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**Antimicrobial Resistance in Irish isolates of Verocytotoxigenic *Escherichia coli* (*E. coli*)-
VTEC.**

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Abstract

This study compared the antimicrobial resistance profiles of *Escherichia coli* O157:H7 isolates (n = 257) recovered from bovine hides, minced beef and human clinical samples in Ireland, to those profiles of a range of Irish non-O157 *E. coli* (O111 and O26) isolates (n=31) from a variety of clinical and veterinary sources. Four multi-drug resistant (MDR) *E. coli* O157:H7 food isolates were identified, with resistance to 10 (1 isolate), 6 (1 isolate) and 4 (2 isolates) antimicrobial agents respectively. Two of these isolates (resistant to 7 and 4 antimicrobial classes) were characterised further by molecular methods and found to contain class 1 integrons along with a β -lactamase-encoding *tem-1* gene. Transfer of antimicrobial resistance (ampicillin, streptomycin and sulphonamides), the *tem-1* gene and markers (*int1*, *qacE Δ 1*, *sul1*) characteristic of class 1 integrons were evident in one MDR isolate (resistant to 4 antimicrobial classes) when conjugation and transformation experiments were performed. A clinical isolate and a veterinary isolate of the O111 serotype were MDR and resistant to 4 and 3 antimicrobial classes respectively. These data suggests that the prevalence of antimicrobial resistance among the three VTEC serotypes examined in this study is low. These organisms may become a public health risk should they enter the food chain.

1 Introduction.

Cattle are considered to be a reservoir host of *Escherichia coli* O157:H7 and contaminated foods of bovine origin are important vehicles of human infection. Infection can manifest itself in severe and often fatal clinical symptoms, notably haemolytic uremic syndrome (HUS), which is characterised by the simultaneous occurrence of haemolytic anaemia, thrombocytopenia and renal failure (Weir, 2000). The low infectious dose and high virulence of *E. coli* O157:H7 make infections severe and life threatening, particularly for young children, the elderly and immuno-compromised individuals.

Multi-drug resistance (MDR) is more often reported amongst non-pathogenic *E. coli*, compared to the pathogenic verocytotoxigenic *E. coli* (VTEC) organisms (Mizan *et al.*, 2002; Sanchez *et al.*, 2002; Bettelheim *et al.*, 2003), independent of their source(s). Moreover, MDR-VTEC isolates reported now include both O157:H7 (Gallard *et al.*, 2001; Fitzgerald *et al.*, 2003) and non-O157 serotypes, [including O111, O26, O118, O103, O128 and O145] (Maidhof *et al.*, 2002; White *et al.*, 2002; Schroeder *et al.*, 2002b).

The use of antimicrobials in medicine (clinical and veterinary), coupled with their application in animal husbandry (often at a subtherapeutic levels), is regarded as a potential driving force for the selection of antimicrobial resistant bacteria. The increased use of antimicrobial agents has resulted in phenotypic changes, often due to chromosomal mutation(s) or the acquisition of extraneous DNA as part of mobile genetic element(s) such as plasmids or other related structures. Integrons are another type of genetic element, which are now recognised as efficient means by which resistance genes can be acquired through *in vivo* recombination and subsequently disseminated. Integrons can incorporate variable sized open reading frames (ORFs), gene cassettes, that encode antibiotic resistance determinants. One or more cassettes can be recombined within an integron. In addition to the latter, integrons also contain two domains, a 5'-conserved segment (CS) encoding an *intI* recombinase and a 3'-CS

containing two ORFs, *qacEAI* (resistance to quaternary ammonium compounds) and *sulI* (a sulphonamide resistant gene).

Increasing resistance to therapeutically valuable antimicrobial agents among pathogenic bacteria may lead to an increase risk of therapeutic failure. For *E. coli* O157:H7 infections, the use of antimicrobial therapy remains controversial, as these agents can lead to bacterial cell wall disruption and the eventual release of shiga toxins *in vivo* (Takahasdi *et al.*, 1997; Mack, 2000). Conversely, it has been reported that certain antimicrobials may also suppress the release of these toxins, thereby preventing the onset of haemolytic uremic syndrome (HUS) (Shioma *et al.*, 1999; Murakami *et al.*, 2000). Any change in susceptibility of the pathogens may comprise the use of antimicrobial agents.

In this study we examined the antimicrobial resistance profiles of 288 VTEC isolates cultured from the human food production, processing, distribution and the consumption chains, [i.e. veterinary, abattoir, retail, and human (clinical) sources] in Ireland. A genetic basis for the resistance profile was established for two MDR isolates (resistant to 10 and 6 antimicrobial agents) and their ability to transfer antimicrobial resistance was investigated.

2. Materials and Methods

2.1 Bacterial Strains.

VTEC isolates (288) examined in this study included *E. coli* O157:H7 (204) isolated from the hide of beef carcasses in Irish abattoirs (O'Brien *et al.*, 2005), *E. coli* O157:H7 (45) isolated from Irish ground beef (Cagney *et al.*, 2004) and clinical *E. coli* O157:H7 (8) obtained from a hospital in the Dublin area. In addition, this study also included non-O157 isolates, *E. coli* O111 (16) and *E. coli* O26 (15), cultured from a variety of Irish clinical and veterinary sources.

2.2 Antimicrobial Susceptibility Testing.

The resistance profiles of all VTEC isolates (288) were determined against a panel of 15 antimicrobial agents as described by Walsh *et al.*, (2001), using the standardised Bauer-Kirby agar disc diffusion technique. Lawn cultures of each isolate were prepared on Mueller Hinton Agar (Oxoid) following the NCCLS guidelines (Anon, 2004). Antimicrobial containing discs (Oxoid) used, included ampicillin (Amp 10 µg); cefachlor (Cec 30 µg); cefixime (Cfm 5 µg); chloramphenicol (C 30 µg); ciprofloxacin (Cip 5 µg); doxycycline (Do 30 µg); kanamycin (K 30 µg); minocycline (Mh 30 µg); moxalactam (Mox 30 µg); nalidixic acid (Na 30 µg); norfloxacin (Nor 10 µg); streptomycin (S 10 µg); sulfonamides (Su 300 µg); tetracycline (T 30 µg) and trimethoprim (W 5 µg) were carefully applied onto the lawn cultures and incubated for 24 h at 37°C. Zone diameters were measured and the susceptibility (or resistance) of each isolate to the panel of agents was determined. The controls strains used were *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Any isolate was

resistant to 3 or more different antimicrobial classes (Miragaia *et al.* 2002) was defined as multi-drug resistant (MDR).

2.3. Genomic DNA isolation.

Genomic DNA was purified from each isolate using the Wizard Genomic DNA purification kit (Promega, Madison, WI), according to the manufacturer recommendations. Following recovery of the template DNA, the corresponding DNA concentration was determined spectrophotometrically as previously described (O'Mahony *et al.*, 2005). The integrity of the purified template DNA was assessed by conventional agarose gel [1.5%, (w/v)] electrophoresis in 1 X tris-EDTA-acetic acid (TAE) buffer containing 0.5 µg/ml ethidium bromide (EtBr). DNA preparations were stored at 4°C.

2.3. PCR analysis.

Variable regions containing the characteristic gene cassette(s) associated with class 1 integron structures were amplified, as described previously (O'Mahony *et al.* 2005). Primer sets and thermal cycling conditions are outlined in Tables 1 and 2. The conserved structures of class 1 integrons, 5'- and 3'-CS domains were also assessed by PCR.

2.4. Amplification of β -lactamase-encoding *tem* gene by PCR.

Template DNA from the two MDR isolates (resistant to 7 and 4 antimicrobial classes) were examined for the presence of the *tem*-encoding β -lactamase, using the PCR primers and cycle conditions described in Table 1 and 2.

2.5. DNA sequence analysis of β -lactamase-encoding *tem-1* gene

Amplicons of interest were extracted directly from the agarose gel using a QIAGEN gel extraction kit (QIAGEN, West Sussex, UK). The extracted DNA fragment was purified and quantified (as described above) and sequenced commercially (Qiagen, Hilden, Germany). Amplicons were sequenced in both directions once. Sequence text files were subsequently obtained and used to search the current GenBank databases using the BLAST suite of programs (Altschul *et al.*, 1997). CLUSTALW amino acid sequence alignments were produced for comparison (Thompson *et al.*, 1994).

2.6. Investigation of antimicrobial transfer from 2 MDR *E. coli* O157:H7 isolates (resistant to 10 and 6 antimicrobials)

Each of the 2 MDR *E. coli* O157:H7 (donor stains) and recipient strain of *Salmonella* Derby were cultured in duplicate in 10 ml of LB Broth (Miller) for 24 h at 37°C. The recipient *S. Derby* strain was previously shown to be susceptible to all antimicrobials, to which the 2 donor MDR strains were resistant. *S. Derby* did not contain the β -lactamase gene *tem* or a class 1 integron. The overnight cultures were centrifuged at 15,000 g for 3 min and each pellet was resuspended in 3 ml sterile water. Each of the 2 MDR donor strains was combined separately with the *S. Derby* recipient strain in a 1:1 ratio. One hundred μ l aliquots were then plated out onto 10 previously dried Tryptone Soya Agar (Oxoid) plates and incubated for 24 h at 37°C. The resultant cultures were restreaked onto Xylose Lysine Desoxycholate (Oxoid), which after 2 h at 25°C was overlaid with TSA containing 100 μ g/ml⁻¹ ampicillin and incubated for a further 24 h at 37°C. Isolates of typical *Salmonella*

morphology (black colony with halo) were restreaked on XLD containing $100 \mu\text{g/ml}^{-1}$ ampicillin and incubated at 37°C for 24 h. Further confirmation tests were carried out on the resultant cultures.

Plasmid purification of the 2 MDR *E. coli* O157:H7 strains was carried out using a standard kit (Pure Link Hi Pure Plasmid Maxiprep kit, Invitrogen, USA). The resultant plasmid was then transformed into competent strains of *S. Derby* by heat shocking at 42°C for 2 min and followed by growth on TSA containing $100 \mu\text{g/ml}^{-1}$ ampicillin in accordance with Ausubel *et al.* (2002).

The transconjugant and transformed isolates were tested to ascertain their newly acquired antibiotic resistance profiles, by disc diffusion testing in accordance with the NCCLS standard. The DNA of these strains was once again purified and screened by PCR for the *tem* gene and class 1 integron associated genes as previously described.

3. Results

3.1. Antimicrobial Profiles.

The resistance profiles of the VTEC isolates examined are presented in Table 1. Four (1.4%) of the 288 VTEC isolates tested were found to be MDR. Among the *E. coli* O157:H7 isolates, 2 MDR isolates were cultured from retail mince-beef (ground beef). One was resistant to 10 antimicrobials (AmpCDoKMhNaSSuTW) belonging to 7 different antimicrobial classes and the other to 6 antimicrobials (AmpDoMhSSuT) belonging to 4 different antimicrobial classes. It was noted that considerable cross-resistance existed between tetracycline, doxycycline and minocycline.

All remaining *E. coli* O157 isolates from hides or from clinical sources (Table 1) were not classed as MDR since they were resistant to fewer than two antimicrobial classes. Two *E. coli* O111 isolates examined were MDR, one clinical (AmpNaSW) and one veterinary isolate (AmpSSu). Only 2 out of 15 *E. coli* O26 isolates (clinical and veterinary) examined showed antimicrobial resistance (tetracycline), all other isolates were found to be sensitive to all the antimicrobials used in this study.

The highest prevalence of resistance in the *E. coli* O157 minced beef isolates were to the antimicrobials, streptomycin (36% of all minced beef isolates), nalidixic acid (29%), doxycycline (9%) and tetracycline (9%). None of the 45 minced beef isolates were found to be resistant to ciprofloxacin, norfloxacin and moxalactam. Similarly, the highest prevalence of resistance in *E. coli* O157 bovine hide isolates was to streptomycin (8% of bovine hide isolates), followed by sulfonamides (6%) and kanamycin (6%). Streptomycin resistance was common (10%) in the clinical isolates of *E. coli* O157 (8), O111 (7) and O26 (6) examined, followed by ampicillin (5%), nalidixic acid (5%) and trimethoprim (5%). Clinical isolates were found to be completely susceptible to 11 of the examined antimicrobials. The highest prevalence of resistance amongst *E. coli* O111 (9) and *E. coli* O26 (9) veterinary isolates was also to streptomycin (17%), followed by sulfonamides (11%) and kanamycin (11%). All veterinary isolates were found to be susceptible to 9 of the antimicrobials tested. Overall the highest prevalence of resistance in all of the 288 isolates tested was found to the antimicrobial streptomycin (8%), followed by nalidixic acid (6%), sulfonamides (3%), kanamycin (2%) and tetracycline (2%). All 288 VTEC isolates were found to be susceptible to cefixime and ciprofloxacin.

3.2. Investigation of 2 MDR isolates for resistant isolates for integrons and β -lactamase-encoding tem-1 genes by PCR.

The genetic basis for antimicrobial resistance and transfer were explored in the 2 MDR *E. coli* O157:H7 mince beef isolates (resistant to 7 and 4 antimicrobial classes). Two MDR isolates of *E. coli* O157:H7 (resistant to 7 and 4 antimicrobial classes) were analysed for the presence of class 1 integrons and *tem-1*-encoding ORFs by PCR. Data obtained showed that 2 of the minced beef MDR *E. coli* O157:H7 isolates contained class 1 associated gene cassette structure. Both MDR isolates produced an amplicon of approx. 500 bp after agarose gel electrophoresis. These two MDR isolates were also found to contain *int1*, *qacEΔ1*, *sul1* markers characteristic of class 1 integrons (data not shown). In addition, these isolates were found to contain the *tem-1* gene. The *tem-1* gene confers resistance to β-lactam agents.

Sequence analysis of the putative *tem-1* gene amplified from the *E. coli* O157:H7 which was resistant to 7 antimicrobial classes, showed 98% sequence identity to *tem-1* from a *Serratia marcescens* isolate (accession number AB103506), a *Salmonella* Enteritidis isolate (accession number AB103092) and a plasmid (pINSRA99) located *tem-1* gene from *E. coli* (accession number AJ437107). BLAST searches with the putative *tem-1* gene from the *E. coli* O157:H7 MDR isolate (resistant to 4 antimicrobial classes), also confirmed the presence of a *tem-1* gene in this isolate. In the latter case, this *tem-1* displayed a 97% identity to other *tem-1* genes found within *E. coli* expression vectors such as pKM263-2xHMK (accession number AY428066).

3.3. Investigation of antimicrobial transfer from 2 MDR *E. coli* O157:H7 isolates (resistant to 7 and 4 antimicrobial classes))

The MDR *E. coli* O157:H7 strain resistant to 4 antimicrobial classes (AmpDoMhSSuT) transferred ampicillin resistance by conjugation with the *S. Derby* recipient strain. Ampicillin

resistance was also transferred by plasmid isolation from this MDR strain and transformation into a competent *S. Derby* strain. Disc diffusion showed that resistance to streptomycin and sulphonamides was transferred on both occasions, along with ampicillin resistance.

Resistance to tetracycline, minocycline or doxycycline was not transferred. PCR confirmed the presence of the *tem* gene and class 1 integron associated markers (*int1*, *qacEΔ1* and *sul1*) in the recipient strain, which was not present in *S. Derby* prior to conjugation or transformation.

The transfer of antimicrobial resistance was not evident by mating or transformation in the strain of *E. coli* O157:H7 resistant to 7 antimicrobial classes (AmpCDoKMhNaSSuTW).

4. Discussion

E. coli is commonly found in the gastrointestinal tracts of humans and animals. Various selective pressures in these environments favour the development, persistence and dissemination of robust strains some of which may be resistant to antimicrobial agents (Schroeder *et al.*, 2002a; 2002b). In this study, we observed antimicrobial resistance to several classes of antibiotics {including β - lactams, aminoglycosides, sulfonamides, quinolones, tetracyclines, cepheems, benzene derivatives (chloramphenicol) and macrolides}, and our observations are supported by those of others (Maidhof *et al.*, 2002; White *et al.*, 2002; Schroeder *et al.* 2002a; 2002b; Magwira *et al.*, 2005), confirming that resistance to a broad variety of antimicrobials classes can occur in VTEC. Among the 288 VTEC isolates in this collection (1.4% of which were MDR), two of 45 *E. coli* O157:H7 isolates and 2 strains of *E. coli* O111 exhibited an MDR phenotype, suggesting that MDR may be emerging across the range of VTEC (including O157 and non-O157) serotypes. This is in agreement with the literature (Schroeder *et al.*, 2002a; 2002b; White *et al.*, 2002), which reported a MDR *E. coli*

O118:H6 isolate carrying resistance to 8 antimicrobials (Maidhof, 2002). One of the MDR isolates from this study was resistant to 10 antimicrobial agents (AmpCDoKMhNSSuTW) belonging to 7 antimicrobial classes and the other to 6 antimicrobial agents, belonging to 4 antimicrobial classes. Multi-drug resistance to six or seven antimicrobial agents has been previously reported in *E. coli* O157:H7 (Giammanco *et al.*, 2002; Miwa, *et al.* 2002; Golding and Matthews, 2004). Detection of an isolate simultaneously resistant to 10 antimicrobials, especially from a retail meat source is a concern, as it may pose a risk to public health were these isolates to gain entry to the food chain. The therapeutic value of antimicrobial use in the case of VTEC infection requires careful consideration, however there is cautious optimism that with further research and observation, some antimicrobials be useful in the treatment of VTEC-related infections.

Further investigation was carried out on the two MDR *E. coli* O157:H7 strains (resistant to 7 and 4 antimicrobial classes), to identify the mechanisms responsible for resistance at the genotypic level. Both strains were found to contain class 1 integrons. These genetic structures have been previously reported in antimicrobial resistant *E. coli* (Du *et al.*, 2005; Box *et al.*, 2005) and VTEC isolates (Ahmed and Shimamoto, 2004; Ahmed *et al.*, 2005).

A β -lactamase gene *tem-1* was identified in both MDR *E. coli* O157:H7 isolates. The *tem-1* gene amplified from the *E. coli* O157:H7 resistant to 7 antimicrobial classes, showed 98% identity to a *S. Enteritidis* isolate and a similar gene located on a plasmid from *E. coli* and similarly the *tem-1* gene from the *E. coli* O157:H7 isolate resistant to 4 antimicrobial classes showed a 97% identity to other *tem-1* genes found within *E. coli* expression vectors. These results suggest that the *tem-1* is widely distributed in *Salmonella* and *E. coli*. This is in agreement with the literature, which suggests that *tem-1* is the most common plasmid-

mediated enzyme and confers resistance to ampicillin mainly in Gram-negative enteric organisms (Larson and Reuben, 2002).

This study showed the transfer of antimicrobial resistance by conjugation and transformation from one MDR strain of *E. coli* O157 to a *Salmonella* strain. The transfer of antimicrobial resistance in *Enterobacteriaceae* has been previously documented (Blake *et al.* 2003; Maisonnveuve *et al.* 2000; Yates *et al.* 2004). Singh *et al.* 2005 reported the transfer of antimicrobial resistance and class 1 integrons from 5 VTEC strains during conjugation. Resistance to 3 antimicrobials (ampicillin, streptomycin and sulphonamides) were transferred in this study. Similarly, Singh *et al.* 2005 reported the transfer of ampicillin, streptomycin and sulphonamides, among the antimicrobial resistance markers transferred during conjugation. The transfer of antimicrobial resistance (ASSu) and a class 1 integron markers during conjugation and transformation suggested that this resistance was plasmid-mediated. No evidence was found that antimicrobial resistance could be transferred from the other MDR *E. coli* O157 strain (resistant to 10 antimicrobial agents), suggesting that resistant in this instance may be chromosomal or possibly efflux pump related (Li *et al.*, 2004). This isolate is currently the subject of further research.

The overall prevalence of antimicrobial resistance in the types of VTEC examined in this study are low, however any increase in the numbers of these isolates being reported would pose serious clinical and therapeutic challenges in the future. VTEC typically colonise the gastro-intestinal tract of ruminant animals, which is an environment with the potential for considerable gene transfer within the *Enterobacteriaceae* family. Multi-drug resistance among foodborne *Enterobacteriaceae* poses a serious challenge to all public health professionals. Vigilant surveillance of these pathogens must be a food safety priority.

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TABLE 1. Antibiotic Resistance Profiles of Verocytotoxigenic *E. coli* (*E. coli* O157, *E. coli* O111 and *E. coli* O26), isolated from bovine hide, retail, clinical human and bovine veterinary sources.

Serotype	Source	No of <u>isolates</u> analysed	No and % of resistant isolates	R-type¶
O157	Minced Beef	45	1 (2%)	AmpCDoKMhNaSSuTW
O157	Minced Beef		1 (2%)	AmpDoMhSSuT
O157	Minced Beef		2 (4%)	DoST
O157	Minced Beef		12 (27%)	NaS
O157	Minced Beef		1 (2%)	CecSu
O157	Bovine Hide	204	2 (1%)	NaS
O157	Bovine Hide		1 (0.5%)	AmpCec
O157	Bovine Hide		1 (0.5%)	AmpK
O157	Bovine Hide		1 (0.5%)	MoxSu
O157	Bovine Hide		1 (0.5%)	KSu
O157	Bovine Hide		1 (0.5%)	SSu
O157	Bovine Hide		1 (0.5%)	Cec
O157	Bovine Hide		1 (0.5%)	K
O157	Bovine Hide		1 (0.5%)	Mh
O157	Bovine Hide		1 (0.5%)	Nor
O157	Bovine Hide		1 (0.5%)	S
O157	Bovine Hide		1 (0.5%)	T
O157	Clinical	8	1 (13% %)	S
O111	Clinical	7	1 (14%)	AmpNaSW
O111	Veterinary	9	1 (11%)	AmpSSu
O111	Veterinary		1 (11%)	KSSu
O111	Veterinary		1 (11%)	KS
O111	Veterinary		1 (11%)	Mh
O26	Clinical	6	none detected	none detected
O26	Veterinary	9	2 (22%)	T

Notes:

¶ (Amp) ampicillin, (C) chloramphenicol, (Cec) cefachlor, (Do) doxycycline, (K) kanamycin, (Mh) minocycline, (Mox) moxalactam, (Na) nalidixic acid, (Nor) norfloxacin, (S) streptomycin, (Su) sulfonamides, (T) tetracycline and (W) trimethoprim

Multi-drug resistant strains (resistant to 3 or more different antimicrobial classes) are shown in **bold**.

TABLE 2. Sequences and corresponding citations for oligonucleotide primers

Genes		Sequence 5'→3'	References
<i>intl1</i>	Fwd	GGC ATC CAA GCA GCA AGC	Levesque <i>et al.</i> , 1995
	Rev	AAG CAG ACT TGA CCT GAT	
<i>sull</i>	Fwd	CTT CGA TGA GAG CCG GCG GC	Sundstorm <i>et al.</i> , 1998
	Rev	GCA AGG CGG AAA CCC GCG CC	
<i>qacEΔ1</i>	Fwd	ATC GCA ATA GTT GGC GAA GT	Stokes and Hall, 1989
	Rev	CAA GCT TTT GCC CAT GAA GC	
<i>Tem</i>	Fwd	TTG GGT GCA CGA GTG GGT TA	Arlet and Philippon, 1991
	Rev	TAA TTG TTG CCG GGA AGC TA	

TABLE 3. Thermocycler amplification conditions

Genes	Denaturation	Annealing	Extensions	No. of Cycles
<i>intl1</i>	95°C-1 min	55°C-1 min	72°C-2 min	30
<i>sull</i>	95°C-1 min	65°C-1 min	72°C-2 min	30
<i>qacEΔ1</i>	95°C-1 min	55°C-1 min	72°C-2 min	30
<i>Tem</i>	94°C-1 min	54°C-1 min	72°C-30 sec	30