotic resistance genes should be added to the genetic profile such as those that code for carbapenamases. In order for this to be achieved, certain limitations need to be overcome. This method takes advantage of the use of channels that multiplex real-time PCR machines use to detect fluorescent chemistry such as the one used in this study. The PCR instrument used here (Pikoreal ThermoFisher Scientific, UK) has four channels, and therefore can only multiplex four different products in one well. However, advancements in technology has led to the development of real-time PCR instruments that can multiplex to a much higher level, such as the Quant Studio 12K flex qPCR instrument that can multiplex up to 21 genes at a time. It was also mentioned that optimization is required for use in low income countries. With the development of microfluidics and biosensors, it is possible that the time to detection for this method could be reduced further and incorporated into a small stand-alone device that could be used in the field, in doctor's offices etc. The invention of new types of DNA polymerases that are much cheaper to manufacture, could mean that this method becomes far cheaper to run and attainable to low income countries.

Currently, this method requires pathogen identification and culture. Ideally this method should be optimized for use straight from urine, and there are now DNA extraction kits that can potentially do this. This still leaves the issue of pathogen identification, as urine samples can contain a large number of contaminants and bacteria that are not causing harm. Therefore, it is suggested that this method be combined with one that can identify the

causative agent. Research has already gone some way in developing qualitative real-time PCR methods that can identify pathogens, with a semi-quantitative method (a threshold for Ct method indicates infection) having already been developed.

Ideally the results from this method, where possible, should be automatically uploaded to surveillance databases, in order for real-time surveillance to be achievable.

Alternative forms of PCR include loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA). Whilst LAMP is cost-effective and rapid, due to the isothermal nature of BST polymerases, primer design is far more laborious and false positives can be a significant problem. In addition, multiplexing LAMP assays is very difficult to achieve (Mayboroda, et al, 2018). RPA is another isothermal form of PCR, than can achieve results in as little as ten minutes. However, currently all RPA kits are produced under the license of TwistDx Limited (Cambridge, UK), meaning that optimisation of assays is more difficult (Mayboroda, et al, 2018). If the issues with these methods can be overcome, they would become ideal for the development of diagnostics.

5.7.3 Cranberry

Further research on the use of cranberry as an alternative to antibiotics/in combination with antibiotics should be conducted. This should involve testing more virulence and antibiotic resistance genes; a microarray analysis may be more useful in doing this. This should also be combined with phenotypic and *in vivo* studies, to give a broad overview of the effect that cranberry treatment has. Other bacterial strains and species as well those that are antibiotic resistant and non-resistant, that cause UTI should be studied to see if the effect of cranberry

is broad-spectrum. Cranberry should be studied for the effect on other infections. For example, could it be used to treat wound infections in the same way as honey?

In this study, only one reference gene was able to be used. Multiple reference genes increase the accuracy of relative expression normalization and allows for the quality control on the stability of the reference gene expression. Therefore, the use of one reference gene is a limitation of this study.

This study has focused on ESBL-producing UTIs. This is only one of numerous antibiotic resistant infections that are of major concern, and it is likely that many more will emerge in the future. Many more studies like this one are required all over the world in order to tackle antibiotic resistance.

5.8 Concluding Remarks

This study shows that antibiotic resistance is a complicated and multifaced problem that requires more than one effort, in combination. However, results from this study demonstrate that it is possible to make a difference to antibiotic resistance. Here the prevalence of ESBLs in the local area was studied, and suggestions have been made to improve surveillance. A rapid qualitative real-time PCR method to detect ESBLs in UTIs was developed that, with optimization, could see a real benefit to patients. The effect of the cranberry product Cysticlean® was tested by qRT-PCR and it was found that it reduced the expression of virulence genes associated with UTI infection and the antibiotic resistance gene CTX-M.

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Appendix A

Experimental Development and Rationale

6 Experimental Development and Rationale

6.1 Isolate Collection

Bacterial isolates (n=236) of *Enterobacteriaceae* isolated from urinary tract infections in the form of bacteria on agar plates, were obtained from the Leicester Royal Infirmary hospital (Leicester, England) on a weekly basis. These isolates were then sub-cultured onto Müller-Hinton agar (Oxoid, Basingstoke, UK) plates for 24 hours at 37°C. Bacterial isolates were stored at -20°C in 50% glycerol stocks. Glycerol stocks were made by adding 250 µl distilled water to 250 µl glycerol and autoclaving. 500 µl of overnight nutrient broth (Oxoid, Basingstoke, UK) culture was then added. A total of 181 of these isolates were used in the Real Time-PCR assay development.

Five control isolates were obtained from Public Health England, that can be found in table 6-1. These were stored on cryogenic protect beads (Scientific Laboratory Supplies, Loughborough, UK). One set was stored at -20°C for working stocks and one set was stored at -80°C for creation of future working stocks. These control isolates were used in both the prevalence study and the development of the qualitative real-time PCR assay. NCTC 13353 was also used in the qRT-PCR study.

6.1.1 Bacterial Strains

Table 6-1 Five control isolates were obtained from Public Health England used in this study. Strains, the species of bacteria and the antibiotic resistance gene expressed are shown.

Strain name	Antibiotic resistance gene expressed	Bacteria
NCTC 13353	CTX-M-15	*Escherichia coli*
NCTC 13351	TEM-3	*Escherichia coli*
NCTC 13368	SHV-18	*Klebsiella pneumoniae*
NCTC 13442	OXA-48	*Klebsiella pneumoniae*
NCTC 10418	Negative	*Escherichia coli*

6.2 Recipes

This study used several different solutions for the growth and maintenance of bacteria or during RNA extraction and RNA or DNA detection. The formulation of each solution is outlined in table 6-2.

Table 6-2 The formulations of the different media used in this study. Ethylenediaminetetraacetic acid (EDTA).

Name	Formulation g/litre
Nutrient broth	1.0 “Lab Lemco” powder, 2.0 yeast extract, 5.0 peptone, 5.0 sodium chloride
Muller Hinton agar	Beef, dehydrated infusion from - 300.0, Casein hydrolysate - 17.5, Starch - 1.5, Agar - 17.0
Minimal medium	Disodium Phosphate Heptahydrate 25.6, Monopotassium Phosphate, Sodium Chloride 1, Ammonium Chloride, 20 mL of 20% D-Glucose solution, 2 mL of 1.0 M MgSO4 solution, 0.1 mL of 1.0 M CaCl2 solution
PBS	Sodium chloride - 8.0, Potassium chloride - 0.2, Disodium hydrogen phosphate - 1.15, Potassium dihydrogen phosphate - 0.2
LB broth (miller)	Tryptone - 10, NaCl - 10, Yeast Extract – 5.0
MacConkey agar	Peptone - 20.0, Lactose - 10.0, Bile salts - 5.0, Sodium chloride - 5.0, Neutral red - 0.075, Agar - 12.0
10x TBE buffer	89mM Tris, 89mM Boric acid, 2mM EDTA

6.3 DNA, RNA and Plasmid Extraction

6.3.1 DNA Extracted from Colonies

DNA for use in 2.1.2 and 3.1.1 was extracted by the boiling method (figure 6-I). DNA extractions were stored at -20°C.

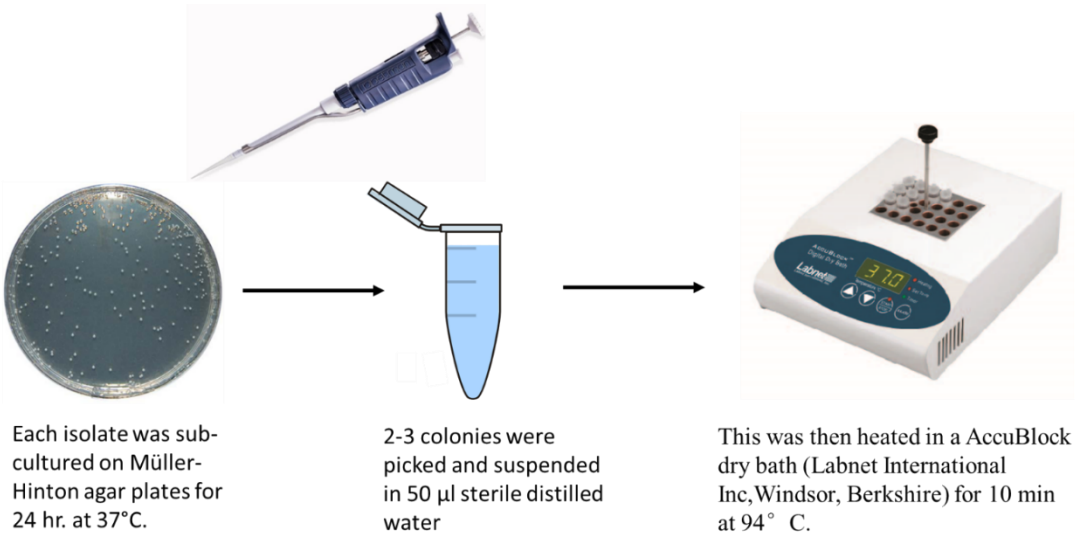

Figure 6-I The boiling method used to extract DNA from bacterial isolates. Each isolate was sub-cultured onto Muller Hinton agar and incubated for 24 hr at 37 °C. Colonies were then picked from the agar plate and suspended in sterile distilled water and mixed by vortexing. This suspension was then heated in a dry bath for 10 min at 94 °C before being used in PCR reactions.

6.3.2 Plasmid Extraction for Replicon Typing

Each isolate was sub-cultured in LB broth overnight at 37°C. Plasmids were extracted using the Illustra PlasmidPrep Mini Spin Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). One colony was suspended in 10 ml nutrient broth (Scientific Laboratory Supplies, Loughborough, UK) and grown overnight at 37°C. 1.5ml of this culture was transferred into a microcentrifuge tube and centrifuged at full speed for 30 seconds using a Sigma 1-14 microcentrifuge (Sigma Zentrifugen, Osterode am Harz Germany). The supernatant was discarded, and the pellet was re-centrifuged at full speed

for 30 seconds. A pipette was used to remove any remaining supernatant. A total of 175 µl of lysis buffer type 7 was added to the pellet and resuspended by vortexing using a Fisherbrand™ ZX3 Vortex Mixer (Fisher Scientific, Loughborough, UK). A total of 175 µl of lysis buffer type 8 was then added and this time mixed by inversion until the solution became clear. Vortexing would have sheared the genomic DNA and contaminated the final purification. It was also important to complete both lysis steps within 5 minutes, as the NaOH will denature plasmids above this time. The resulting mixture was then centrifuged at full speed for 4 minutes. The supernatant was then transferred to a collection tube, containing one Illustra plasmid mini column and centrifuged at full speed for 30 seconds. Flow through was then discarded. The column was then washed with 400 µl of lysis buffer type 9 and centrifuged at full speed for 30 seconds. Flow through was discarded once again. 400 µl of wash buffer type 1 was added to the column and centrifuged for 1 minute. Flow through and collection tube was discarded. The Illustra plasmid mini column was transferred into a fresh Microcentrifuge tube and 100 µl of elution buffer type 4 was added directly to the centre of the column. This was then incubated for 30 seconds at room temperature and then centrifuged at full speed for 30 seconds. The flow through was then stored at -20 °C.

6.3.3 Purified DNA extraction

DNA was extracted using the Illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK). One colony was suspended in 10 ml nutrient broth (Scientific Laboratory Supplies, Loughborough, UK) and grown overnight at 37°C. 1 ml of this culture was transferred into a Microcentrifuge tube and centrifuged at full speed for 30 seconds. The supernatant was discarded. 40 µl of lysis buffer type 2 was added and mixed by vortexing until completely resuspended. 10 µl of proteinase K was added and mixed by vortexing for 10 seconds. 10 µl of lysis buffer type 3 was added and vortexed for 10 seconds to mix and then centrifuged for 5 seconds at 1000 x g to collect the sample at the bottom of the tube. This was then incubated for 7 minutes in a dry bath at 55 °C. After the incubation, the sample was vortexed and centrifuged for 5 seconds at 1000 x g to collect the sample in the bottom of the tube. This was then incubated for another 8 minutes and centrifuged again for 5 seconds at 1000 x g. 500 µl of lysis buffer type 4 was added and mixed by vortexing for 10 seconds, then incubated at room temperature for 5 minutes. After the incubation step, the sample was vortexed and centrifuged for 5 seconds at 1000 x g, then incubated again for 5 minutes at room temperature. The samples were then added to a collection tube, containing a bacteria mini column and centrifuged for 1 minute at 11000 x g. The flow through was discarded. 500 µl of lysis buffer type 4 was added to the column and centrifuged for 1 minute at 11000 x g, and the flow through was discarded. 500 µl of wash buffer type 6 was added to the column and centrifuged for 3 minutes at 16000 x g. The bacteria mini column was then transferred to a fresh Microcentrifuge tube and 200 µl of elution buffer type 5, pre-heated to 70 °C in a dry bath was added directly to the centre of

the column. This was then incubated for 1 minute at room temperature and centrifuged at 1000 x g for 1 minute. The flow through was then stored at -20 °C.

6.3.4 RNA Extraction Method Development for RT-qPCR

Different growth stages were tested to see which gave the best results. $X10^6$, $x10^7$, $x10^8$ and $x10^9$ CFU/ml were tested. $X10^9$ CFU/ml gave the best results. RNA was extracted by the method below and the resulting RNA was analysed for quality by agarose gel electrophoresis using the method outlined below. Upon analysis of the gel result, there appeared to be DNA contamination in the RNA product (figure 6-II). In order to improve this, the standard lysis time and DNase was doubled to 10 min. DNA contamination on the gel disappeared.

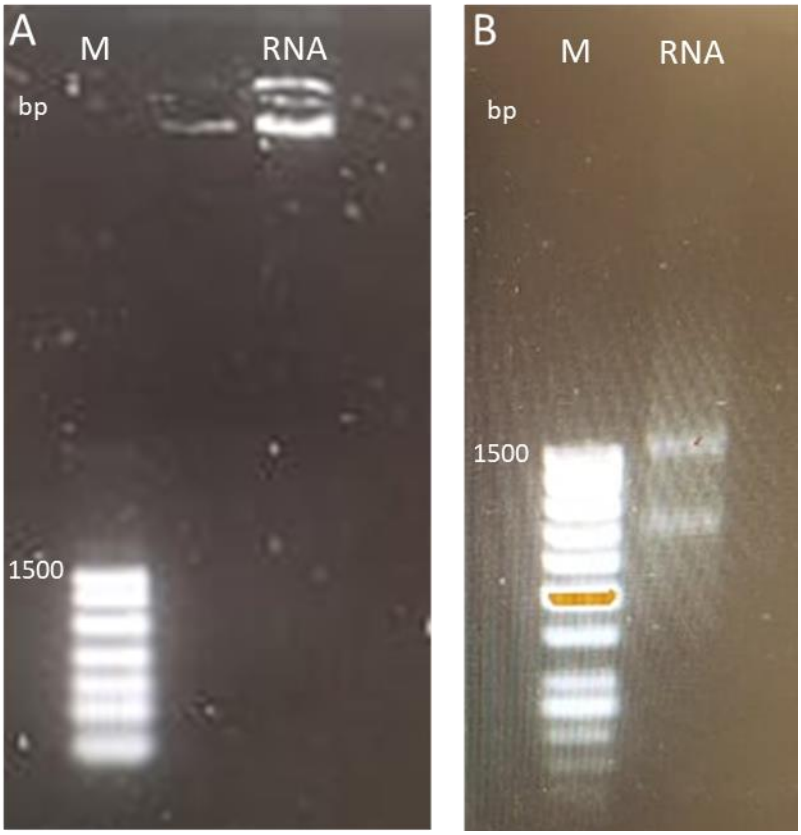

Figure 6-II Optimisation of RNA extraction protocol. A) shows the first attempt at RNA extraction. DNA contamination appears to be highly present, in the form of a bright band in the well of the gel. B) shows RNA extraction after the doubling of the combined lysis and DNase time from five to ten minutes. M indicates the DNA ladder. The band in the well of the gel has disappeared and there are two distinct bands clearly present.

The first run on the qPCR with the 16S gene showed quite a high Ct (figure 6-III), so it was determined that less RNA was needed in the reaction.

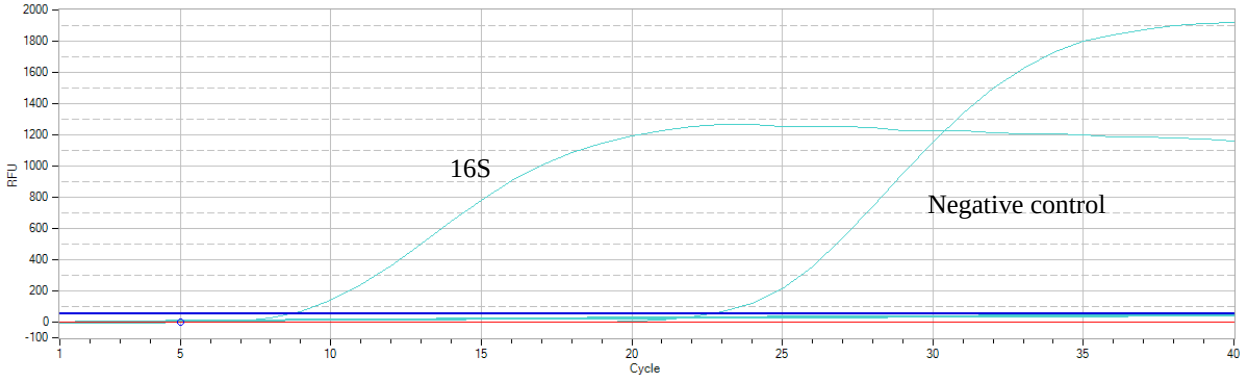

Figure 6-III Initial run of RT-qPCR protocol with the 16S gene and a negative control. It was determined that the Ct of this first run was too high, and less RNA was need in future experiments.

During the MIC testing, it became quite clear that the cranberry extract would be an issue in qPCR testing, due to its colour and viscosity, and this would need to be filtered out. Filtering did significantly reduce the concentration of RNA achieved, however as the concentration needed to be reduced anyway, this was not an issue. Filtering did not appear to have an effect on the quality of RNA. The untreated and treated samples yielded slightly different RNA concentrations, and this was calculated so that the same amount of RNA was added in the RT-qPCR reaction.

In the final protocol, one colony of NCTC 1553 CTX-M-15 producing *E. coli* bacteria was added to 10ml minimal medium and incubated at 37°C with shaking overnight. In one tube, 160 µl of bacterial culture was resuspended in 16 ml fresh medium and the contents of one Cysticlean tablet was added. For the untreated sample, 100 µl of bacterial culture was added to 10 ml minimal medium. A negative control consisted of 10 ml minimal medium only. These were incubated for 4 hours at 37°C with shaking.

Resulting bacterial cultures were filtered, firstly through a Whatman 1 membrane, to remove the cranberry, and then through a Whatman 0.45 membrane, to allow the bacteria to adhere to the filter. 0.45 filters were then placed in 10ml PBS and vortexed at full speed for 2 mins, to remove the bacteria from the filter. 1 ml of this PBS was removed and centrifuged at full speed for 10 mins. The PBS was removed without disturbing the resulting pellet, washed with 50µl Phosphate Buffered Saline (PBS) and vortexed to resuspend. The suspension was centrifuged again at full speed for 1 min. As much PBS as possible was removed and the remaining pellet was placed on ice. 49.5µl of the DNA lysis solution and 0.5µl of the DNase solution was added, mixed by pipetting and incubated for 10 min at room temperature. 5µl of the stop solution was added, mixed by pipetting and incubated for 2 mins at room temperature. Extracted RNA was stored at -20°C in aliquots and never thawed more than once. RNA stabilizers such as RNA protect, are not required with the cells to Ct kit.

6.3.5 DNA and RNA Quantification

DNA and RNA concentration were measured using a Qubit 3 fluorometer (figure 6-IV) (Thermofisher Scientific, Paisley, UK).

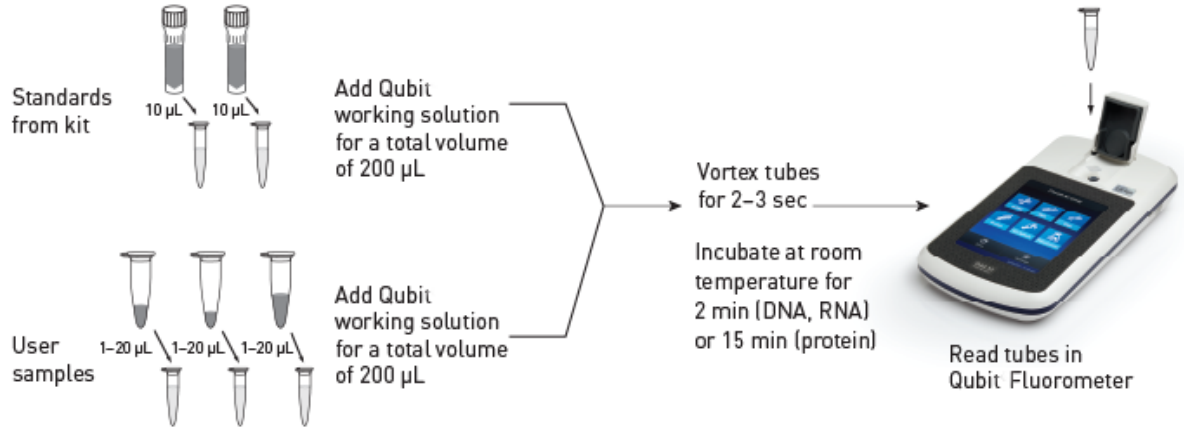

Figure 6-IV The Qubit™ kit protocols for the detection of DNA and RNA. dsDNA BR Assay Kit was used to detect the concentration of DNA and the Qubit™ RNA HS Assay Kit was used to detect the concentration of RNA. DNA or RNA was mixed with the Qubit working solution (1:200 assay reagent with dilution buffer), vortexed and incubated at room temperature for 2 min before reading on the instrument.

6.3.6 Determination of Quality of RNA Extracted for RT-qPCR

In order to determine the quality of RNA extracted, RNA was run on gel electrophoresis immediately after extraction. Approximately 2µg of RNA (measured with the Qubit 3 fluorometer) was mixed with the same amount of RNA loading dye in a Microcentrifuge tube. This was then heated in the heat block alongside 4µl of RiboRuler High Range RNA Ladder, ready-to-use (Thermofisher, Paisley, UK) in a separate tube, for 10 min at 70°C. Both tubes were then placed in ice for 3 mins and spun briefly using the Labnet Prism mini centrifuge (Fisher Scientific, Loughborough, UK) before being loaded onto a 1% agarose gel (made using protocol in 6.5.3) and run at 110 volts for 60 mins.

6.4 Primer Design

6.4.1 End-Point PCR

Primer sequences were found using NCBI GenBank (National Center for Biotechnology Information, Bethesda, USA) and primers were designed using the Primer-Blast tool. Primer-Blast can be accessed here: https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi. GenBank can be accessed here: https://www.ncbi.nlm.nih.gov/genbank/. Primers were then checked for specificity by the BLAST blastn suite (National Center for Biotechnology Information, Bethesda, USA). BLAST can be accessed here: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&SHOW_DEFAULTS=on&BLAST_SPEC=MicrobialGenomes. Primers were then checked using the OligoAnalyzer 3.1 (Integrated DNA Technologies Inc, Illinois, USA) for self-dimers, heterodimers, hairpins, GC content, annealing temperature and palindromes. The OligoAnalyzer can be found here: https://eu.idtdna.com/calc/analyzer. In general, primers for end-point PCR should be 18-22 bp in length, have a GC content of 40-60% and the forward and reverse primers should have similar annealing temperatures. Primers for multiplex PCRs were also ran through the ThermoFisher Multiple Primer Analyzer, to check for primer-dimer formation. The Multiple Primer Analyzer can be found here: https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html.

Primers were ordered from Integrated DNA Technologies, at a concentration of 25 nm, and diluted with sterile distilled water to 100 µM concentrations.

6.4.2 Qualitative Real-Time PCR – Plexor

Primers were adapted from (Singh, et al., 2016). Iso-dC and fluorescence labels were added to each of the forward primers using the IDT custom DNA oligos website (Integrated DNA Technologies Inc, Illinois, USA). Forward primers were ordered at a concentration of 100 nM and purified by HPLC. Reverse primers were ordered at a concentration of 25 nM with standard desalting. All primers were diluted to 100 µM with MOPS/EDTA buffer (supplied with Plexor qPCR System, Promega, Southampton, UK).

6.4.3 RT-qPCR – Taqman

Taqman chemistry was chosen for its high specificity, when compared to SYBR green. qPCR primers and Taqman probes were designed using the PrimerQuest Tool (Integrated DNA Technologies) using custom design parameters. Primers were designed to be 20-24 bp, with a melting temperature of approximately 60 °C. The GC content was designed to be 40-60%, however ideally it should be 50%. Ideally, the last five bases should be made up of 3 A/T and 2 G/C. Taqman probes were designed to be 20-30 bp in length, with a melting temperature of 7-10 °C higher than that of the primers, with a GC content of 35-65%. The 5’ end of the probe should ideally be around 5 bases away from the 3’ end of the forward primer. A G base should be avoided at the 5’ end. Ideally the probe should have fewer Gs than Cs. Both primers and probes should avoid >4 repeats of the same base e.g. AAAAAAAA, as well as palindromes e.g. AGGAGGAGGA. The amplicon should be small, around 70-150 bp. The OligoAnalyzer 3.1 was used to identify and possibility of

secondary structure and primer dimers. Whilst non-specific products do not produce results in Taqman qPCR, they should be avoided as they reduce the efficiency of the reaction. The FAM fluorophore was added to the 5' end of the probe and the Black Hole Quencher®-1 was added to the 3' end using the custom DNA oligo tool (Integrated DNA Technologies). Probes were ordered as a 100nm scale and purified by HPLC. Primers were ordered as a 25nm scale by standard methods. Both primers and probes were ordered as "lab ready", 100 µM in IDTE, PH 8.0.

6.5 Development and Optimisation of PCR Assays

6.5.1 Detection of the ESBLs: TEM, SHV, OXA, CTX-M

As the end-point PCR machine does not have a gradient PCR option to help determine the optimum annealing temperature of multiplex primers, touchdown PCR was used to aid optimization of the first assay. Optimisation of primer concentration was achieved by means of a primer concentration gradient using the format in figure 6-V. A 400 nM final concentration was determined to give optimal results.

	Forward Primer (nM)											
Reverse Primer (nM)		50		100		200		400		600		800	
	50												
	100												
	200												
	400												
	600												
	800												
	NTC												

Figure 6-V Primer concentration gradient plate format. Primer concentration gradient was used to optimise the concentration of both the forward and reverse primers.

DNA concentration was also optimized and a range of 1 µl to 2.5 µl was tried. It was found that 2.5 µl gave the best results (figure 6-VI). This could be because colonies were used rather than purified DNA.

The PCR amplification protocol was adapted from (Al-Mayahie, 2013) with the addition of touchdown annealing temperatures.

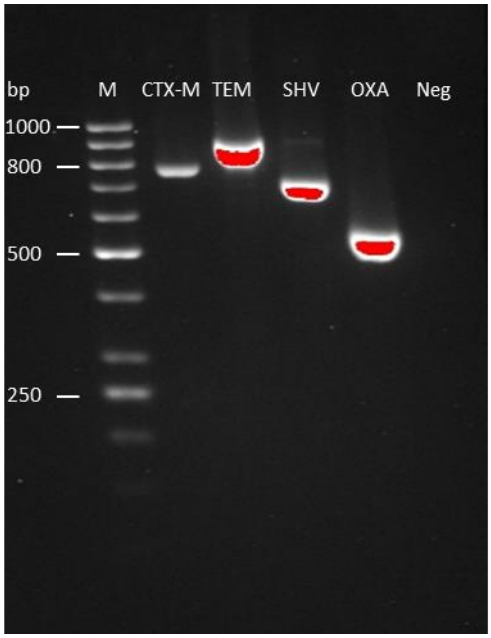

Figure 6-VI Agarose gel showing multiplex PCR results for TEM, SHV, OXA and CTX-M primers. DNA was isolated from the control isolates obtained from Public Health England. All genes were detected at the expected bp size. Neg indicates the negative sample and no bands were observed. M indicates the DNA ladder used, GeneRuler 50 bp DNA Ladder (Thermofisher, UK). Products and ladder ran on 2% Agarose gel.

6.5.2 Multiplex PCR-based Replicon Typing

Detection of plasmids was by means of adapting the replicon typing assay designed by (Carattoli, 2005). The annealing temperature was raised from 60°C to 65°C and the extension time in each cycle was reduced from 1 min to 30 sec. Results can be seen in figure 6-VII.

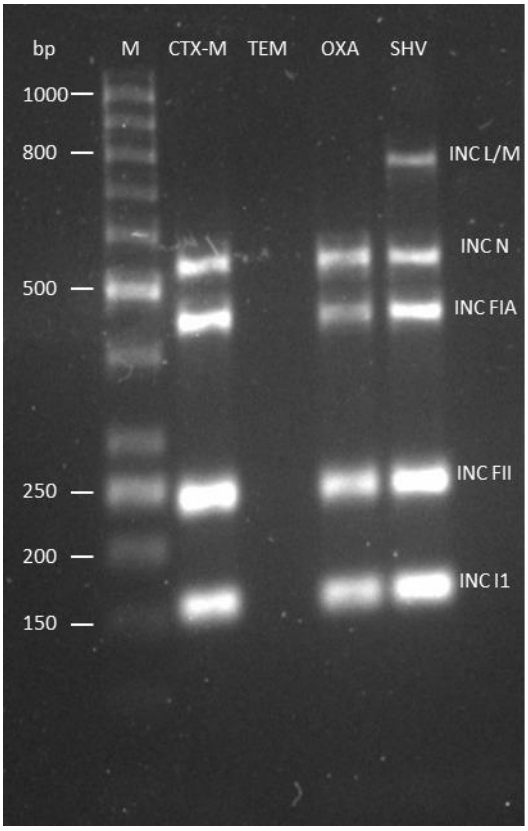

Figure 6-VII Plasmid replicon-typing of control ESBL producing isolates by multiplex PCR. Plasmid DNA was isolated from the control isolates obtained from Public Health England (table 1). Plasmids INC N, INC FIA, INC FII and INCI1 were detected in three of the control bacterial isolates (CTX-M, OXA, SHV), however, no plasmid was detected in the control TEM bacterial isolate. INCL/M was only detected in the control SHV bacterial isolate, all genes were detected at the expected bp size. M indicates the DNA ladder used, GeneRuler 50 bp DNA Ladder (Thermofisher, UK). Products and ladder ran on 2% Agarose gel.

6.5.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyse the results of PCR products. A 10x Tris/Boric Acid/EDTA (TBE), Nucleic Acid Electrophoresis Buffer (BioRad, Hertfordshire, UK), diluted to 1x with distilled water, was used for gel electrophoresis. The Sub-Cell® GT Cell gel tank was used to carried out the electrophoresis. UltraPure™ Agarose, (ThermoFisher Scientific, Paisley, UK) was mixed with the SYBR™ Safe DNA Gel Stain in 0.5X TBE to make the following percentages and melted carefully using a microwave. To decide upon the percentage of gel required, the table below was used.

Table 6-3 Agarose concentrations used for electrophoresis gels. Concentrations of agarose used for electrophoresis in this study, the amount of agarose required to achieve this concentration in a 50 ml gel and the range of resolution each concentration is suited to.

Agarose concentration (w/v%)	Resolution Range	Agarose for 50 ml gel (g)
0.8	500bp-12kb	0.4
2	400bp-10kb	0.5
1.5	200bp—bp-4kb	0.75
2	100bp-2kb	1
4	10bp-400bp	2

A total of 5 µl of PCR product was loaded into each well. Loading buffer was not required for the end-point PCR products, as the 5x green buffer contained in the GoTaq® G2 DNA Polymerase kit (Promega, Southampton, UK) acts as a suitable loading buffer. Different voltages and times were required for different products. Generally short amplicons were run on a high voltage for a short amount of time. For reactions containing multiple products, i.e. multiplex PCR, gels were run at a low voltage and long length of

time to optimise resolution of products. The gels were visualised using a Gel Doc EZ (BioRad, Hertfordshire, UK) with the Image Lab software (BioRad, Hertfordshire, UK).

6.6 Qualitative Real-Time PCR - Plexor

Qualitative real-time PCR conditions such as annealing temperature, primer concentration and DNA concentration were optimised using the protocol laid out in 6.5.1 in simplex (figure 6-VIII), and once satisfied, in multiplex (figure 6-IX). The amplicons of the control isolates were initially tested using high resolution melting curve analysis, first in simplex and then multiplex to determine the melting point of the amplicons.

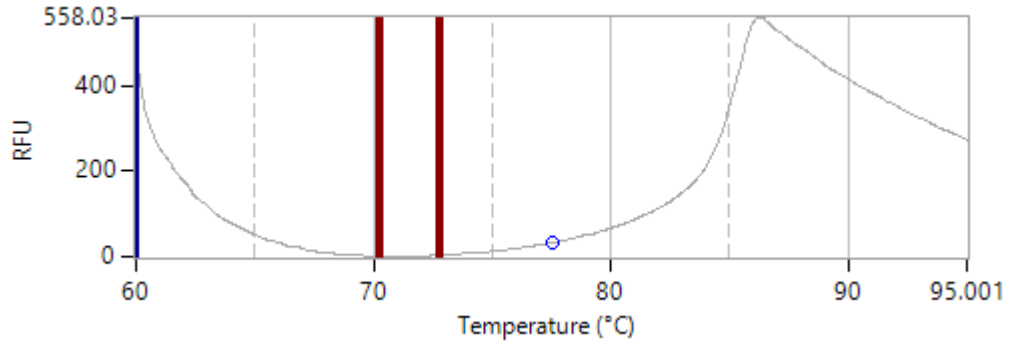

Figure 6-VIII Example melt curve analysis from Real-time PCR for the CTX-M primers in simplex reaction. One clear peak indicates only one product was produced during the reaction. RFU relative fluorescence units.

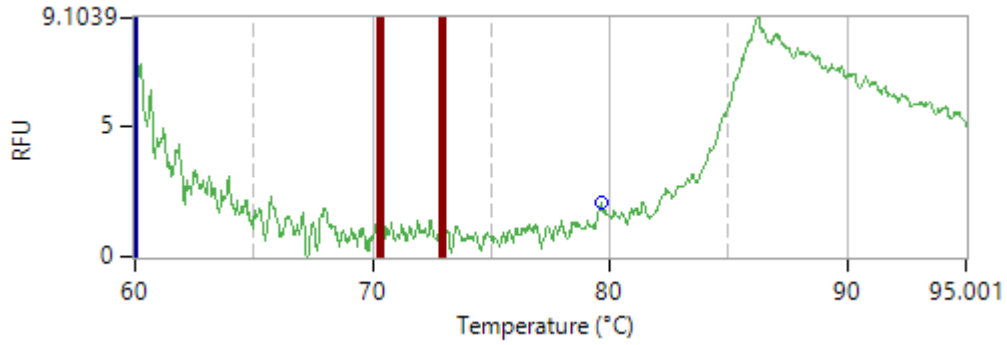

Figure 6-IX Example melt curve analysis from real-time PCR results for the CTX-M primers in multiplex reaction. One clear peak indicates only one product was produced during the reaction. RFU relative fluorescence units.

The high-resolution melt curves gave a temperature to use for the simultaneous detection method. Initially, 2 °C increments after the annealing stage was used. An increase in temperature increments between 84°C and 86°C was implemented to aid resolution between the amplicon melting points of CTX-M and TEM as they were quite similar. It was found that simultaneous detection before cycle 16 was unnecessary, and so it was removed. In the initial stages, DNA extracted by methods explained in 6.3.3 was used, and then DNA extracted from colonies, outlined in 6.3.1 were used instead. Results were compared and no significant difference was seen. This meant that DNA extracted from colonies used in 3.1.1, could be used in this experiment, rather than extracting purified DNA from all the patient isolates.

The results for the development of the qualitative real-time PCR were ran on an agarose gel, which can be seen below.

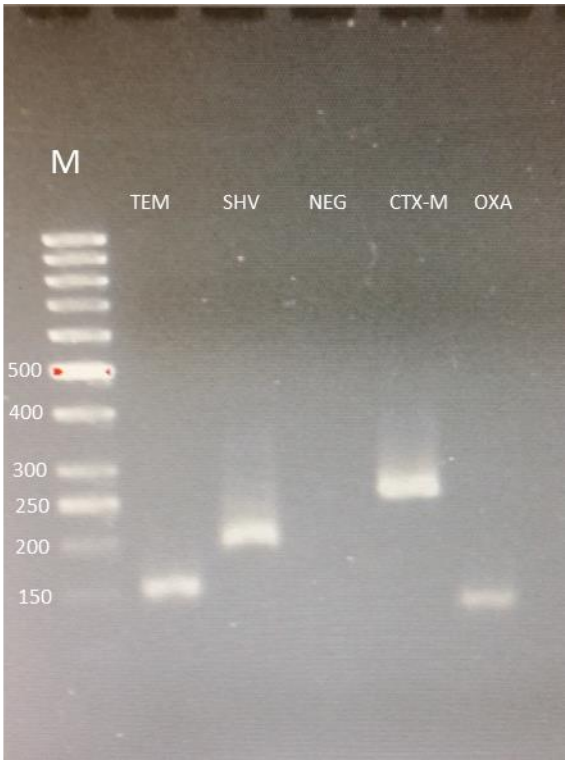

Figure 6-X Agarose gel results for the multiplex qualitative real-time PCR assay. All genes were detected at the expected bp size. 1 band was observed for each primer pair indicating primer specificity. M indicates the DNA ladder used, GeneRuler 50 bp DNA Ladder (Thermofisher, UK). Products and ladder ran on 2% Agarose gel.

Results for patient isolates were compared to results achieved previously by phenotypic testing and multiplex end-point PCR.

6.7 Development of RT-qPCR assay

6.7.1 Minimum Inhibitory Concentration for RT-qPCR

In order to determine the concentration of cranberry to use in the RT-qPCR experiment, the minimum inhibitory concentration was determined. The macrodilution method was used in this study. Five tablets were dissolved in 20ml minimal medium (M9 Minimal Salts, 5X, Sigma Aldrich, Dorset, UK) to create a 60mg/ml stock solution. Minimal medium was chosen as it contains a limited amount of nutrients, aiming to simulate the urinary tract

environment. This was then serially diluted in duplicate to produce the following concentrations: 30, 15, 7.5, 3.75, 1.87, 0.93, 0.46, 0.23, 0.11, 0.05, 0.03 mg/ml.
Two single colonies were added to 10 ml minimal medium and 100 µl was added to each dilution. A positive control consisted of minimal medium and *E. coli*. A negative control consisted of minimal medium only. All dilutions were incubated overnight at 37°C with shaking. Each dilution was then plated out on MacConkey agar and grown for 24 hours at 37°C. Growth was compared between dilutions and positive control.

6.7.2 Choice of RT-qPCR Method

The Cells-to-CT™ 1-Step TaqMan™ Kit was chosen for the RT-qPCR method. This kit performs expression analysis from cultured cells, without extensive RNA purification. The mastermix contains a reverse transcriptase, allowing for a 1-step RT-qPCR. Laborious cDNA conversion is eliminated, as RNA is the input material into the qPCR reaction. The Cells-to-CT™ mastermix contains reverse transcriptase, allowing cDNA conversion during qPCR. Reducing the number of steps in a protocol, reduces the chances of contamination or handling errors. The whole process can be carried out in a single tube, which also reduces chance of contamination. Applied Biosystems have compared their product against purified RNA and found that the performance was equivalent.

6.7.3 Primer Specificity

RT-qPCR primers were checked for specificity by end-point PCR using extracted DNA mentioned in 6.3.3 (figure 6-XI). The presence of primer dimers in addition to the band of interest on gels was accepted, as they do not produce results in RT-qPCR, however they were avoided as much as possible as they reduce the efficiency of the reaction. Cycling parameters were as follows: initial denaturation at 95 °C for 20 s, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min.

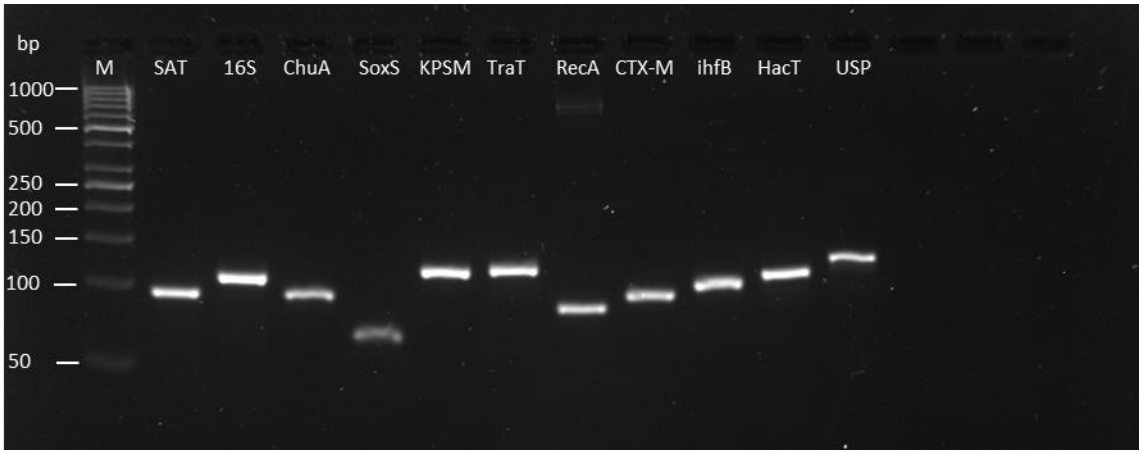

Figure 6-XI Agarose gel results for the primer specificity for the genes investigated by RT-qPCR. DNA was isolated from the CTX-M control isolate obtained from Public Health England. All genes were detected at the expected bp size. 1 band was observed for each primer pair indicating primer specificity. M indicates the DNA ladder used, GeneRuler 50 bp DNA Ladder (Thermofisher, UK). Products and ladder ran on 4% Agarose gel.

6.7.4 No Reverse Transcriptase

A no reverse transcriptase control was used in triplicate by adding Taqman RT-qPCR mix after the reverse transcription step in the PCR cycle, therefore this RNA had not undergone cDNA conversion.

6.7.5 Validation of Reference Genes

Six genes (16S, RecA, idnT, cysG, hcaT and idfB) were chosen for potential suitability as a reference gene by running qPCR with treated and un-treated samples in triplicate. A change in Ct between the untreated and treated samples of less than 0.5 was accepted. Only one gene (16S) was determined as a suitable reference gene. Due to time constraints, no more genes were tested for suitability as reference genes. The five genes that had failed as reference genes, were then treated as genes of interest or discarded.

6.7.6 Primer Efficiencies

Primer efficiencies were calculated using serial dilutions of amplicons (diluted 1:1000) in triplicate and by plotting the resulting data to produce standard curves for each primer set (figure 6-XII). The amplification efficiency = [10(-1/slope)] – 1. A correlation coefficient (R2) of 0.99 or greater was preferred. An amplification efficiency of 80-120% was preferred. The efficiency of figure 6-XII below was 118.9%.

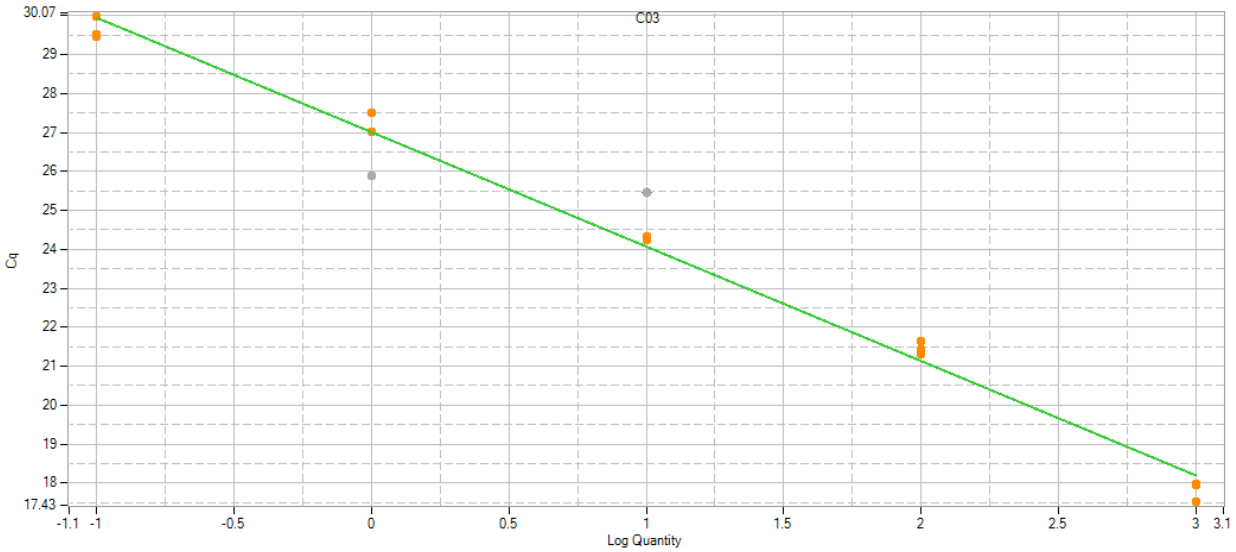

Figure 6-XII Example standard curve for the HcaT gene primers. Primer efficiencies were calculated using serial dilutions of amplicons 1:1000. In this example two anomalies were removed from the calculations.

Table 6-4 The efficiencies of the primers used in the development of the RT-qPCR study Primer efficiencies were calculated using serial dilutions of amplicons 1:1000. An efficiency of 80-120% was accepted..

Primer	Efficiency %
16S (reference gene)	84.3
RecA	111.1
CTX-M	99.7
SAT	111.6
TraT	94.3
ChuA	115.6
KPSM	101.7
SoxS	103.0
USP	90.2
HcaT	118.9
IhfB	115.3

6.8 Choice of Statistical Methods

6.8.1 Prevalence Study

It was determined that my data was non-parametric, and I was looking to test for frequency distribution of my ESBL types, therefore the Chi squared test was chosen. The Spearman Rank Correlation test was chosen for its ability to investigate relationship between the ESBL types and plasmids.

6.8.2 Qualitative Real-Time PCR assay

Calculating the sensitivity, specificity and likelihood ratios for a new diagnostic method is common practice in the field. A number of journals request that this has been completed before submitting a paper suggesting a new diagnostic method.

6.8.3 Statistical Analysis of RT-qPCR

6.8.3.1 Modified delta delta Ct method

The ΔΔCt equation was used with a modification to account for primer efficiencies. The equation can be found below.

6.8.3.2 Abbreviations

Average Ct = AvCt

Average Ct taking into account efficiency = AvCtE

Change in Ct = ΔCt

6.8.3.3 Calculations

$$AvCt = \frac{raw\ data\ 1 + raw\ data\ 2 + raw\ data\ 3}{3}$$

$$AvCtE = average\ Ct * \left(\log\frac{(1 + \frac{efficiency}{100})}{(\log(2))}\right)$$

$$\Delta Ct\ experimental = AvCtE\ (experimental) for\ gene\ being\ tested -$$

$$AvCtE\ (control) for\ gene\ being\ tested$$

$$\Delta Ct\ control = AvCtE\ (experimental) for\ reference\ gene - AvCtE\ (control) for\ reference\ gene$$

$$\Delta\Delta Ct = \Delta Ct\ experimental - \Delta Ct\ control$$

$$Expression\ fold\ change = 2\text{^}\Delta\Delta Ct$$

6.8.3.4 T-test

Data was determined to be parametric, and independent. Therefore, the independent t-test was applied.