THE EMERGENCE OF BETA - LACTAMASE PRODUCING *Escherichia coli* AND THE PROBLEMS IN ASSESSING THEIR POTENTIAL CONTRIBUTION TO FOODBORNE ILLNESS: A REVIEW

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Abstract

Antimicrobial agents have been in use for therapeutic purposes for over a century, with most of the development occurring in the latter half of the twentieth century. Penicillin was the first of the naturally occurring antimicrobials to be used in medicine and its structure includes a beta-lactam ring. Further compounds, such as the cephalosporins, were discovered and these also included beta-lactam structures. Subsequently bacteria which were resistant to these compounds were found, and their resistance was due to their production of enzymes, beta-lactamases, which hydrolysed the beta-lactam ring. Synthetic derivatives of the beta-lactam antimicrobials were developed to render them recalcitrant to beta-lactamases but enzymes with a broader substrate range evolved, and were categorised as extended substrate beta-lactamases (ESBL). Since the antimicrobials had a significant role to play in human medicine the emergence of ESBL caused significant concerns. Further, similar antimicrobials were used by veterinarians, raising the prospect that bacteria in the commensal flora of livestock could acquire ESBL resistance properties and exchange them via genetic exchange. Thus, pathogenic bacteria present in livestock could become resistant to antimicrobials with adverse consequences should zoonotic infections occur. In this review we consider the emergence of ESBL, the problems involved in detecting and reporting such properties, and consider the consequences for consumers of potentially contaminated food products.

Key words: beta-lactamase, emerging, Escherichia coli, foodborne, pathogen.

INTRODUCTION

Therapeutic antimicrobial agents have been used in human health care for over a century following the discovery of the antisypohilitic activity of the synthetic compound arsphenamine in 1909. Subsequently a wide range of therapeutic compounds was discovered, including the penicillins, cephalosporins and other naturally occurring compounds. The first of the antibiotics to be used in medicine was penicillin, synthesised by a fungus (*Penicillium* spp.) and the chemical structure of this compound is characterised by the presence of a beta-lactam ring. Subsequently other beta-lactam antibiotics were discovered, such as the cephalosporins. The range of beta-lactam like compounds available was subsequently expanded by further discovery and by chemical modification of naturally occurring compounds.

Antimicrobial resistance to beta-lactams
At present there is a wide range of penicillins, cephalosporins, monobactams and carbapenems available for therapeutic use.

Broadly speaking, all of the compounds act by the inhibition of bacterial cell wall biosynthesis but may be vulnerable to inactivation due to being cleaved by one of the beta-lactamase class of enzymes. To maintain the antimicrobial activity of the therapeutic agents, beta-lactam agents may be combined with compounds such as clavulanic acid, which can inhibit the activity of some beta-lactamases. However, restoring the clinical activity of beta-lactams is very complex due to the very large number of such compounds in use, the small number of specific enzyme inhibitors available, and large number of beta-lactamase enzymes known to exist.

Beta-lactam antimicrobial agents were widely applied in human and animal healthcare but resistance to them soon emerged as a clinical problem, being reported within two years of their widespread use. For several decades new beta-lactam agents with enhanced activity were regularly developed and introduced, however, this stream of new products has slowed, almost to a halt, in recent years. The most important mechanism conferring bacterial resistance to beta-lactam antibiotics is the production of one or more enzymes capable of hydrolysing, i.e. breaking open, the beta-lactam ring, rendering the antibiotic ineffective. In view of their properties these enzymes are termed beta-lactamases.

In 1965 Datta and Kontomichalou reported in Nature that *Escherichia 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 patient's name, and other TEM enzymes were discovered in due course (Bush et al., 1995). The genes coding for beta-lactamases were subsequently named by using the abbreviation *bla* with the specific gene type being added. In the case above the gene was denoted as TEM, hence this group of enzymes were referred to as bla_{TEM}. In addition, the abbreviation was followed by a number denoting the specific enzyme, in the format bla_{TEM-1}. The bla_{TEM} genes were found to be carried on transposable elements (Blanc et al., 2006; Partridge and Hall, 2005) i.e. sections of DNA, and these elements can be readily transferred between members of the *Enterobacteriaceae* (the family of enteric, or gut, bacteria to which *E. coli* belongs) (Bradford, 2001; Partridge and Hall, 2005; Sirot et al., 1991; Thong and Modarressi, 2011). Further, transposons are also known to be transferred between unrelated species of bacteria (Bradford, 2001).

Less than ten years after TEM-1 was discovered a different lactamase enzyme was reported, and was later named SHV-1, derived from sulfhydryl 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). The beta-lactamase SHV gene is denoted bla_{SHV}. Again, numbers are appended to the definition to define specific enzymes within this group.

A key feature of the first TEM and SHV enzymes 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.

Emergence of extended spectrum beta-lactamase (ESBL)

Third generation cephalosporins (3GC), were introduced in the 1980s (Paterson and Bonomo, 2005) and were also referred to as extended spectrum cephalosporins. Due to their efficacy they were widely used from the early 1980s onward (Bryan, 1991).

However, soon after the 3GC were introduced resistance to them was reported in species such as *E. coli*. The mechanism for this resistance was based on beta-lactamase enzymes able to hydrolyse a wider (or extended) range of substrates than seen previously, including new cephalosporins. The term 'extended spectrum beta-lactamase' was coined (Philippon et al., 1989) to describe the beta-lactamases conferring resistance to 3GC, normally abbreviated to ESBL.

The first ESBLs were seen to be encoded by variants of the widely disseminated bla_{TEM} and bla_{SHV} genes (Bradford, 2001; Partridge and Hall, 2005). Derivatives of the bla_{TEM} and bla_{SHV} genes are now numbered in their hundreds, and differ subtly in their properties

(Bourouis et al., 2015; D'Andrea et al., 2013). For most of the 1980s and 1990s these enzymes were the main focus of concern in relation to ESBLs.

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 *Escherichia coli*, *Proteus mirabilis* and *Salmonella typhimurium* suggesting that it was mobile within the *Enterobacteriaceae* family. The resistance noted in *S. marcescens* was also carried on a plasmid, which could also be transferred to the other *Enterobacteriaceae* studied, but some elements were lost in transfers to *E. coli*. The ESBL enzyme was not equally efficient in its hydrolysis of 3GC, conferring significantly greater resistance to cefotaxime than ceftazidime, hence it was designated a „cefotaximase" and the gene designated bla_{CTX}. The work of Knothe et al. (1983) therefore raised the prospect of non-pathogenic organisms transferring 3GC resistance on a plasmid to pathogenic organisms, e.g. *S. typhimurium.*

Briefly, the history of ESBL enzymes can be summarised as progressing from infrequent reports of ESBL enzymes, mainly bla_{TEM} and bla_{SHV} variants, through to the reporting of widespread dissemination of this resistance in hospital settings. Early dissemination was probably due to a widespread failure of authorities to identify this resistance phenomenon because of deficiencies in clinical laboratory detection procedures. Subsequently the bla_{CTX} ESBLs emerged with near worldwide dissemination through hospitals (Paterson and Bonomo, 2005), nursing homes (Rooney et al., 2009), the general community and the environment, including food animals (Blanc et al., 2006).

In human healthcare the dissemination of this resistance is linked specifically to that of *E. coli* clonal group ST131 (ST indicates DNA sequence type) (Clermont et al., 2009) which frequently carries this resistance (Morris et al., 2012; Rooney et al., 2009). More than 10% of all *E. coli* isolated from blood culture in Ireland carry ESBL (http://www.hpsc.ie/AboutHPSC/AnnualReports/File,15139,en.pdf).

Antimicrobial resistance in the food chain

Therapeutic antimicrobial agents were not only applied in human medicine, but also in veterinary medicine. In addition, antibiotics were found to be effective growth promoters, especially with poultry, and supplemented feeds were used from the 1950s onwards (Jones and Ricke, 2003). This later led to concerns that antimicrobial resistance (AMR) could become widespread in the microflora of animals, and subsequently consumers could be exposed to these organisms via meats. A seminal work in this regard was the UK Report on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine, aka the Swann report, published in 1969 (Soulsby, 2007). Others have also highlighted concerns (Bensink and Bothmann, 1991), especially the possibility of foodborne pathogens, such as *Salmonella* spp., acquiring AMR and compromising normal antimicrobial therapy.

ESBLs have been extensively reported in *Salmonella enterica* where this type of resistance significantly complicates the clinical management of cases of invasive salmonellosis which require systemic treatment. Although antimicrobial use in animals is accepted as selecting for resistant bacteria in animals, noted in the Swann Report, and although it is apparent that antimicrobial resistance is found in zoonotic pathogens such as *Salmonella* and *Campylobacter*, there is great uncertainty regarding the overall extent to which antimicrobial resistance found in human isolates can be attributed to antimicrobial use in animals (Torneke et al., 2015). One element of this uncertainty relates to the extent to which antimicrobial resistant *E. coli* (AREC), and other *Enterobacteriaceae* from the gut of animals, are transferred to humans in the food chain, and to what extent, if any, this may contribute to AMR problems in relation to *E. coli* associated urinary tract infections, blood infections and other associated invasive infections in people.

The use of antimicrobials for growth promotion in healthy animals was banned by the European Union, with effect from 1st January 2006 (http://europa.eu/rapid/press-release_IP-05-

1687_en.htm). However, this ban does not apply in many other parts of the world which export foodstuffs to the EU (Maron et al., 2013). This was highlighted in late 2015 when extensive colistin use in pig production in China, led to the associated emergence of transferrable colistin resistance in *Enterobacteriaceae*, with subsequent reports of worldwide dissemination (Liu et al., 2016). Furthermore, antimicrobial agents continue to be used in very large quantities for the prevention, and treatment, of infection in food producing animals. The EU also enforces 'washout' periods following antimicrobial administration to animals but it is important to note, however, that whilst washout of the antimicrobial agents will occur, this does not apply to the AMR bacteria, which can be much more persistent (Soulsby, 2007).

In the longer term the removal of antimicrobials from use in food animals can have benefits: Agersø and Aarestrup (2013) reported that a voluntary ban on cephalosporin use in Danish pig production had effectively reduced the numbers of extended-spectrum cephalosporinase-producing *Escherichia coli* detected in slaughter pigs.

The potential problems arising from antibiotic resistant zoonotic infections was addressed by the 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.“

In consequence both the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) work together to produce an annual report on AMR in key zoonotic pathogens isolated in member countries from animals and humans: Antimicrobial resistance surveillance in Europe

(Available from http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/Pages/publications.aspx). The gravity of the problems associated with AMR in food animals was further underlined when EFSA and ECDC combined with the European Medicines Agency (EMA) to produce the 'ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals' (Available at: http://ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-JIACRA-report.pdf).

The report cited results based on indicator organisms which were seen to be carrying resistance genes, which could then be passed to pathogenic organisms. This is significant since traditionally food microbiology sought primarily 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 AMR of zoonotic bacteria, such as salmonellas, could be enhanced by genetic material supplied by other *Enterobacteriaceae*. 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.

ESBL *E. coli*

E. coli is a very complex heterogeneous species of bacteria and a variety of typing methods have been used to sub classify members of the species. Traditionally phenotypic methods were used, such as serotyping (based on O and H antigens), phage typing (based on susceptibility to panels of bacteriophage) and antibiotic resistance patterns. More recently molecular methods based on DNA have been developed to allow different levels of discrimination (Thakur et al., 2010; Leverstein-van Hall et al., 2011). *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 (Chelariu et al., 2017). 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.

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). OXA lactamases were named for their ability to hydrolyze oxacillin. In relation to antimicrobial resistance in the food chain, and its link with human data, one challenge is that many studies of ESBL carrying organisms do not fully characterise the ESBL. This is illustrated in a review of the antimicrobial resistance 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.

Detection of ESBL in foodstuffs
Studies to determine the presence of ESBL *E. coli* in foods, or in animals destined for foodstuffs, have taken many different approaches (Mateescu et al., 2014; Sala et al., 2012). 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 (Alvarez-Fernandez et al., 2013; Chaisatit et al., 2012; Lyhs et al., 2012; Schwaiger et al., 2012). 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. a 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.

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 *E. coli* 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 2 mg/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 1 mg/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 8 mg/l vancomycin and 0.25 mg/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 medium 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 medium, which is diagnostic for *E. coli*, was used and supplemented with 2 mg/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 *E. coli* 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 might 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 5 mg/l cefoperazone, a third-generation cephalosporin to which ESBL organisms are resistant and mCCDA contains 32 mg/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

Whilst study of the isolation procedures used to obtain ESBL *E. coli* shows 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 antimicrobial susceptibility of bacteria have been developed for use in medicine. The Clinical and Laboratory Standards Institute (CLSI) has promulgated standardised methods to determine antimicrobial resistance (AMR). Nowadays, however, in Europe the methods and interpretive criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org) is the most widely accepted approach and it informs the joint EFSA/ECDC summary report. Following revisions in interpretive criteria in recent years there is now very close agreement between CLSI and EUCAST in relation to antimicrobial susceptibility testing of *E. coli* and methods for confirming ESBL production in *E. coli*.

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 (Table 1). It is important to note that the AMR tests, and ESBL confirmation

methods, listed in Table 1 are indicative of the work undertaken and not exhaustive.

When *E. coli* are confirmed as producing ESBL then further studies to define the specific type of beta-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.

Characterisation of ESBL

The costs of the work required to define precisely which ESBL is being produced by an organism is significant, and it should be noted that only 25% of the studies listed in Table 1 undertook this task, even in part.

This is due to the diversity of the enzymes within each group. Using DNA amplification, it is possible to differentiate between the main groups: bla_{TEM}, bla_{SHV} and bla_{CTX-M}, and to some degree differentiate within the subgroups, e.g. bla_{CTX-M} groups 1-9 but DNA sequencing of their genomes is often required to specifically differentiate them. Working with *E. coli* isolated from eggs, Egea et al. (2011) used specific primers to identify ESBL as belonging to the CTX-M-9, SHV and CTX-M-1 groups.

Table 1. Methods used to define the antimicrobial resistance (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[2]	+	E-test
Overdevest et al., 2011	Chromogenic agar		E-test, dHPLC[5], microarray, sequencing
Schwaiger et al., 2012	Broth		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.

The amplicons produced by the primers were then sequenced to more narrowly define the ESBL. Shahada et al. (2013) isolated *Salmonella* spp. and *E. coli* from broilers, then used ten primer pairs to identify ESBL as TEM, TEM-52, SHV, CMY-2 or one of six CTX groups. For the latter groups, organisms carrying the genes bla_{TEM}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, and $bla_{CTX-M-9}$ were identified using primers that amplified a partial sequence of the target genes in the initial screening. Specific primers to amplify the entire sequence of the target genes were then used to obtain amplicons for the DNA sequencing analysis.

Thus the identification of specific ESBL requires sophisticated technology, and ultimately is based on DNA sequencing. Some of the different approaches which can be applied to the identification of ESBL are illustrated in the study of Overdevest et al. (2011) where all *E. coli* isolates were tested for bla_{CTX-M} by using PCR (with amplicons being identified using denaturing high-performance liquid chromatography), then screened for other ESBL genes by using a micro-array test that was designed to detect single nucleotide polymorphisms in essential bla_{TEM} and bla_{SHV} genes, variant genes, and bla_{CTX-M} group genes ($bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, and $bla_{CTX-M-8/25}$). On the basis of microarray results, organisms carrying bla_{SHV}, bla_{TEM}, and bla_{CTX-M} genes were identified and the genes amplified using specific primers and PCR. PCR amplicons were then selected and sequenced after purification.

Agersø et al. (2012) also used microarray, PCR, and sequencing technology, but initially genotyped their *E. coli* isolates into two groups based on AMR. Two ESBL phenotypes were commonly found in the reservoirs studied (pigs and pork) and isolates were characterised as one of these: ESBL$_M$ (resistant to cefoxitin and cefepime), and ESBL$_A$ (resistant to cefoxitins and reduced in susceptibility to cefepime;

resistance to ceftazidime and/or cefotaxime and synergy to clavulanic acid) or unclear interpretation. $ESBL_M$ isolates were first screened for bla_{CMY-2} by PCR and sequencing; if negative, the isolates were further screened for up-regulated *ampC* by PCR and sequencing. The rest of the $ESBL_{MS}$ were screened using two different microtube DNA array systems and based on the array result, PCR and sequencing were done. $ESBL_A$ isolates were screened for bla_{CTX-M} by PCR and further sequenced for determination of the bla_{CTX-M} type. If the isolates were negative for bla_{CTX-M}, they were screened for bla_{TEM} and bla_{SHV} by PCR followed by sequencing.

Isolates negative for these two PCRs were examined by DNA array and, based on the result, PCR and sequencing were done to determine the exact gene responsible for resistance to cephalosporins. Isolates with an unclear phenotype were screened for up-regulation of the *ampC* gene and for the presence of bla_{CTX-M} genes. For isolates negative for up-regulated *ampC* or bla_{CTX-M} genes, the DNA array system was used followed by PCR and sequencing.

Agersø et al. (2012) recommended that standard surveillance methods should be supplemented with methods based on selective enrichment when monitoring ESBLs. They also noted that the detection of ESBL genotypes in pigs or broiler meat which were also found in humans indicated that food-producing animals may be the origin, in at least part, of human cases. Hence information on ESBL genotype may be valuable for source attribution.

It is of interest to note that when Shahada et al., (2013) studied the distribution of ESBL in *Salmonella* and *E. coli* isolated from broilers in Japan, they found three groups: TEM, SHV and CTX, but they also screened for seven plasmids associated with ESBL (IncP, IncI1-Iy, IncFIB, IncFIC, IncK, IncB/O and IncY). Thus, the phenotypic property was defined, but also the genotypic means of dissemination was identified.

From the studies of (Agersø et al., 2012 and Overdevest et al., 2011) reported above, in a simplified fashion, it can be seen that the identification of ESBL is complex and requires considerable resources. Once phenotyping has confirmed the presence of ESBL then genotyping, in some form or another, will be required to identify the specific ESBL. Such an identification may be to the level of group, i.e. TEM, SHV or CTX, or continue to the identification of the precise gene carried by the organism, such as $bla_{CTX-M-1}$. In view of the costs and complexity of the procedures involved, detailed characterisation of ESBL is unlikely to be commonly undertaken until inexpensive tools are available. A consequence of this is to limit the number of studies providing detailed information on ESBL isolated. Further, it is important to define the transferable DNA element carrying the resistance gene, in order to fully understand the risk posed by the bacterial host, and be able to attribute its source.

Determining that *E. coli* carrying ESBL are present in foodstuffs does not in itself confirm a risk to people. The potential for such foods to act as vectors and carry the resistant bacteria, in a viable state, to humans where they cause infection must be determined. Even if the *E. coli* is unable to colonise transference of resistance to the resident gut flora must be considered. Hence detailed characterisation of both foodborne and human *E. coli* with ESBL must be undertaken, in order to allow comparison. The study of (Leverstein-van Hall et al., 2011) undertook such a comparison and concluded that their findings were suggestive for transmission of ESBL genes, plasmids and *E. coli* isolates from poultry to humans, most likely through the food chain.

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, whilst Mateescu et al. (2014) studied both *E. coli* and *S. enterica*.

Also, as new ESBL enzymes arose the nomenclature was changed (Bush et al., 1995), hence synonyms exist for some enzymes, complicating the search for specific ESBLs. Overall, the history of foodborne ESBL is complicated by the restrictions under which studies were undertaken, often leading 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., 2011; Jakobsen et al., 2010; Lyhs et al., 2012; Obeng et al., 2012). Further, carriage of such antimicrobial resistance will compromise treatment of the animals themselves (Olsen et al., 2014), and the elimination of ESBL would benefit animals 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: „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. Hence zoonotic transfers are considered probable, and in 2015 a Food Safety Authority of Ireland report (available at https://www.fsai.ie/publications_AMR) concluded that '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.

Overall, in the preparation of this review almost all of the scientific articles referring to the study of antimicrobial resistance in foodstuffs contained terms referring to meats. Thus, there has been a preponderance of study on potential zoonotic transfers.

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 of chicken, beef and pork, and these were the subject of study in the study of retail meats on retail sale on the island of Ireland, in the project of which this report is a part. However, salads have yielded AREC (Egea et al., 2011), and, as noted above, (Franz

and van Bruggen, 2008) vegetables can be vectors for pathogenic *Enterobacteriaceae.*

CONCLUSIONS

Since people are homoeothermic, then bacteria which can colonise them must be mesophilic, and such organisms are normally found associated with other homoeothermic animals, such as those farmed to provide meats. The use of antibiotics in animal husbandry will be one of the factors leading to antimicrobial resistant bacteria being present on, or in, food animals. During the slaughter of these animals the bacteria can be transferred to edible products, and subsequently obtain access to the consumers homes. There is also the possibility of direct transfer of such organisms to those involved in farming, slaughtering, and processing such animals (Sharp et al., 2014).

Although *Escherichia coli* is considered ubiquitous, isolates from different meats are seen to have different phenotypes (Johnson et al., 2009). Thus demonstrating a zoonotic infection in people requires a detailed knowledge of *E. coli* from people, and food animals. Given advances in the sequencing entire bacterial genomes this will be possible. Further, the specific mechanisms which confer antimicrobial resistance properties are many and varied, as discussed above, but these can be defined using whole genome sequencing. Thus the risks posed to people by foodborne bacteria carrying antimicrobial resistance factors will be ultimately defined, given sufficient study.

Currently, however, there is a need to define the prevalence of organisms such as ESBL *E. coli* in retail meats and other foodstuffs, and undertake characterisation of the bacterial phenotype, and define, as far as resources will allow, the type of ESBL carried. Such study will allow more detailed future work to be targeted at the *E. coli* which appear to constitute the greatest risk to the human population. This in turn will allow prophylactic measures to be aimed at the foodstuffs of greatest concern.

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