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Assessment of antibiotic resistant *Escherichia coli* in meat production systems

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Abbreviations

AFBI	Agri-Food and Business Institute
AMR	Antimicrobial Resistance
AREC	Antibiotic resistant *Escherichia coli*
Bla	beta-lactamase encoding
BPW	Buffered Peptone water
3GC	Third Generation Cephalosporins
CLED	Cysteine Lactose Electrolyte Deficient Agar
CTX	Cefotaxime
CP	Ciprofloxacin
CHCA	Cyano-4-Hydroxycinnamic Acid
EC Broth	*Escherichia coli* broth
E. coli	*Escherichia coli*
ECOFF	Epidemiological Cut Off
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ESBL	Extended Spectrum Beta Lactamase
FSAI	Food Safety Authority of Ireland
IS	Insertion Sequences
IOI	Island of Ireland
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
MDR	Multi-Drug Resistant
MHA	Mueller- Hintin Agar
MP	Meropenem
NI	Northern Ireland

PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
ROI	Republic of Ireland
STEC	Shiga Toxin-producing *Escherichia coli*
TBX	Tryptone Bile X-Glucuronide
TN	Composite Transposons
TSA	Tryptone Soy Agar
TSB	Tryptone Soya Broth
VTEC	Verocytotoxigenic *Escherichia coli*
WGS	Whole Genome Sequencing
mCCDA	modified Charcoal Cefoperazone Deoxycholate Agar
MLST	Multilocus sequence typing

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1 Executive summary

Antimicrobial Resistance (AMR) is considered one of the major public health challenges of this century. AMR organisms are detected with increasing frequency in humans, animals and the environment. It is accepted that this process of selection for antimicrobial resistance in human and animal domains is driven by antimicrobial use in the relevant domain. There is clear evidence that classical foodborne zoonotic pathogens, such as *Salmonella* spp. and *Campylobacter* spp., which have acquired antimicrobial resistance, transfer to humans and cause illness. The situation is much more uncertain with regard to the potential relationship between antimicrobial resistant *Escherichia coli* (*E. coli*) in food animals and food and its transfer to the human population. The possible relationships are complex because in addition to the potential for transfer of antimicrobial resistant *E. coli* between humans and animals there is also the potential for transfer of antibiotic resistant genes between human and animal *E. coli* and between *E. coli* and other *Enterobacteriaceae*. These possible relationships are important because *E. coli,* a normal element of the flora of the gastrointestinal tract of animals and humans, is also the leading cause of some of the most common (urinary tract) and some of the most serious (blood stream) human infections.

Of particular concern is, the trend of increasing resistance to fluoroquinolones (e.g. ciprofloxacin) and third generation cephalosporins (e.g. cefotaxime) in *E. coli*, associated with invasive disease in humans. The problem of resistance to third generation cephalosporins in *E. coli* is closely related to the spread of a group of genes that code for enzymes known as Extended Spectrum Beta-Lactamases (ESBLs). Studies in some countries have suggested that there is considerable similarity between ESBL producing *E. coli* in food and that associated with disease in humans in that country, however, this issue has not been the subject of any major study on the IOI.

Acquired resistance to antimicrobial agents in *E. coli*, especially to fluoroquinolones and cephalosporins, is driving increased reliance on **carbapenem antimicrobials** (e.g. meropenem). These latter agents are widely regarded as critical reserve agents for treatment of human infection with antimicrobial resistant *E. coli* and other *Enterobacteriaceae*.

This study aimed to determine the prevalence of *E. coli* resistant to these three key classes of antimicrobials (fluoroquinolones, cephalosporins and carbapenems) in raw meat on retail sale on the IOI. Furthermore, the study aimed to characterise the isolates detected using phenotypic and molecular methods. This data was then compared to some data from previous studies *of E. coli*

associated with human infection to assess, in so far as possible, similarities between meat isolates of Antimicrobial Resistant *E. coli* (AREC) and human associated isolates of AREC.

This study aimed to determine the prevalence of E. coli resistant to these three key classes of antimicrobials (fluoroquinolones, cephalosporins and carbapenems) in raw meat on retail sale on the IOI

Using a statistically derived sampling plan, 600 samples of raw meats were purchased from retail outlets across the IOI, comprising equal numbers of beef, chicken, and pork. These samples were screened to detect the presence of AREC, using nutrient broth supplemented with different antibiotics. The broth was supplemented with one antibiotic from either the fluoroquinolone class of antibiotics (ciprofloxacin), the cephalosporin class of antibiotics (cefotaxime) or one from the carbapenem class of antibiotics (meropenem). This method selected bacteria which exibited resistance to the aforementioned classes of antibiotics.

Sampling was conducted from November 2013 to September 2014 and from 600 valid samples almost 500 presumptive AREC isolates were obtained, and sent to NUIG where 485 were confirmed as *E. coli* using Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) spectroscopy. Fourteen per cent of beef samples yielded AREC, 99 per cent (%) of chicken and 32.5% of pork. All 485 *E. coli* were then characterised in terms of clinical AMR using a panel of 13 compounds. Overall, 143 isolates were confirmed as ESBL *E. coli,* isolated from: beef 1; chicken 130; and pork 12. A total of 326 *E. coli* isolates were obtained which, although not ESBL, were resistant to at least one antimicrobial used. Ciprofloxacin resistance was common, particularly in chicken. Resistance to nalidixic acid was much more common than resistance to ciprofloxacin, but such resistance in almost all cases represents a genetic step towards ciprofloxacin resistance. Resistances to up to 10 antimicrobials were found in some isolates. It is reassuring, and an important baseline, that we have demonstrated that carbapenem resistance was not detected in any *E. coli* isolates from this representative survey.

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ESBL producing *E. coli* were studied to define their specific enzyme type. This was compared with pre-existing data on ESBL producing *E. coli* associated with human infection on the IOI. The current data available indicate that the majority of ESBL producing *E. coli* in retail meat on sale on the IOI are not very similar to ESBL producing *E. coli* that are associated with most human disease.

Overall, we find little evidence that ESBL producing *E. coli* in retail meat on IOI is a major direct contributor to the growing problem of ESBL producing *E. coli* infections in people. This is most likely because any such transfer that does occur is obscured by the burden of transmission between humans. Nevertheless in keeping with the One Health concept, which recognises, recognises that the health of humans is connected to the health of animals and the environment, all antimicrobial resistance is interconnected at some level. Because of the potential for rapid dissemination of antimicrobial resistant faecal flora once established within a species (human or animal) rare inter-species transfer events may be important in seeding new resistance phenomena within a species. The high frequency of fluoroquinolone and cephalosporin resistance in *E. coli* particularly in poultry meat is therefore a concern and should prompt a review of the extent to which these agents are used and the pathways that facilitate transmission of such organisms in meat production.

The high frequency of fluoroquinolone and cephalosporin resistance in E. coli particularly in poultry meat is therefore a concern

Key project recommendations

Recommendation 1: Measures to control fluoroquinolone and cephalosporin resistant *E. coli* related disease in the human population on IOI at present should focus on antimicrobial use and also human-human transmission of antimicrobial resistant *E. coli* rather than on control of fluoroquinolone and cephalosporin resistant *E. coli* in raw meat.

Although actions that focus on control of fluoroquinolone and cephalosporin resistance in raw meat in isolation are unlikely to have a major impact on the overall frequency with which such organisms cause infection in the human population these measures may be important in reducing the potential for seeding the human population with new variants of resistant bacteria or new resistance genes.

Recommendation 2: The causes of the high prevalence of antimicrobial resistant *E. coli* in chickens and pigs merit further study.

Recommendation 3: As it is likely that the prevalence of AREC in retail meat reflects a high prevalence of such resistance in the animals from which the meat is derived the implications of such resistance for animal health and veterinary practice merit further study.

2 Introduction & background to the project

AMR is considered one of the major public health challenges of this century. Antimicrobial resistant organisms are detected with increasing frequency in humans, animals and the environment. It is accepted that this process of selection for AMR in human and animal domains is driven by antimicrobial use in the relevant domain. There is clear evidence that classical foodborne zoonotic pathogens, such as *Salmonella* spp. and *Campylobacter* spp., which have acquired antimicrobial resistance, transfer to humans and cause illness. The situation is much more uncertain with regard to the potential relationship between antimicrobial resistant *E. coli* in food animals and food, and antimicrobial resistance in *E. coli* and other *Enterobacteriaceae* in the human population. The potential relationships are complex because in addition to the potential for transfer of antimicrobial resistant *E. coli* between humans and animals there is the potential for transfer of antibiotic resistant genes between human and animal *E. coli* and between *E. coli* and other *Enterobacteriaceae*. These potential relationships are important because whilst *E. coli* is a normal element of the flora of the gastrointestinal tract of animals and humans it is also the leading cause of some of the most common (urinary tract) and some of the most serious (blood stream) human infections. Of particular concern is the trend to increasing resistance to fluoroquinolones (e.g. ciprofloxacin) and third generation cephalosporins (e.g. cefotaxime) in *E. coli* associated with invasive disease in humans.

Resistance to third generation cephalosporin antibiotics in *E. coli* is closely related to the spread of a group of genes that code for enzymes, known as Extended Spectrum Beta-Lactamases (ESBLs). ESBLs are a group of evolving Beta-lactamase enzymes which confer resistance to a range of antibiotics such as penicillins and cephalosporins. The enzymes hydrolyze the antibiotics and render them ineffective. It must be noted that ESBL's have no effect on carbapenem antibiotics

Studies in some countries have suggested that there is considerable similarity between ESBL producing *E. coli* in food and that associated with disease in humans in that country however this issue has not been the subject of any major study on IOI. Acquired resistance to antimicrobial agents in *E. coli*, in particular acquired resistance to fluoroquinolones and cephalosporins is driving increased reliance on carbapenem antimicrobials (e.g. meropenem). However, these agents are widely regarded as critical reserve agents for treatment of human infection with AREC and other *Enterobacteriaceae*.

This forms the background to this study which aims to assess the prevalence of *E. coli* resistant to these three key classes of antimicrobials (fluoroquinolones, cephalosporins and carbapenems) in raw meat on retail sale on the IOI , and to subsequently assess the relationship between any such isolates detected in meat and *E. coli* detected in humans with infection.

E. coli are a very diverse group of bacteria. The species is comprised of several phylogroups, some of which are associated primarily with humans and human infection, and others that are associated primarily with animals. Each phylogenetic group also encompasses significant diversity. It has become apparent in recent decades that there simialr groups within phylogroups known as clonally related groups. An important example of this phenomenon is the clonal complex ST131 (sequence type 131) which is a subset of phylogroup B2. The ST131 clonal group has been very successful in global dissemination and now accounts for a very high proportion of all cephalosporin resistant ESBL *E. coli* associated with human colonisation and infection on IOI. *E. coli* can be classified into major phylogroups and ST131 can be differentiated from other B2 clonal groups by validated PCR.

The potential for a given *E. coli* isolate to be associated with disease is related to the expression of genes that enhance the ability to adhere to and invade tissues and to elaborate toxins. Therefore, this project aimed to assess the pathogenic potential of AREC, particularly those producing ESBL's, in foods derived from animal production systems and to define the potential risks posed by these organisms to the consumer on the IoI.

Likewise, AMR in *E. coli* is dependent on the expression of specific genes that encode for resistance to one or more antimicrobial agents. Amongst the most common and important resistance genes are those which code for beta-lactamase enzymes that destroy penicillins and/or cephalosporins. Of particular interest for this project are the ESBLs that inactivate third generation cephalosporins such as cefotaxime. There are a several known ESBL variants but a small number of families account for the majority of ESBL associated infection in humans. ESBL encoding genes transfer between different strains of *E. coli* and between *E. coli* and other enteric bacteria. It was therefore possible that *E. coli* from raw meat and from human infection could differ in terms of their phylogroup or sequence type group, but they could share common ESBL encoding genes. To address this possibility, the ESBL genes in the *E. coli* from raw meat were characterised to compare with existing data on predominant genes in strains associated with human infection.

The project commenced with a structured literature search of the subject area and a report on the findings was submitted to ***safe***food with the information obtained used to inform the subsequent isolation and characterisation studies. Based on population distribution, sampling of fresh raw meats from retail outlets on the IOI was undertaken over a 12 month period. Samples were shipped to the Food Science Branch at AFBI in Belfast, and analysed within 24 hours of arrival at AFBI.

Samples were enriched in three selective broths designed to select for organisms resistant to three different clinically significant types of antibiotics. Whilst the aims of the project were centred on ESBL producing organisms, it was considered that the relatively small costs of extending the study to include media selective for organisms carrying fluoroquinolone resistance, or carbapenemases would add significantly to the value of the work. The isolates able to grow in the antibiotic supplemented broth were regarded as being resistant and were then streaked onto a diagnostic medium to allow selection of *E. coli*.

Presumptive AREC were screened genotypically, by Polymerase Chain Reaction (PCR), to detect genes indicative of pathogenicity before being despatched to NUIG where isolates were confirmed as *E. coli* by MALDI-TOF spectroscopy. Confirmed *E. coli* were tested for susceptibility to a selected panel of antibiotics. Using BioNumerics software a preliminary similarity matrix was calculated based on the data collected. Subsequently AREC isolates obtained during the survey were evaluated, and with the agreement of ***safe*food,** a subset was selected and studied in detail to include:

- Determination of membership of the ST131 clonal group that predominates in humans (PCR based method)
- Characterization of the common beta-lactamase encoding (*bla*) genes associated with ESBL producing organisms – differentiation between CTX-M, SHV and TEM, subdivision within CTX-M (PCR based with follow on sequencing if required)
- Pulsed Field Gel Electrophoresis (PFGE) (Pulse Net XbaI) of *E. coli* - compare patterns with each other and with patterns from human isolates;
- Whole genome sequencing (WGS) and bioinformatic analysis was performed on selected isolates to provide the most definitive characterisation of isolates

These studies constituted a thorough definition of the phenotypic and genotypic properties of the isolates, allowing the similarities of the isolates to be statistically defined. This provided a basis for comparing the similarity of isolates with each other and with previously characterised human isolates and for evaluation of the resistance gene profile of ESBL-producing bacteria in the food chain for consumers on the IOI. This will support studies of the origins and dispersal mechanisms of the AREC, and consideration of approaches to limit exposure by consumers to ESBL-producing bacteria.

3 Project aims and objectives

The main project objectives and tasks were:

1. To undertake structured literature review.

2. To carry out a survey of beef (n=200), pork (n=200) and chicken (n=200) samples at retail sale on the IOI for the presence of antibiotic resistant *Escherichia coli* (AREC). Isolates to be selected on the basis of resistance to one of three types of antibiotic: third generation cephalosporins, fluoroquinolones and carbapenems. Presumptive *E. coli* to be selected following growth on a diagnostic medium, Tryptone Bile X-Glucuronide (TBX) medium, to give presumptive AREC isolates.

3. Presumptive AREC isolates to be initially phenotyped by standard biochemical tests to confirm that they are *E. coli*, and then further defined by determining their susceptibility to a panel of 13 antibiotics with methods as per The European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards. The antibiotics to be tested as follows: ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracycline, trimethoprim, nalidixic acid, kanamycin, ciprofloxacin, ceftazidime, gentamycin and cefotaxime. AREC resistant to ceftazidime and/or cefotaxime to be further studied to determine ESBL status.

4. All AREC to be genotyped using appropriate PCR protocols to examine phylogenetic diversity, to and to detect the presence of virulence factors and antimicrobial resistance determinants.

5. All available data to be analysed by the AFBI Biometrics Division to identify any associations between food production systems, the presence of antibiotic resistant organisms and their pathogenic potential. Cluster analysis of all isolates based on their phenotyping and genotyping to be undertaken using BioNumerics software. Based on these analyses a sub-set of AREC isolates to be subjected to in-depth genotyping to define, in greater detail, the risk to consumers on the IOI that they present.

6. The project team to review all results and their statistical analyses, to determine the risk to consumers presented by AREC in the food chain.

7. The sources of the risks identified by project team, in the context of animal and food production, to be investigated by consulting relevant literature and experts.

8. A final report to be produced and submitted to *safe*food.

4 Literature review

Method

The literature review relating to the presence of AREC was undertaken using a variety of academic and other bibliographical sources (e.g. the Thompson ISI databases, PubMed Net and Dialog Select) to identify relevant information from experimental, survey and other work, worldwide, relating to AREC in meat production systems. The project team used their network of established international collaborators to identify publications that are either in press or in the grey literature.

To ensure objectivity and assure the quality of the literature review it was undertaken using a systematic approach with a scored assessment of each paper's quality using the Oxford System (Jadad et al. 2000; Jadad and Murray, 2007). The search string chosen, after evaluation, was:

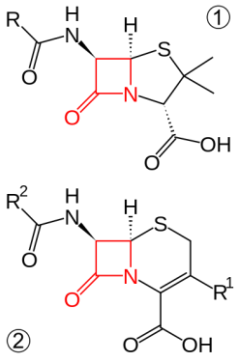
Topic=("escherichia coli") AND Topic=("meat") AND Topic=("antibiotic" OR "antimicrobial") AND Topic=("resistance") AND Topic=("retail").

This yielded 97 documents which were the basis of the draft document, but other publications were added to the pool as the study progressed.

Background

The first antibiotic to be used in medicine was penicillin and the structure of this compound is characterised by the presence of a β-lactam ring, (Figure 1) Subsequently other β-lactam antibiotics were discovered, such as cephalosporin (Figure 1) and modified versions of existing antibiotics synthesised.

Figure 1: Penicillin (1) and cephalosporin (2) with β lactam ring in red



These antibiotics were widely applied in human medicine but eventually bacterial resistance to the compounds occurred . One mechanism conferring bacterial resistance to beta lactam antibiotics was the production of an enzyme capable of hydrolysing or breaking open the ring, rendering the antibiotic ineffective. In 1965 Datta and Kontomichalou reported in Nature that *E. coli* isolated from a patient named Temoneira, in Athens, could hydrolyse penicillin due to production of a beta lactamase. The enzyme was designated TEM-1, from the patients name, and other TEM enzymes were discovered in due course (Bush et al. 1995). The genes coding for beta lactamases were subsequently referred to by the abbreviation *bla* and hence the gene for TEM was denoted bla_{TEM}. The bla_{TEM} genes were found to be carried on transposable elements (Partridge and Hall 2005; Blanc et al. 2006) which meant that the enzymes could be exchanged between members of the *Enterobacteriaceae*.

Bacterial transposable elements are normally one of two types; insertion sequences (IS) and composite transposons (Tn). The latter are normally being simply referred to as transposons and some can code for antibiotic resistance (Blot 1994). Horizontal transfer of antibiotic resistance by transposons in *Enterobacteriaceae* is well documented (Sirot et al. 1991; Bradford 2001; Partridge and Hall 2005; Thong and Modarressi 2011).

Less than ten years after TEM-1 was discovered a different lactamase enzyme was reported, and was later named SHV-1, derived from sulphydryl variable because it was thought that the inhibition of SHV activity by *p*-chloromercuribenzoate was variable according to the substrate used for the assay (Paterson and Bonomo 2005). In the current nomenclature the beta-lactamase SHV gene is denoted bla_{SHV}.A key feature of the first TEM and SHV enzymes to be discovered was that they hydrolysed penicillins such as ampicillin, but not the newer cephalosporin beta lactams such as cefotaxime. Consequently, they can be regarded as having a narrow substrate range. Also they could be inhibited by compounds such as clavulanic acid, which acts as a competitive inhibitor of beta-lactamases

Since antibiotics of clinical significance, i.e. penicllins and cephalosporins, were being rendered ineffective by bacteria carrying enzymes such as TEM-1 and SHV-1, amongst others, research was undertaken to develop antibiotics resistant to beta lactamases. This led to the development of third generation cephalosporins (3GC), which were introduced in the 1980s (Paterson and Bonomo 2005). These were also referred to as extended spectrum cephalosporins. Examples of such antibiotics are cefotaxime, cefpodoxime, ceftriaxone and cephalothin. This class of antibiotics was found to be extremely effective and was therefore widely used from the early 1980s onward (Bryan 1991).

However, after 3GC were introduced, resistance to the drugs began to be noted. In Germany, Knothe et al. (1983) studied 3GC resistant *Klebsiella pneumoniae* and *Serratia marcescens*, isolated from patients in a Frankfurt clinic, and showed that the resistance to cefotaxime in *K. pneumoniae* was plasmid based. Further, laboratory studies showed that the plasmid in *K. pneumoniae* was readily passed to *E. coli*, *Proteus mirabilis* and *Salmonella* Typhimurium suggesting that it could be mobile within the *Enterobacteriaceae* family. The resistance noted in *S. marcescens* was also found to be carried on a plasmid, which could also be transferred to the other *Enterobacteriaceae* studied, but some elements were lost in transfers to *E. coli*. In view of the ability of this lactamase to hydrolyse cefotaxime it was subsequently termed CTX, giving rise to the gene being designated bla_{CTX}. The discovery of CTX raised the prospect of non-pathogenic organisms carrying resistance to 3GC and being able to transfer the plasmid to pathogenic organisms, such as *S.* Typhimurium.

To more accurately describe the beta lactamases conferring resistance to 3GC the term 'extended spectrum beta lactamase' was coined (Philippon et al. 1989); normally abbreviated to ESBL. Initially ESBL were only found in *Enterobacteriaceae* isolated in hospitals or similar environments, but subsequently they have been found to be more widely distributed. This report deals principally with ESBL *E. coli* which is discussed in more detail below.

Antibiotic Resistant Bacteria in the Food Chain

The benefits of antibiotic therapy were not only applied in human medicine, but also in veterinary medicine. In addition, when incorporated into animal feeds, antibiotics were found to act as effective growth promoters, especially with poultry, and such supplemented feeds began to be widely used from the 1950s onwards (Jones and Ricke 2003). This later led to concerns that antibiotic resistance could become widespread in the microflora of animals, and subsequently consumers could be exposed to these organisms via meats (Bensink and Bothmann 1991). One particular concern was the possibility of foodborne pathogens, such as *Salmonella* spp., acquiring antibiotic resistance and hence compromising normal antimicrobial therapy. Should salmonellas acquire ESBL activity, they would be resistant to 3GC which are the drugs of choice for treating salmonellosis in children (Zhao et al. 2009).

The removal of antibiotics from routine use has been seen to directly affect the prevalence of antimicrobial resistant bacteria in the animals concerned. Agersø and Aarestrup (2013) reported that a voluntary ban on cephalosporin use in Danish pig production had effectively reduced extended-spectrum cephalosporinase-producing *E. coli* in slaughter pigs.

The potential problems arising from antibiotic resistant zoonotic infections was addressed by the European Union (EU) over ten years ago, in Zoonosis Directive 2003/99/EC, which requires member states to undertake monitoring of antimicrobial resistance. The document also stated: 'The alarming emergence of resistance to antimicrobial agents (such as antimicrobial medicinal products and antimicrobial feed additives) is a characteristic that should be monitored. Provision should be made for such monitoring to cover not only zoonotic agents but also, in so far as they present a threat to public health, other agents. In particular, the monitoring of indicator organisms might be appropriate. Such organisms constitute a reservoir of resistance genes, which they can transfer to pathogenic bacteria.'

The latter reference to indicator organisms constituting a reservoir of resistance genes, which could then be passed to pathogenic organisms, is significant since traditionally food microbiology sought to isolate and identify specific pathogenic bacteria from foodstuffs. The presence of antimicrobial resistance genes, carried on mobile genetic elements which can be readily exchanged between members of the *Enterobacteriaceae*, means that the pathogenic potential of zoonotic bacteria, such as salmonellas, may be underestimated. Characterisation of a *Salmonella* spp. isolated from a

foodstuff could show that it is antibiotic sensitive, and will therefore respond to normal drug therapy. However, the consequences of the drug therapy could be to increase the pathogenic potential of the *Salmonella*. Exposure of a bacterial population to an antibiotic leads to increased production, and export, of the plasmid carrying the resistance gene with consequent horizontal gene transfer (Svara and Rankin 2011). Hence, should indicator organisms carrying a plasmid with an ESBL gene be present in the same foodstuff, then there is the potential for an ESBL *Salmonella* spp. arising in an infected host (Carattoli 2009), which could be resistant to the normal therapy.

E. coli is used as an indicator organism, since it is ubiquitous in the gastrointestinal tract of humans (Sedgley and Samaranayake 1994) and farm animals (Pointon et al. 2012). This ubiquity means that the use of antibiotic therapy in animals and humans will expose the *E. coli* of the host to the drugs. It is therefore unsurprising that antibiotic resistant *E. coli* have arisen since virtually all cases of antibiotic therapy will lead to the host's gut flora being exposed to the agent, with a consequent evolutionary pressure to develop/acquire drug resistance. Since *E. coli* is also a pathogen (Schroeder et al. 2003) studies into its drug resistance have been undertaken (Bush 2013; Kluytmans et al. 2013) but approaches have differed, leading to a lack of consistent data.

Extended Spectrum Beta-Lactamase (ESBL) *Escherichia coli* (*E. coli*)

Types of ESBL

Classification schemes to categorise the myriad of beta-lactamase enzymes were elaborated some decades ago. These classification schemes are continuously evolving in the light of new discoveries and have been moved to websites accessible online, as have AMR definitions (see http: //www.eucast.org). As early as 1995, 18 types of TEM and 5 types of SHV had been described as ESBL (Bush et al. 1995), but by 2012 more than 1,300 distinct beta lactamase enzymes had been identified (Bush 2013). This proliferation is greatest within the TEM and OXA families (Bush 2013) but the consequence is that many studies of ESBL carrying organisms do not fully characterise the ESBL. This is illustrated in a review of the AMR of emerging foodborne pathogens, where Koluman and Dikici (2013) considered two *Enterobacteriaceae*, *Salmonella* and verotoxin producing *E. coli*, but could not indicate the prevalence of ESBL in these organisms due to limited information in the source material.

Accordingly the general property of carriage of an ESBL will be considered here, and a detailed consideration of the evolution and divergence of these enzymes will be considered beyond the scope of this review.

Isolation of ESBL from foodstuffs

Studies to determine the presence of ESBL in foods, or animals destined for foodstuffs, have taken many different approaches. Some of the methods applied for the isolation of ESBL *E. coli* from foodstuffs, or livestock, are considered here. Some studies investigated *E. coli* isolates which had been obtained as a result of previous surveys or statutory analytical work. This approach was taken in China (Xia et al. 2011) and Germany (Kaesbohrer et al. 2012). Other workers have taken samples of foodstuffs and prepared an initial suspension which was then directly plated onto a selective and diagnostic medium from which presumptive *E. coli* could be obtained (Chaisatit et al. 2012; Lyhs et al. 2012; Schwaiger et al. 2012; Alvarez-Fernandez et al. 2013). The isolates were confirmed as *E. coli* prior to being studied to determine their antibiotic resistance, but it should be noted that these four studies all used different selective media.

Direct plating has the advantage that organisms representative of the total flora will be chosen for detailed study, because the isolates selected should be those most common in the target population. However, should the AREC not be the dominant *E. coli*, then they may not be detected. When initial culture is on media containing the antimicrobial of interest (e.g. cephalosporin) the intention is to maximise detection of such organisms in a sample, in the event that they are present as a minority of the population, by suppression of susceptible organisms which would otherwise mask their presence.

In Canada Cook et al. (2011) used such a two-step enrichment procedure to obtain *E. coli* isolates for subsequent study and, as salmonellas were also being sought, samples were initially incubated in buffered peptone water (BPW). After incubation in the non-selective BPW samples were subcultured into the selective EC (*E. coli*) broth and subsequently streaked onto a diagnostic medium, eosin methylene blue. Hence the final isolates chosen would reflect their ability to grow profusely in the two broths, rather than their population in the original sample. This procedure is also time consuming and requires the use of three media to obtain isolates, which must then be screened for resistance to anti-microbials. Hence it is a cumbersome and expensive method to detect AREC.

As a simpler isolation procedure, several groups seeking to isolate AREC placed their sample directly into a broth selective for *E. coli* or similar organisms, such as MacConkey broth (Zhao et al. 2012) F1000 reference), Gram negative broth (Obeng et al. 2012), or EC broth (Shahada et al. 2013). Again, this allowed *E. coli* numbers to increase markedly, prior to streaking onto a solid diagnostic medium from which *E. coli* could be isolated for study. Only when pure cultures of *E. coli* had been obtained were the isolates screened to determine their antibiotic resistance properties. However, should AREC grow more slowly than other *E. coli* in the sample, or comprise a small proportion of the total *E. coli* population in the sample, it is possible that they might not be detected such procedures.

Since the significant property of AREC is their resistance to antimicrobials then this property can be exploited in the isolation procedures. In a study to detect ESBL in chicken meat, (Leverstein-van Hall et al. 2011) samples were incubated overnight in BPW, then streaked onto a commercial diagnostic ESBL medium. Mesa et al. (2006) also incubated samples in BPW then streaked directly onto a solid selective medium, in this case MacConkey agar supplemented with 2mg/l cefotaxime, to isolate ESBL *E. coli*. Egea et al. (2011) used a similar BPW recovery incubation, the solid selective medium was MacConkey agar supplemented with 1mg/l cefotaxime or ceftazidime. Overdevest et al. (2011) applied a two step enrichment when detecting ESBL *E. coli*; samples were incubated overnight in non-selective broth (in this case tryptone soy broth, TSB), then a subculture was placed in TSB supplemented with 8mg/l vancomycin and 0.25mg/l cefotaxime before being streaked onto a chromogenic ESBL agar. Thus a repair stage was followed by enrichment of presumptive ESBL bacteria which were then selected from the diagnostic medium.

Agersø et al. (2012) combined a selective enrichment with a selective diagnostic medium to obtain ESBL from meat. Presumptive ESBL-producing *E. coli* were isolated by adding the meat to MacConkey broth supplemented with 1 mg/L ceftriaxone. After incubation an aliquot was streaked on MacConkey agar supplemented with 1 mg/L ceftriaxone, incubated overnight at 44°C, and a maximum of three colonies were subcultured.

Costa et al. (2010) adopted a simpler approach by only using one medium onto which homogenised samples were plated directly. Levine agar, which is diagnostic for *E. coli*, was used and supplemented with 2mg/l cefotaxime. Growth in the presence of the antibiotic indicated presumptive-ESBL organisms were present, hence presumptive ESBL *E. coli* could be detected.

Overall, no single methodology has emerged as the most appropriate procedure for isolating ESBL from foodstuffs. Several of the studies noted above were designed to investigate the total flora of *E. coli*, and characterise representative examples of the population. Thus, if ESBL comprised a minority of the population they would not be detected. However, as for any study, the method chosen must be fit for the specific purpose of the study to be undertaken. Since cost will always be a significant factor in research, a three step process involving repair in a non-selective broth, selective enrichment, then detection on a diagnostic medium will be at a disadvantage.

However, the standard procedures for isolating *Campylobacter* spp. from foodstuffs (ISO 10272-1:2006) are based on a two-step procedure: the sample is incubated in a selective enrichment broth, i.e. Bolton broth, then streaked onto modified charcoal cefoperazone deoxycholate agar (mCCDA). It should be noted that Bolton broth contains 5mg/l cefoperazone, a third generation cephalosporin to which ESBL organisms are resistant and mCCDA contains 32mg/l cefoperazone. ESBL *E. coli* have been reported as proliferating during these isolation procedures (Jasson et al. 2009; Moran et al. 2009)

despite the fact that both the broth and plates are incubated under low oxygen, high CO_2 conditions at 41°C. This suggests that a simplified selection procedure for AREC could be based on the use of an enrichment broth containing an appropriate antibiotic, and an appropriate selective/diagnostic solid medium.

Confirmation of ESBL from foodstuffs

Whilst study of the isolation procedures used to obtain ESBL *E. coli* show that a wide range of methodologies have been applied, the characterisation of the antimicrobial resistance of these organisms is more consistent. This is due to the fact that standard procedures for determining the antibiotic resistance of bacteria have been developed for use in medicine. The Clinical and Laboratory Standards Institute (CLSI) has promulgated standardised methods to determine AMR with interpretation in European countries normally being based on the breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org). Workers in the US and other countries have used CLSI guidelines.

The use of CLSI defined methodologies to detect AMR is seen to be relatively common Table 1 Disc diffusion was applied in 38% of cases whilst automated broth dilution systems were used in 25% of cases, i.e. Agersø et al. (2012), (Cook et al. 2011), Xia et al. (2011) and Zhao et al. (2012). Since disc diffusion analysis can be undertaken with minimal equipment, and hence capital costs, its more frequent use is not unexpected. Specific discs which combine amoxicillin with clavulanate were commonly applied to allow the confirmation of ESBL. Clavulanate inhibits ESBL and therefore increased sensitivity to amoxicillin should then be observed, in comparison to exposure to amoxicillin alone, if ESBL is present. This effect can also be observed using other methodologies noted for the AMR test (**Error! Reference source not found.**) Note that the AMR tests, and ESBL confirmation methods, listed in **Error! Reference source not found.** are indicative of the work undertaken and not exhaustive.

Error! Reference source not found.. **Methods used to define the AMR of *Escherichia coli* isolates obtained from foodstuffs. Where Clinical and Laboratory Standards Institute (CLSI) methodology was specifically mentioned in the article this is denoted by +. The E-test is a commercial product to determine AMR, sold by bioMérieux.**

Reference	AMR test	CLSI	ESBL confirmation
Agersø et al. 2012	Broth	+	Am/clav[4], discs, PCR, microarray, sequencing
Alvarez-Fernandez et al. 2013	Disc[1]	+	Am/clav
Chaisatit et al. 2012	Disc	+	Am/clav
Cook et al. 2011	Broth[2]		Am/clav
Costa et al. 2010	Disc	+	Am/clav
Egea et al. 2011			PCR, sequencing
Kaesbohrer et al. 2012	Broth	+	Implicit in AMR results
Leverstein-van Hall et al. 2011	Previous studies		Microarray
Lyhs et al. 2012	Disc		Am/clav
Mesa et al. 2006	Disc	+	E-test
Obeng et al. 2012	Agar[3]	+	E-test
Overdevest et al. 2011	Chromogenic agar		E-test, dHPLC[5], microarray, sequencing
Schwaiger et al. 2012	Broth[2]		Am/clav
Shahada et al. 2013	Disc	+	PCR
Xia et al. 2011	Broth	+	Am/clav
Zhao et al. 2012	Broth		Am/clav

[1]Disc diffusion, [2]Broth dilution, [3]Agar dilution. [4]Amoxicillin+clavulanate, [5]denaturing high performance liquid chromatography.

When *E. coli* are confirmed as producing ESBL then further studies to define the specific type of β lactamase may be undertaken. However, this cannot be done by phenotyping due to the very similar properties of some ESBL, and DNA-based studies are required

Zoonotic transfer of ESBL *E. coli*

Tracing the appearance of foodborne ESBL *E. coli*

The evolution of an organism and its ability to proliferate or persist in certain environments can normally be discovered by reviewing the literature relevant to the specific organism. For example Franz and van Bruggen (2008) reviewed the ecology of *E. coli* O157:H7 and *Salmonella enterica* in the primary vegetable production chain. In principle, this would require a literature search to find publications in which the target organisms were associated with the vegetable production chain. However, *E. coli* O157:H7 is a specific sub-group with a species whilst most of the pathogenic salmonellas considered were also subspecies: *Salmonella enterica* subsp. *enterica*. In marked contrast to obtaining studies of specific bacterial groups in a given environment, the study of ESBL is a study of the property of specific antibiotic resistance due to the ability of bacteria to produce enzymes. The genes for these enzymes may be on plasmids (Shahada et al. 2013) or transposable elements (Partridge and Hall 2005). As noted above, studies can vary widely in terms of the information reported on ESBL, which may be produced by a wide range of different species, and genera. For example Taguchi et al. (2012) simply reported the prevalence of ESBL and AmpC phenotypes of *Salmonella enterica*, collected from retail chicken over a five year period.

Further, as new ESBL enzymes have been recognised and classified the nomenclature has been changed (Bush et al. 1995), and therefore synonyms exist for some enzymes which complicates the search for specific ESBLs. Overall, tracing the history of foodborne ESBL has to take cognisance of the restrictions under which studies were undertaken, which frequently led to relatively limited information on the type of ESBL being published.

Foodborne Infections

As noted above, the confirmation of zoonotic transfer requires that both the *E. coli* genotype, and the specific ESBL enzyme(s) must be confirmed as occurring both in animals and people. This imposes a high burden of proof , requiring more detailed research (Young et al. 2009) and few examples exist in the literature. However, such infections have been suggested as possible by several studies (Jakobsen et al. 2010; Jakobsen et al. 2011; Lyhs et al. 2012; Obeng et al. 2012). It should also be noted that carriage of such antimicrobial resistance will compromise treatment of the animals themselves (Olsen et al. 2014), and the elimination of ESBL in would benefit animals (in this case poultry) as well as reducing the possibility of zoonotic infection.

Whilst data from previous studies indicates similarities between ESBL in animals and man, the transmission routes have yet to be defined (Sharp et al. 2014). However, the subject is of great significance with the UK Government producing a report in 2015 entitled 'Antimicrobials in agriculture and the environment: reducing unnecessary use and waste' (Available at http://amr-review.org) indicating that the potential for zoonotic infections must be addressed. Amongst other conclusions the report noted that 'countries need to come together and agree to restrict, or even ban, the use of antibiotics in animals that are important for humans'. It is therefore implicit that zoonotic transfers are considered probable.

In the same year the Food Safety Authority of Ireland (FSAI) produced a report: 'Potential for Transmission of Antimicrobial Resistance in the Food Chain' (available at https://www.fsai.ie/publications_AMR) which concluded that ' although the relative importance of the food chain is uncertain, use of antimicrobials in food production and the potential for spread of bacteria from host-to-host through the food chain are areas of concern'.

Hence the potential for foodborne infections is accepted as a threat, but the complex nature of the routes of transmission, and the manner in which both the bacteria, and the resistance elements, require characterisation at the molecular level to allow identification has limited progress in this field. However, the rapid strides made in DNA sequencing technology mean that whole genome sequencing can provide definitive information on bacteria, which could be used to track resistant strains (Koser et al. 2014). Relating sequence data to phylogenetic properties of pathogens has been described (Gordon et al. 2014; Tyson et al. 2015; Zhang et al. 2015) Thus the tools to define the types of zoonotic transfer which can occur, and therefore allow the risks to be assessed, are available and it remains for appropriate studies to be undertaken to allow the risks posed by antimicrobial resistant *E. coli* in foodstuffs to be defined.

In preparing this review 169 articles were obtained using the search procedure noted above. These articles were searched to find the term 'antimicrobial' in the abstract and this was then combined with other terms to give the numbers of references noted in Table 1 below:

Table 1: number of published articles combining the words 'antimicrobial' and meat related terms found during literature search.

Term	Number of articles
Meat	62
Chicken	41
Beef	35
Pork	32
Lamb	3

Milk was only cited in two articles, as a source of antimicrobial resistant bacteria. Thus the majority of antimicrobial resistant bacteria are associated with the meats chicken, beef and pork, and these were the subject of study in the study of retail meats on retail sale on the IOI, in the project of which this report is a part. However, salads have yielded AREC (Egea et al. 2011).

5 Survey of retail meats to obtain presumptive antimicrobial resistant *E. coli* (AREC)

Method

All microbiological media were Oxoid (Basingstoke, UK) unless otherwise stated.

The distribution of premises to be sampled in the ROI was based on population distribution using the four EU election constituencies as sampling areas. Equal numbers of samples were taken within each of Connaught-Ulster, Munster, Leinster and Dublin. Within Northern Ireland (NI) the sample distribution was based on the distribution of population, and mainly consisted of larger outlets. A list of postcodes of retailers was drawn up to ensure sampling was appropriately distributed.

Trained sampling officers were used to collect samples and no more than four samples of each raw meat type were permitted to be collected in each retail premises. Standard sampling forms were completed by the sampling officers, and sample diversity was ensured by sampling officers consulting sample processor codes and 'use by' dates. The temperatures of all samples was measured using infra-red thermometers (Fluke Foodpro infra-red thermometer, Fluke UK Ltd, Norwich, UK). Samples were then packed in insulated containers (Biotherm 45, DGP Intelsius Ltd, Elvington, UK) with refrigerant materials, as per the protocols developed for previous research projects. A Tinytag Transit 2 temperature logger was included in each box of samples (Gemini data Loggers (UK) Ltd, Chichester, UK). Samples from south-west Ireland were sent by overnight courier (TNT) to Belfast and analysis of these samples commenced within 36 hours of being purchased, for all other samples analysis began within 24h. Prior to analysis, the temperature logger data was examined and samples rejected if the temperature fell below 0°C, or exceeded 8°C for a significant period (exceeding 2 hours). All samples, including labels, were photographed for verification purposes.

From each sample, three aliquots of 10g were excised and added to 100ml of tryptone soya broth (TSB) supplemented with:

Cefotaxime (CTX): 0.5mg/l

Ciprofloxacin (CP): 0.064 mg/l

Meropenem (MP): 0.25mg/l

The concentration chosen was based on being twice the epidemiological cut off values (ECOFF) data from the EUCAST website. It was then verified using a trial evaluating growth of resistant and susceptible organisms using impediometry. The broths were incubated overnight at 37˚C, then streaked onto tryptone bile x-glucuronide medium (TBX) and again incubated overnight at 37˚C. Appropriate negative and positive controls were used. From each sample one typical *E. coli* colony were selected and streaked to purity on tryptone soy agar (TSA) and again incubated overnight at 37˚C. Purified cultures were checked for the ability to produce indole at 44˚C, and the ability to ferment lactose. Presumptive *E. coli* were harvested into 1ml of nutrient broth with 10% glycerol and stored at -80˚C. In addition, they were harvested into set buffer prior to having DNA extracted using a phenol/chloroform procedure. The extracted DNA was used to screen isolates for the presence of potential pathogenicity factors using the PCR methodology of Paton and Paton (1998).

Results

The survey aimed to obtain 200 retail samples each of three types of raw meat: beef, chicken and pork. Sampling was conducted from November 2013 to September 2014 and 610 samples were purchased, with 600 being valid samples. Most unacceptable samples were from supermarkets and were basted, hence fell outside the sample requirement of being only raw meat.The 600 samples yielded 499 presumptive antibiotic resistant *E. coli* (AREC), each isolate apparently resistant to one of the three antibiotics of interest, Table 2

Table 2: Total number of presumptive *Escherichia coli* isolates obtained from retail meats (n=200 per meat) sold on the IOI, after enrichment in antibiotic supplemented broths

	Number of isolates from meats			
Antibiotic	Chicken	Pork	Beef	Total
Cefotaxime	182	15	9	206
Ciprofloxacin	191	61	21	273
Meropenem	15	3	2	20
Total	388	79	32	499

One isolate, from beef, was seen to have pathogenic properties, possessing shiga toxin type 2 and was subsequently identified as *E. coli* O157 by NUIG staff. No other isolates appeared to carry the virulence genes assessed for by the PCR method of Paton and Paton (1998).

6 Species identification & determination of antimicrobial resistance (AMR)

Background

Definitive identification of species was undertaken using MALDI-TOF mass spectrometry. MALDI-TOF is designed for the rapid and accurate identification of microorganisms with the equipment used, the MALDI-TOF Biotyper (Brucker), identifying microorganisms by analyzing the expression of their intrinsic proteins using mass spectrometry. The method and instrument have been validated for species identification in accordance with the requirements of the ISO15189 standards. An individual colony from a culture plate or 1 µl aliquot of extract is applied to the target plate. A low-mass organic compound, called a matrix is added to the sample and allowed to dry. This matrix is essential for the successful ionization of the clinical sample as it supplies protons for the ionization of the clinical material. The sample matrix is irradiated for a short period of time using a UV laser beam. The matrix is evaporated releasing positively charged proteins in a so called 'soft' ionization process. This soft ionization process allows the analysis of large bio-molecules, including ribosomal proteins. Once ionized, proteins within the clinical specimen are measured in the context of a spectrum-of-mass-to-charge ratio *m/z* (a measurement of how quickly charged ions from the clinical sample move through the time of flight tube and reach the detector). The MALDI Biotyper generates a characteristic species-specific mass and intensity spectrum of the mainly ribosomal proteins ('protein fingerprint'). This spectrum is compared to a database of defined reference spectra leading to the identification of the microorganism.

Species identification

1. The test organism was cultured overnight on nutrient agar at 370C +/- 20C.
2. A small amount of a single colony was applied directly on a MALDI target plate in a thin film ("smear preparation").
3. The thin microbial film is overlaid with 1 μl of a saturated alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (50% acetonitrile, 2.5% triflouroacetic acid) and air dried.
4. The plate was then loaded into the MALDI-TOF instrument.
5. A work sheet of sample IDs and corresponding positions on the plate was prepared using the 'MALDI Biotyper RTC Wizard'.
6. Sample analysis was monitored using the FlexControl program.

Results

All 499 isolates were analysed and 485 confirmed as being *E. coli.* The remainder were mainly *Acinetobacter baumannii* (n=7) or *Pseudomonas aeruginosa* (n=7).

Determination of AMR

Methods

1. Each isolate was initially cultured on cysteine lactose electrolyte deficient agar (CLED) agar at 37°C +/- 2°C for 18-24hours to ensure purity.
2. A single colony was subcultured from the CLED agar onto nutrient agar at 37°C +/- 2°C for 18-24hours.
3. A suspension of the test organism was prepared in saline and adjusted to be equal in turbidity to a 0.5 McFarland standard (1 X 10^8 CFU/ml).
4. A sterile swab was used to swab the surface of Mueller Hinton Agar (MHA) plates with the suspension.
5. The antibiotic discs listed above were applied using a disc dispenser. The range of antibiotics tested represent six antimicrobial classes and eight antimicrobial categories as defined by Magiorakos *et al.* (2012).
6. Following incubation at 37°C +/- 2°C for 18-24hours the diameter of each zone of inhibition was recorded.

7. The results were assessed in accordance with EUCAST interpretive criteria [EUCAST, 2014]. Where EUCAST interpretive criteria for certain antibiotics were not available, CLSI criteria were applied [CLSI, 2014].
8. *E. coli* ATCC 25922 was used as a control.
9. ESBL production was confirmed by the combination disk method using cefpodoxime alone and in combination with clavulanic acid. Where the diameter of the zone of inhibition surrounding the Cefpodoxime/ Clavulanic acid containing disk was > 5mm compared to that surrounding the Cefpodoxime disk this was confirmation of ESBL production. *Klebsiella pneumoniae* ATCC 700603 was used as a control for screening for ESBL production.

Table 3: Antibiotic discs used to characterise *E. coli* isolates from raw retail meats.

Antibiotic (Disc content)	Supplier
Ampicillin (10µg)	Oxoid
Cefpodoxime (10µg)	Mast Diagnostics
Cefotaxime (5µg)	Oxoid
Ceftazidime (30µg)	Oxoid
Cefoxitin (30µg)	Oxoid
Meropenem (10µg)	Oxoid
Ertrapenum (10µg)	Oxoid
Nalidixic Acid (30µg)	Oxoid
Ciprofloxacin (5µg)	Oxoid
Gentamicin (30µg)	Oxoid
Streptomycin (10µg)	Oxoid
Kanamycin (30µg)	Oxoid
Tetracycine (30µg)	Oxoid
Trimethoprim (5µg)	Oxoid
Choramphenicol (30µg)	Oxoid
Cefpodoxime (10µg) and Clavulanic acid (1µg)	Mast Diagnostics

Table 4: Number of isolates non-susceptible to individual antimicrobial classes and agents

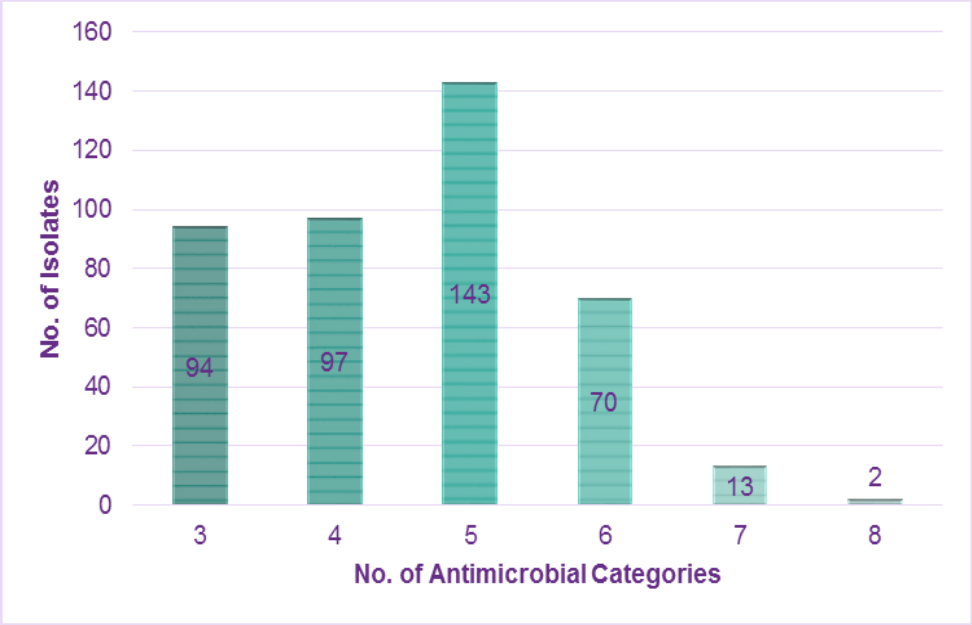
Antimicrobial Class/Agent	No of resistant isolates
Beta-lactams	
Ampicillin	429
Cefpodoxime	249
Cefotaxime	238[1]
Ceftazidime	111
Cefoxitin	92
Meropenem	0
Ertapenem	5[2]
Quinolones/Fluoroquinolones	
Nalidixic Acid	311
Ciprofloxacin	101
Aminoglycosides	
Gentamicin	69
Streptomycin	296
Kanamycin	55
Tetracyclines	
Tetracycline	339
Folate pathway inhibitors	
Trimethoprim	17
Phenicols	
Chloramphenicol	100

[1]Of which 143 were extended-spectrum beta-lactamase producers. The mechanism of cefotaxime resistance in the remaining 95 isolates was not fully characterised but is likely to be related to high level expression of intrinsic chromosomal *bla*$_{AmpC}$.

[2] The ertapenem resistant isolate were tested for carbapenemase production and carbapenmease genes. These were not detected. The resistance is therefore most likely related to a combination of non-carbapenemase enzyme (e.g. AmpC) and reduced permeability. The isolates remained susceptible to meropenem.

Four hundred and nineteen isolates were multi-drug resistant (MDR) based on the categorisation of non-susceptible to at least one antimicrobial agent in three or more antimicrobial categories (Magiorakos, 2011) (Figure 2).

Figure 2: Proportion of isolates that were multi-drug resistant and number of antimicrobial categories



One hundred and forty-three (29.5%) *E. coli* were confirmed as ESBL producers, of which 130 were isolated from chicken, 1 from beef and 12 from pork. All ESBL producers were susceptible to cefoxitin, ertapenem and meropenem and resistant to ampicillin, cefpodoxime and cefotaxime.

One hundred and eight (22.2%) *E. coli* isolates were resistant to ciprofloxacin. Of these *E. coli,* 83 were isolated from chicken, four from beef and 20 from pork. Fourteen (two pork, 12 chicken) of these were also ESBL producers. It is also important to note that an additional 203 isolates (311 in total) were resistant to the quinolone nalidixic acid indicating that they have moved one step towards ciprofloxacin resistance.

7 Detection of virulence factors

Method

DNA was extracted from all presumptive *E. coli* (n=499) using chloroform/phenol and subjected to the multiplex PCR protocol of Paton and Paton (1998) to detect the pathogenicity factors STX1, STX2, *eae* and *hly*. See Appendix 1 1 for full details of the method.

Results

No isolates from meropenem broths carried the virulence factors detected by this assay, but one of 206 isolates from cefotaxime carried *eae*, whilst this gene was found in 13 isolates from cefotaxime. The eae gene encodes for an adhesin that plays a role in attachement of *E. coli* to epithelium although *E. coli* isolates containing the eae gene in isolation are not vero/shiga toxigenic *E. coli* (VTEC/STEC). In addition one beef isolate from cefotaxime carried STX2, and was identified as being from serogroup O157. This pattern is typical of the virulence profile classically associated with VTEC related diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome.

8 Cluster analysis & detailed determination of antimicrobial resistance

Method

Cluster analysis was performed using BioNumerics, with all data obtained in the above studies, a total of 25 parameters.

The resulting similarity matrix resolved the isolates into two principal clusters, based on the antibiotics used in the enrichment process. No other factors gave significant resolution of the isolates, therefore no other significant associations were seen.

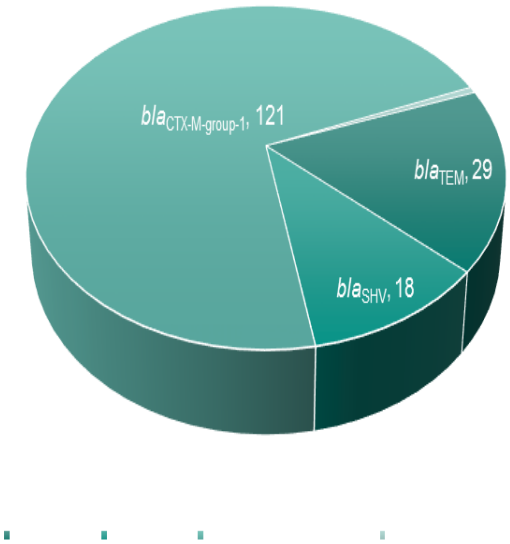
Based on the team's assessment of the available data all ESBL producing *E. coli* and ciprofloxacin resistant *E. coli* (n = 251) were phlyogrouped by PCR using specific primers and protocols as previously described (Clermont, 2012). The majority of isolates were members of phlyogroup B1 (n = 111/46%) (Table 5) which is an animal associated phylogroup not frequently associated with human infection.

Table 5: Phylogrouping of ESBL producing and ciprofloxacin resistant isolates

Phylogroup	ESBL producers	CIP Resistant isolates	ESBL producer and CIP Resistant
A	13	0	3
C	2	16	0
A or C	0	16	0
B1	86	35	8
B2	9	4	0
Clade I or II	3	7	1
D	2	0	1
E	8	0	1
D or E	0	2	0
F	13	19	0
Unknown	7	25	0
Total	143	108	14

All ESBL producing *E. coli* (n = 143) were tested for the presence of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and $bla_{\text{OXA-1}}$ by PCR using specific primers and protocols as previously described (Woodford, 2006, Dallenne, 2010). All isolates harboured one or more of the beta-lactamase encoding genes with $bla_{\text{CTX-M Group-1}}$ predominating (n = 121/85%). One isolate harboured a $bla_{\text{CTX-M Group-9}}$ gene and 33 isolates harboured both $bla_{\text{CTX-M Group-1}}$ and bla_{TEM}. Eighteen isolates harboured bla_{SHV} alone, 29 isolates harboured bla_{TEM}, 3 isolates harboured harboured both bla_{SHV} and bla_{TEM} and $bla_{\text{OXA-1}}$ was not present in any isolate. For comparison the predominant $bla_{\text{CTX-M Group}}$ associated with ESBL *E. coli* in humans in Ireland is $bla_{\text{CTX-M}}$ group 1.

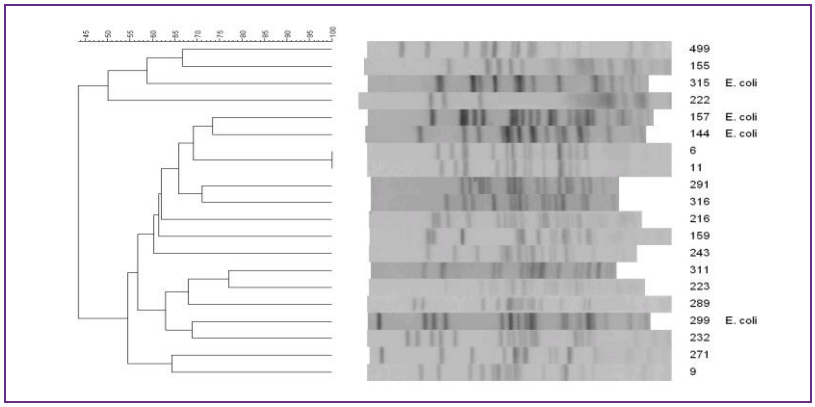
Figure 3: Proportion and type of ESBL encoding genes detected in *E. coli* (n-143)



All phlyogroup B2 isolates were assessed for membership of the pandemic clonal group *E. coli* ST131 by PCR using specific primers and protocols as previously described (Clermont, 2009). No ST131 isolates were detected. For purposes of comparison ST131 accounts for the great majority of ESBL *E. coli* associated with human infection and colonisation on IOI

Twenty isolates were selected for PFGE analysis based on phylogroup, antibiogram and presence of antimicrobial resistance encoding genes. PFGE of the 20 *Escherichia coli* isolates using *Xba*I generated 19 major patterns (Figure 4). These findings suggest there is significant diversity among ESBL producers isolated from meat samples by PFGE. However, preliminary comparisons with PFGE profiles of ESBL producing *E. coli* isolated from human specimens over the same time period do not reveal any significant similarities.

Figure 4: Dendrogram of *XbaI* PFGE patterns illustration relationship between ESBL-producing *E. coli* isolated from retail meat on IOI



Whole genome sequencing to determine phylogenetic relatedness of isolates.

Ninety-six isolates of *E. coli* were selected for WGS based on the following criteria: production of an extended spectrum beta-lactamase; ciprofloxacin resistance; membership of phylogroup B2, D or unknown; harbouring a bla_{SHV} gene, resistance to ceftazidime and/or antimicrobial susceptibility profile. DNA was prepared using the Epicentre Masterpure DNA purification kit and submitted for sequencing to the High throughput Genomics Group, University of Oxford. Sequence analysis involved assembly of the sequences generated and input together with metadata to the BIGSdb database - http://pubmlst.org/software/database/bigsdb/. BIGSdb is software designed to store and analyse sequence data for bacteria and was developed and is maintained by the Maiden group at Oxford. Access to BIGSdb allows for detailed examination of our own collection and comparison of our sequence data to other data archived in the database. Currently there is very little WGS data available on antimicrobial resistant isolates collected from foods. Sequence data generated could subsequently be examined as part of a more comprehensive further study to understand the role food plays in the dissemination of these important antimicrobial resistant organisms. The WGS analysis indicated considerable diversity amongst the AREC isolates with 44 known MLST types (and two previously undescribed) and 24 ribosomal MLST types. MLST types were observed in more than one category of meat product so that there was not a clear relationship between specific *E. coli* subtypes and particular animal species. When focusing specifically on the bla_{CTX-M} positive ESBL producing isolates the pattern was likewise one of significant diversity in terms of MLST type and rMLST.

9 Project discussion and key findings

Ciprofloxacin resistant and cefotaxime resistant *E. coli* are very present in uncooked meats but with prevalence highest in poultry, lower in pig meat and lowest in beef. This project represents the most comprehensive study to-date to assess the prevalence of *E. coli* resistant to key antimicrobial agents in raw meat on retail sale on the IOI. There are few comparably comprehensive studies internationally.

Ciprofloxacin resistant and cefotaxime resistant E. coli are very present in uncooked meats but with prevalence highest in poultry, lower in pig meat and lowest in beef

The sampling process was designed to ensure that the data is representative of the island as a whole and that three major meat groups are equally represented. The methods were designed to detect *E. coli* with low level resistance to ciprofloxacin, cefotaxime and meropenem.

Meropenem resistant E. coli are not present in meat at present and that the small number of ertapenem resistant E. coli detected are not related to carbapenemase production.

The methods provide a template for comparable studies in other parts of Europe. The WGS data generated will be a valuable resource for additional studies allowing comparison with an emerging library of data. The key findings are that ciprofloxacin resistant and cefotaxime resistant *E. coli* are very present in uncooked meats but with prevalence highest in poultry, lower in pig meat and lowest in beef. It is an important baseline to establish that meropenem resistant *E. coli* are not present in meat at present and that the small number of ertapenem resistant *E. coli* detected are not related to carbapenemase production. It is not surprising that antimicrobial resistant *E. coli* are more common in meat from intensively farmed animals where antimicrobial use is typically more intense and there is greater opportunity for spread of microorganisms than in meat from extensively farmed animals such as beef. Extended Spectrum Beta-Lactamase producing *E. coli* which are a significant human health concern accounts for a high proportion of cephalosporin resistant *E. coli* in meat.

Further characterisation indicates the antimicrobial resistant *E. coli* isolated from meat are diverse and generally different from the principle *E. coli* type associated with ESBL mediated resistance in humans in Ireland and the United Kingdom. However the gene (blaCTX-M 1) encoding ESBL production in these meat derived *E. coli* is commonly detected in human isolates therefore further work on gene transfer to *E. coli* populations in the human gut are warranted. There is a risk of ingesting viable AREC for a person purchasing meat that contains antimicrobial resistant *E. coli* depends on handling and food preparation practices. It is important therefore to continue to inform the public regarding safe practices in relation to handling, storage and preparation of uncooked meat.

Antimicrobial resistant E. coli isolated from meat are diverse and generally different from the principle E. coli type associated with ESBL mediated resistance in humans

Apart from any implications for human health the prevalence of ciprofloxacin and cefotaxime resistance in meat has implications for animal health care. Growing resistance to these agents in animals will result in reduced efficacy when these agents are used to treat animals which may impact on animal welfare and on the efficiency of food production if alternative approaches to management are not available and implemented. This could potentially lead to demand for use of newer classes of antimicrobial agents in animals driving forward the cycle of selection for resistance.

10 Project conclusions

E. coli resistant to critical clasess of antimicrobial agents (ciprofloxacin and cefotaxime) are commonly present in meat on retail sale on the island of Ireland, in particular in poultry meat.

Although the type of *E. coli* carrying ESBL in meat do not appear to represent a major contributor to the *E. coli* types carrying ESBL in the human population in Ireland the type gene encoding the ESBL is similar and given that antimicrobial resistance genes transfer between *E. coli* strains this means that a contribution of meat to dissemination of antimicrobial resistance genes in the human population cannot be dismissed.

As it seems likely that the prevalence of antimicrobial resistance in raw meat reflects the prevalence in the animal species from which the meat is derived these findings may also have very significant implications for veterinary medicine and food animal production.

11 Added value and anticipated benefits of research

This study combined the strengths of a group expert in applied microbiology, and the undertaking of microbiological surveys of foodstuffs, and a medical microbiology group with significant expertise in antimicrobial resistance. The synergies which emerged have increased the skill sets of both groups and initiated a dialogue which will continue long after the study has formally concluded. The expertise at AFBI ensured that the set of cultures was collected in a rigorous manner, and hence that the resulting collection of AREC, can be studied in the future, confident in their provenance. The extension of the initial aim, to collect solely ESBL producing *E. coli*, to a collection of isolates with presumptive carbapenemase and resistance to ciprofloxacin, was extended at the behest of Prof Cormican. This more than doubled the number of isolates collected, at a modest cost in media and staff time and therefore markedly added value to the study.

Further added value can be seen in the establishment of a large culture collection at NUIG which will be available for further study and will therefore continue to inform the topic of antimicrobial resistance for years to come.

The whole genome sequences obtained in this study will be deposited in public databases contributing substantially to the limited body of food related WGS data in the public domain and available for analysis in association with other data collections that will emerge in the future, thus adding further value to work described above.

The benefits of the research will accrue to consumers due to the insight provided to human medicine, and also to veterinary medicine. Whilst direct transmission of the AREC present in foods, directly to consumers, was considered unlikely due to the genotypes involved, the veterinary consequences of the very high prevalence seen in broilers will need to be addressed, and identification of this problem is a significant benefit.

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13 Publications

Conference publications

1. Morris, D., S. Kavanagh, L. Moran, C. Kelly, Cormican, M, C. Carroll, Madden, RH. Retail meats - a source of antibiotic resistant *E. coli*? In: Proceedings of the Infectious Diseases Society of Ireland Annual Scientific Meeting, Dublin, May 7th-9th 2015.

2. Morris, D., S. Kavanagh, L. Moran, C. Kelly, Cormican, M, C. Carroll, Madden, RH. Chicken as a source of antimicrobial resistant *E. coli* in the Irish retail market. In: Proceedings of ENVIRON 2015, Sligo, April 8th-10th, 2015.

3. Morris, D., S. Kavanagh, L. Moran, C. Kelly, Cormican, M, C. Carroll, Madden, RH. Chicken in the Irish retail market – a significant source of antimicrobial resistant *E. coli*? In: Proceedings of the Society of General Microbiology (SGM) Annual Conference, Birmingham, March 30th-April 2nd, 2015

4. Moran, L., Cormican, M., Morris, D., Carroll, C., Kelly, C. and Madden, R.H. 2015 Presence of antimicrobial resistant *Escherichia coli* in retail raw meats on the island of Ireland. Proceedings of the 61st International Congress of Meat Science & Technology, Clermont Ferrand, 23rd-28 August

14 Appendix

Appendix 1: Genotyping methodologies used in details characterisation of *E. coli* isolates

Method

Phylogrouping

1. All ESBL producers and ciprofloxacin resistant isolates of *E. coli* were phylogrouped by PCR as previously described (Clermont, 2012).
2. Initially a quadruplex PCR was performed the amplification primers for which are detailed in **Error! Reference source not found.**.
3. The combined amplification of individual genes determined to which of the phylogroups A/C, B1, B2, D/E, F isolates of *E. coli* were assigned.
4. Appropriate positive and negative (sterile water) were included in each PCR assay.

Primers used for phylogrouping – quadruplex PCR

Primer	Nucleotide sequence
Forward chuA.1b primer	5'-ATGGTACCGGACGAACCAAC-3'
Reverse chuA.2 primer	5'-TGCCGCCAGTACCAAAGACA-3'
Forward yjaA.1b primer	5'-CAAACGTGAAGTGTCAGGAG-3'
Reverse yjaA.2b primer	5'-AATGCGTTCCTCAACCTGTG-3'
Forward TspE4C2.1b primer	5'-CACTATTCGTAAGGTCATCC-3'
Reverse TspE4C2.2b primer	5'-AGTTTATCGCTGCGGGTCGC-3'
Forward AceK.f primer	5'-AACGCTATTCGCCAGCTTGC-3'
Reverse ArpA1.r primer	5'-TCTCCCCATACCGTACGCTA-3'

The composition of the reaction mixture and cycling conditions for the quadruplex PCR were as follows:

Reaction Mixture	
10x Reaction buffer	10mM
$MgCl_2$	1.5mM
dNTPs	200 µlM each
Forward primers (x4)	10pmol
Reverse primers (x4)	10pmol
Taq Polymerase 1U	
Sterile Water	To a total volume of 23 µl.
2µl of test and control DNA was added as appropriate	

Cycling conditions:

Temperature and Time	No. of Cycles
94°C for 4 min	1
94°C for 5 sec 59°C for 20 sec	30
72°C for 5 min	1

Electrophoresis of 10µl of PCR products was carried out on a 2% agarose gel in 1xTBE for 90min at 70V using ethidium bromide for staining of the DNA. Gel Doc 2000 software was used to visualise the gel.

This quadruplex PCR does not differentiate between the following phylogenetic groups; A/C and D/E. Where an isolate was assigned to these groups following the initial quadruplex PCR, two further PCR assay were performed to differentiate these.

The composition of the reaction mixture and cycling conditions used for the PCR assay to differentiate the A/C phylogroups were the same as those outlined above for the quadruplex PCR.

The amplification primers used in this assay were as follows;

Primer	Nucleotide sequence
Forward trpAgpC.1	5'-AGTTTTATGCCCAGTGCGAG-3'
Reverse trpAgpC.2	5'-TCTGCGCCGGTCACGCCC-3'
Forward trpBA.f	5'-CGGCGATAAAGACATCTTCAC-3'
Reverse trpBA.r	5'-GCAACGCGGCCTGGCGGAAG-3'

The composition of the reaction mixture used for the PCR assay to differentiate the D/E phylogroups were the same as those outlined above for the quadruplex PCR.

The cycling conditions were as follows:

Temperature and time	No. of cycles
94°C for 4 min	1
94°C for 5 sec 57°C for 20 sec	30
72°C for 5 min	1

The amplification primers used in the D/E PCR assay were as follows:

Primer	Nucleotide sequence
Forward ArpAgpE.f primer	5'-GATTCCATCTTGTCAAAATATGCC-3'
Reverse ArpAgpE.r primer	5'-GAAAAGAAAAAGAATTCCCAAGAG-3'
Forward trpBA.f	5'-CGGCGATAAAGACATCTTCAC-3'
Reverse trpBA.r	5'-GCAACGCGGCCTGGCGGAAG-3'

Positive controls (and source) used in individual PCR assays:

PCR	Positive Controls
Quadruplex phylogenetic group	Group A/C (no. 2: Johnson group, US)
	Group B1 (no. 26: Johnson group, US)
	Group F (no. 35: Johnson group, US)
	Group D/E (no. 43: Johnson group, US)
	Group B2 (no. 53: Johnson group, US)
	Group B2 (no. 55: Johnson group, US)
	D/E phylogenetic group Group D (no. 35: Johnson group, US)
	Group E (no. 31: Johnson group, US)
A/C group phylogenetic group	Group A (no. 141: Johnson group, US)
	Group C (no. 70: Johnson group, US)
Membership of the 025b:ST131 clonal group	Representative of UK Strain A
bla$_{TEM}$, *bla*$_{SHV}$ *and bla*$_{OXA}$	*E. coli* J53 harbouring *bla*$_{TEM-5}$
	E. coli J53 harbouring *bla*$_{SHV-2}$
	Representative of UK Strain A
bla$_{CTX-M\ group8}$ and *bla*$_{CTX-M\ group25}$	*E. coli* harbouring *bla*$_{CTX-M\ group8}$
	E. coli harbouring *bla*$_{CTX-M\ group8}$

Membership of the 025b:ST131 clonal group

The overall method is as outlined in part A.

The amplification primers, composition of the reaction mixture and cycling conditions used for this PCR assay were previously described (Clermont, 2009) and are detailed below.

The amplification primers used in this assay were as follows:

Primer	Nucleotide Sequence
Forward Pab Bspe Primer	5′-TCCAGCAGGTGCTGGATCGT-3′
Reverse Pab Bspe Primer	5′-GCGAAATTTTTCGCCGTACTGT-3′
Forward Trp A Primer	5′-GCTACGAATCTCTGTTTGCC-3′
Reverse Trp A2 Primer	5′-GCAACGCGGCCTGGCGGAAG-3′

The composition of the reaction mixture and cycling conditions were as follows:

Reaction Mixture	
10x Reaction buffer	10mM
$MgCl_2$	1.5mM
dNTPs	200µM each
Forward Pab Bspe Primer	10pmol
Reverse Pab Bspe Primer	10pmol
Forward TrpA Primer	6pmol
Reverse TrpA Primer	6 pmol
Taq Polymerase	1U
Sterile Water	To a total volume of 49µl

Real-Time PCR for detection of antimicrobial resistance encoding genes: *bla*$_{\text{CTX-M Group-1}}$, *bla*$_{\text{CTX-M Group-2}}$ and *bla*$_{\text{CTX-M Group-9}}$.

1. DNA was extracted from the test organisms as outlined above
2. The amplification primers used in this assay are detailed below, (Birkett, 2007)
3. The composition of the reaction mixture is detailed below

Primer/probe	Nucleotide sequence
Forward CTX-M primer	5'-ATG TGC AGY ACC AGT AAR GTK ATG GC-3'
Reverse CTX-M primer	5'-ATC ACK CGG RTC GCC XGG RAT-3'
CTX-M-1 group probe	5'-FAM-CCC GAC AGC TGG GAG ACG AAA CGT-Tamra-3'
CTX-M-2 group probe	5'-JOE-CAG GTC CTT ATC GCT CTC GCT CTG TT-BHQ-2-3'
CTX-M-9 group probe	5'-Cy5-CTG GAT CGC ACT GAA CCT ACT CTG A-BHQ-2-3'

Reagent	Concentration of stock	Volume per rxn (µl)
Reverse CTX-M primer	800nm	0.4
CTX-M Group 1 Probe	200nm	0.1
CTX-M Group 2 Probe	200nm	0.1
CTX-M Group 9 Probe	200nm	0.1
Taq Man Mastermix		12.5
Sterile Water		6.4
Total		**20**

A 20µl volume of this was dispensed into assigned wells of a 96 well plate and 2µl of DNA was added (including the four control samples listed below).

The control strains used in amplification of *bla*$_{\text{CTX-M Group-1}}$, *bla*$_{\text{CTX-M Group-2}}$ and *bla*$_{\text{CTX-M Group-9}}$ by real time PCR are listed below:

Control	Target
E. coli ATCC 25922	Negative Control
E. coli NCTC 13441	CTX-M Group 1
E. coli NCTC 13462	CTX-M Group 2
E. coli U51624	CTX-M Group 9

The well plate was covered with an adhesive sheet. It was then centrifuged for 2 minutes at 2,000 rcf, and placed in the Applied Biosystems 7500 FAST RT-PCR system.

The cycling conditions were as follows:

Temperature and Time	No. of Cycles
50°C for 2 min	1
95°C for 10 min	1
95°C for 15 sec 60°C for 1 min	35

Pulsed-field gel electrophoresis (PFGE)

PFGE analysis of a subset of strains (n = 20) was carried out in accordance with PulseNet standardised protocols (Swaminathan, 2001). Isolates were selected for PFGE analysis based on phylogroup, antibiogram and presence of antimicrobial resistance encoding genes.

Method

1. A suspension of the test isolate was prepared in cell suspension buffer equivalent to a 3 McFarland standard.
2. Four hundred microliters of this cell suspension was mixed with 20µl Proteinase K and 400µl of 1% SeaKem Gold agarose and dispensed into plug moulds.
3. Once set, these were lysed in cell lysis buffer (50mM Tris: 50mM EDTA, pH 8.0 + 1% sarcosyl) plus proteinase K at 54°C for a minimum of 90 minutes.
4. Following lysis, plugs were washed in sterile water and TE Buffer: (10mM Tris: 1mM EDTA, pH8.1).
5. A 2mm slice of each plug (and *Salmonella* Branderup size standards) was restricted with *XbaI* restriction enzyme at 37° C for 90 minutes.
6. Following restriction, electrophoresis was carried out for 20 hours with a switch interval of 2.16s - 54.17s.
7. Following electrophoresis, the gel was stained with ethidium bromide and an image of the gel was captured.
8. PFGE profiles generated were analysed using the Dice coefficient with clustering by the unweighted pair group method with arithmetic averaging (UPGMA).

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