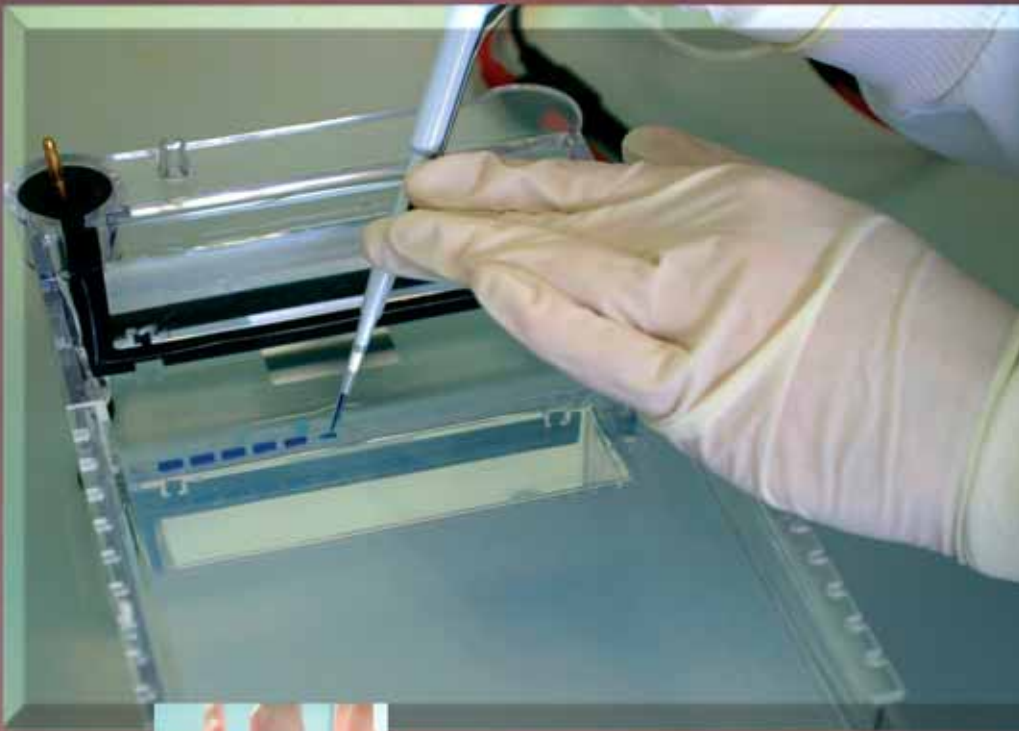


The virulence of *E. coli* O157:H7 isolated from Irish sheep and pigs to humans



THE VIRULENCE OF *E. COLI* O157:H7
ISOLATED FROM IRISH SHEEP AND PIGS
TO HUMANS

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CONTENTS

Summary	1
Introduction	1
Objectives of the project	2
Incidence of <i>E. coli</i> O157:H7 on lamb carcasses, in faeces and fleece	2
Incidence of <i>E. coli</i> O157:H7 on pig carcasses and in faeces	7
Molecular characterisation of <i>E. coli</i> O157:H7 isolates	9
Conclusions	12
Recommendations to industry	12
References	13

SUMMARY

Investigations were carried out at five sheep and five pig export abattoirs situated in the Republic of Ireland to determine the prevalence of *E. coli* O157:H7 in these animals at slaughter. This is the first study for the presence of *E. coli* O157:H7 on sheep and pigs to be carried out in Ireland. Faeces and pre- and post-chill carcass swabs were collected from pigs over a one year period between January and December 2004. Samples were collected from sheep over a 13-month period between February 2005 and February 2006. The pig study recovered *E. coli* O157:H7 from 0.24 % (n=4) of 1680 porcine samples while the sheep study isolated the pathogen from 2.1 % (n=33) of 1600 ovine samples. PCR analysis of *E. coli* O157:H7 isolates determined that they carried the virulence genes *vt1*, *vt2*, *eaeA* and *hlyA* typically associated with clinical illness in humans. The results presented indicate that Irish sheep and pigs are reservoirs for *E. coli* O157:H7 which may be potentially harmful to humans.

INTRODUCTION

Escherichia coli O157:H7 has emerged as a highly-virulent food poisoning pathogen which causes a wide range of symptoms from mild, non-bloody diarrhoea to haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). Pathogenicity of VTEC strains is mainly associated with formation of attaching-and-effacing lesions (*eaeA*), production of one or both verotoxins (*vt1* and *vt2*) and production of haemolysin (*hlyA*) (Paton and Paton, 1998).

Cattle have been reported to be a major reservoir of *E. coli* O157:H7 (Heuvelink *et al.*, 1998; Chapman *et al.*, 2001); however, recent studies suggest that other ruminants, especially sheep, may be important in contributing to human clinical cases (Chapman *et al.*, 2000; Chapman *et al.*, 2001). The presence of *E. coli* O157:H7 has been reported in sheep in the United States (Kudva *et al.*, 1996), United Kingdom (Chapman *et al.*, 2001) and The Netherlands (Heuvelink *et al.*, 1998). Sheep faeces have previously

been linked to two clinical outbreaks, both occurring in Scotland (Licence *et al.*, 2001; Ogden *et al.*, 2002).

Recent research indicates that pigs are also potential carriers of *E. coli* O157:H7 but the incidence is generally much lower than in ruminants. The presence of *E. coli* O157:H7 in pigs has been reported in the United States (Feder *et al.*, 2003), Norway (Johnsen *et al.*, 2001), The Netherlands (Heuvelink *et al.*, 1998) and Chile (Borie *et al.*, 1997). Although pigs are not considered a primary reservoir of the organism, they may play a role as sporadic shedders when cattle are the major reservoir present on the same farm. Studies in Norway and Sweden suggest that keeping pigs and ruminants in mixed farms could be a risk factor for establishing *E. coli* O157:H7 in pigs, especially if there are no hygienic barriers between the different species (Johnsen *et al.*, 2001).

OBJECTIVES OF THE PROJECT

Previous studies on *E. coli* O157:H7 in Ireland have focused on cattle and a nationwide study of the pathogen's presence on pigs and sheep has not been carried out. The objectives of this project were i) to determine the prevalence of *E. coli* O157:H7 on ovine and porcine pre- and post-chill carcasses, in faeces and fleece, ii) to investigate the presence or absence of virulence genes linked to human illness in the isolates obtained and iii) to determine from the virulence profiles of *E. coli* O157:H7 recovered by the present study whether these isolates are likely to cause human clinical infection.

INCIDENCE OF *E. COLI* O157:H7 ON LAMB CARCASSES, IN FAECES AND FLEECE

Five sheep export abattoirs slaughtering 250 sheep/hour were each visited four times over a 13-month period between February 2005 and February 2006. Three of the abattoirs slaughtered both cattle and sheep (A, C, & E) while the remaining two (B & D) slaughtered only sheep. In each abattoir,

animals were chosen at random for sampling. At each visit, 20 pre- and 20 post-chill carcass swabs, 20 faecal and 20 fleece samples were taken to determine the presence of *E. coli* O157:H7. A total of 1600 samples were examined over the course of this study.

Whole body pre-chill carcass swabs were taken from eviscerated animals on the line immediately after final carcass washing with cold chlorinated water. Whole body post-chill carcass swabs were taken from a different set of animals held in a chill for 24 h at a mean temperature of 4°C. Faecal samples were obtained during evisceration by slitting the distal colon with a sterile scalpel and aseptically removing approx 1g of faeces. Fleece samples of approx 10g were taken randomly from the rump area on line before pelt removal. All samples were transported to the laboratory in cool boxes containing ice packs and stored in a chill (1°C) overnight for analysis within 24 h.

For isolation of *E. coli* O157:H7, samples were enriched in a 1:10 dilution of buffered peptone water (BPW) supplemented with the antibiotics vancomycin, cefixime and cefsulodin (VCC). Immunomagnetic separation (IMS) was used to recover *E. coli* O157:H7 from enriched BPW-VCC samples after 6 h incubation at 37°C (Chapman *et al.*, 2000). After IMS processing, bead/sample suspensions were plated in duplicate onto Sorbitol MacConkey agar (SMAC) supplemented with cefixime tellurite (CT) and incubated at 37°C for 24 h. Non-sorbitol fermenting colonies suspected to be *E. coli* O157:H7 were biochemically confirmed on Levines Eosin Methylene Blue agar (EMB), Phenol Red Sorbitol agar supplemented with 4-methylumbelliferyl-B-D-Glucuronide (Oxoid) (PRS-MUG) and Tryptone Tryptophane medium (TT) and all were incubated at 37°C for 24 h. All biochemically confirmed isolates were examined for agglutination with an *E. coli* O157:H7 latex agglutination kit. All presumptive *E. coli* O157:H7 isolates were examined using PCR and phage typing techniques to determine their virulence to humans.

The study detected *E. coli* O157:H7 in 5.75 % (23/400) of fleece samples, 1.5 % (6/400) of pre- and 1 % (4/400) of post-chill carcass swabs. The pathogen

was not isolated from any of the 400 faecal samples examined in this study. The abattoir, isolation date and sample type found positive for *E. coli* O157:H7 are detailed in Table 1. The failure to isolate *E. coli* O157:H7 from faeces in the present study was considered unusual, especially since the pathogen was found on the fleece as well as on carcasses. Previous studies have found the pathogen in sheep faeces (Kudva *et al.*, 1996; Heuvelink *et al.*, 1998; Chapman *et al.*, 2001) but a Norwegian study did not (Johnsen *et al.*, 2001). The lack of recovery of *E. coli* O157:H7 in faeces by the present study may be due to removal of faecal samples from the distal colon compared to other studies that examined faecal samples taken at the rectoanal junction (Low *et al.*, 2005).

An alternative explanation for the absence of *E. coli* O157:H7 from faeces may be related to the methodology used. The enrichment method used in the present study, involving the use of BPW-VCC with incubation at 37°C for 6h, has been used extensively in the isolation of *E. coli* O157:H7 from faeces of cattle and sheep (Chapman *et al.*, 2001; McEvoy *et al.*, 2003; Rey *et al.*, 2003; Ogden *et al.*, 2005). Recently it has been suggested that the isolation of *E. coli* O157:H7 from faeces should be carried out by enrichment in a non-selective medium, such as tryptone soya broth plus phosphate buffer (TSB+PO₄) for 6h at 42°C, followed by IMS (Barkocy-Gallagher *et al.*, 2005). The basis of this and similar techniques is that the sensitivity of the enrichment procedure is improved and that injured and stressed pathogen cells are capable of survival and growth in these conditions, which may not occur in the presence of different cocktails of antibiotics, such as those used in the present study. Low levels of injured *E. coli* O157:H7 cells have recently been detected in bovine faeces (Scott *et al.*, 2006). In addition, it appears that the prevalence of this pathogen is generally more common in bovine than in sheep faeces, making the detection of injured cells very important in indicating the presence of this organism in sheep (Paiba *et al.*, 2002; Keen *et al.*, 2006). The enrichment method used in the present study may not therefore have been sufficiently sensitive to detect low levels of the organism and in particular to detect injured cells.

Table 1 The sheep abattoir, isolation date and sample type positive for *E. coli* O157:H7

<i>Isolate Code</i>	<i>Abattoir</i>	<i>Isolation date</i>	<i>Sample type</i>
1	A	08 Feb 2005	Pre-chill carcass swab
2	A	03 May 2005	Fleece
3	A	03 May 2005	Post-chill carcass swab
4	A	03 May 2005	Fleece
5	A	03 May 2005	Fleece
6	E	18 May 2005	Fleece
7	E	18 May 2005	Fleece
8	C	23 May 2005	Fleece
9	B	29 Jun 2005	Pre-chill carcass swab
10	B	29 Jun 2005	Post-chill carcass swab
11	B	29 Jun 2005	Fleece
12	B	29 Jun 2005	Fleece
13	E	08 Aug 2005	Post-chill carcass swab
14	C	19 Sept 2005	Pre-chill carcass swab
15	C	19 Sept 2005	Pre-chill carcass swab
16	C	19 Sept 2005	Pre-chill carcass swab
17	C	19 Sept 2005	Pre-chill carcass swab
18	B	04 Oct 2005	Fleece
19	B	04 Oct 2005	Fleece
20	B	04 Oct 2005	Fleece
21	B	04 Oct 2005	Fleece
22	B	04 Oct 2005	Fleece
23	B	04 Oct 2005	Fleece
24	E	07 Nov 2005	Fleece
25	C	05 Dec 2005	Fleece
26	C	05 Dec 2005	Fleece
27	C	05 Dec 2005	Fleece
28	C	05 Dec 2005	Fleece
29	C	05 Dec 2005	Post-chill carcass swab
30	B	30 Jan 2006	Fleece
31	B	30 Jan 2006	Fleece
32	B	30 Jan 2006	Fleece
33	B	30 Jan 2006	Fleece

The monthly prevalence of *E. coli* O157:H7 ranged from 0 to 6.25 % with none recovered during the months of March, April and July 2005 and February 2006 (Figure 1). There was no evidence of seasonal variation of the pathogen in the present study. This was not surprising given that the majority of studies on seasonal variation are based on the presence of the organism in animal faeces (Chapman *et al.*, 2000; McEvoy *et al.*, 2003; Kudva *et al.*, 1996; Chapman *et al.*, 2001).

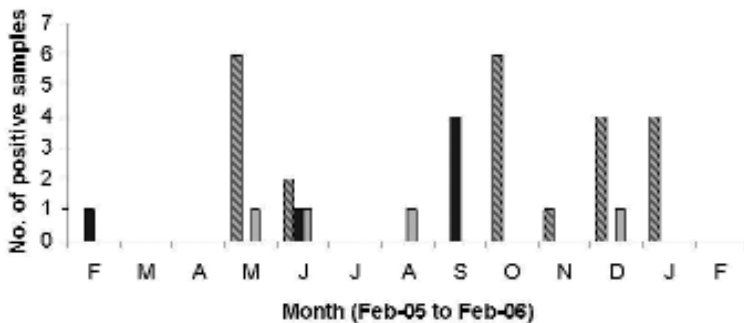


Figure 1: Number of fleece (▨), pre- (■) and post-chill (■) carcass swabs *E. coli* O157:H7 isolated from each month. *E. coli* O157:H7 was not found in any faeces samples.

E. coli O157:H7 was isolated at four of the five abattoirs visited, with abattoir B having the highest number of positive samples, while none were recovered at abattoir D (Figure 2). This absence at abattoir D was unusual and a satisfactory explanation has not been found. The difference may be attributed to a number of factors that varied between the five abattoirs. Samples were taken from three abattoirs that slaughtered both cattle and sheep (A, C & E) while the remaining two abattoirs (B and D) slaughtered sheep only. Other differences between abattoirs included geographic location, animal origin (*e.g.* from mixed or non-mixed farms), animal handling, transport of animals to the factories, lairage design, cleaning and disinfection practices.

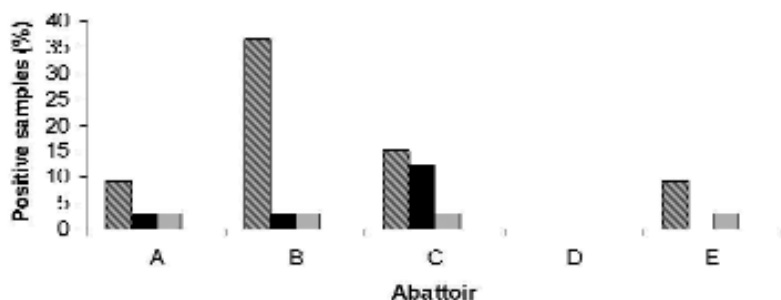


Figure 2: The percentage of fleece (▨), pre- (■) and post-chill (■) carcass swabs found positive for *E. coli* O157:H7 at each abattoir. *E. coli* O157:H7 was not found in any faeces samples.

INCIDENCE OF *E. COLI* O157:H7 ON PIG CARCASSES AND IN FAECES

Four pig abattoirs (A, B, C, D) were each visited four times and a fifth abattoir (E) three times over twelve months from January to December 2004. Four of the abattoirs slaughtered 250 pigs per hour and the fifth 75 pigs per hour. At each visit, 30 faecal samples, 30 pre-chill and 30 post-chill whole body carcass swabs were collected for examination for the presence of *E. coli* O157:H7. In total, 1140 carcass swabs and 540 faecal samples were thus examined.

Whole body pre-chill carcass swabs were taken from eviscerated animals on the line immediately after final carcass washing with cold chlorinated water. Whole body post-chill carcass swabs were taken from a different set of animals held in chill for 24 h at a mean temperature of 4°C. Faecal samples were obtained during evisceration by slitting the distal colon with a sterile scalpel and removing approx 1g of faeces aseptically. All samples were transported to laboratory in cool boxes containing ice packs and stored in a chill (1°C) overnight for analysis within 24 h.

For isolation of *E. coli* O157:H7, samples were enriched in a 1:10 dilution of brilliant green bile broth (BGBB) (Feder *et al.*, 2003). Samples were processed using IMS to recover *E. coli* O157:H7 from enriched BGBB samples after 6 h incubation period at 37°C. Bead/sample suspensions were plated in duplicate onto Sorbitol MacConkey agar (SMAC) supplemented with cefixime tellurite (CT) and incubated at 37°C for 24 h. Suspect non-sorbitol fermenting colonies were biochemically confirmed as described above on EMB, PRS-MUG and TT and checked for agglutination with a *E. coli* O157:H7 latex agglutination kit. All presumptive *E. coli* O157:H7 isolates were examined using PCR, PFGE and phage typing techniques to determine their virulence to humans.

The survey detected *E. coli* O157:H7 in 0.56% (3/540) of faecal samples and in 0.18% (1/570) of post-chill carcass swabs. No *E. coli* O157:H7 was isolated from 570 pre-chill carcass swabs. All samples found to contain *E. coli* O157:H7 were taken during the third quarter of the sampling period from July to September 2004 (Table 2). Of the four isolates recovered, two were found in faecal samples taken during the same visit to abattoir A. The other two isolates recovered from a faecal sample and post-chill carcass swab were isolated during the same visit to abattoir B. The present study did not recover sufficient *E. coli* O157:H7 isolates to establish whether a pattern of seasonal variation exists in pigs.

Table 2 The pig abattoir, isolation date and sample type positive for *E. coli* O157:H7

Isolate Code	Abattoir	Isolation date	Sample type
1	A	17 August 2004	Faeces
2	A	17 August 2004	Faeces
3	B	02 September 2004	Faeces
4	B	02 September 2004	Post-chill carcass

MOLECULAR CHARACTERISATION OF *E. COLI* O157:H7 ISOLATES

Presumptive *E. coli* O157:H7 isolates were subjected to polymerase chain reaction (PCR) analysis to confirm the presence of the O157 antigen and H7 flagellum, and to determine presence or absence of 11 other genes that encode proteins considered to have a role in clinical illness caused by *E. coli* O157:H7. The isolates were examined for a range of genes. The formation of attaching and effacing (A/E) lesions (*eaeA* intimin gene) where the pathogen adheres tightly to the intestinal epithelial cells and production of verotoxins 1 (*vt1* gene) and 2 (*vt2* gene) have been directly linked to haemolytic uremic syndrome and bloody diarrhoea in human patients (Paton and Paton, 1998). The presence of the TIR, *espA*, *espB*, *espF* and *espP* genes belonging to the type III secretion system (TTSS) of *E. coli* O157:H7 was also investigated. The TTSS delivers several effector proteins to the cytoplasm of the host epithelial cells that subvert, inhibit or activate cellular processes and play an essential role in the infection process (Garmendia *et al.*, 2005).

Of the 33 ovine *E. coli* O157:H7 isolates recovered, five carried both the *vt1* and *vt2* genes typically associated with human illness caused by *E. coli* O157:H7 while 24 of the remaining isolates had only the *vt2* gene present. Four isolates did not have either verotoxin gene present while all 33 isolates carried the *eaeA* gene for intimin (Table 3). Examination of the ovine *E. coli* O157:H7 PCR profiles revealed that four isolates had the *vt1* and *vt2* genes missing. In addition, six of isolates also had all of the type III secretion system (TTSS) genes investigated missing (*i.e.* *espA*, *espB*, *espF* and *espP*). Of the six isolates not possessing the *esp* genes, three carried the *vt2* gene which is strongly associated with human illness (Boerlin *et al.*, 1999). However, without the *esp* genes present, these three isolates are unlikely to be pathogenic as they would be unable to successfully attach to the intestinal epithelium and deliver toxin into the host cell. Similarly, the three remaining isolates negative for *vt1*, *vt2*, *espA*, *espB*, *espF* and *espP*, are considered to be non-pathogenic to humans.

Of the four porcine *E. coli* O157:H7 isolates, the virulence profiles for three faecal isolates contained all the genes investigated by PCR while one carcass isolate contained less than half the genes detected in the faecal samples (Table 3). All isolates recovered contained the *vt1*, *vt2* (except the carcass swab isolate), *eaeA*, and *hlyA* genes. A similar genetic profile was found in *E. coli* O157:H7 recovered from pigs in the United States (Feder *et al.*, 2003). Results from the present study indicate that although the incidence in Irish pigs is low, the genetic profile and phage types of the *E. coli* O157:H7 isolates suggest it is potentially pathogenic to humans.

The genetic profile of *E. coli* O157:H7 isolates recovered from both sheep and pigs in the present study were typical of those isolated from Irish patients suffering clinical illness. The predominant *E. coli* O157:H7 PCR profile causing clinical infections in Ireland has the toxin genotype of *vt2* alone, with smaller percentages carrying genes for both *vt1* and *vt2* (Carroll *et al.*, 2005; Garvey *et al.*, 2006). Phage-typing of the ovine and porcine isolates showed that 16 were PT 32 which was the predominant phage type (63.5%) in clinical patients in the Republic of Ireland in 2004 (Carroll *et al.*, 2005) while six isolates were PT 8, which was the second most common phage type (10.5 %) isolated from Irish clinical patients in 2004 (Carroll *et al.*, 2005). These data suggest that the most common phage types found in sheep and pigs in Ireland are also associated with clinical disease in human. Analysis of isolates by pulse field gel electrophoresis (PFGE) demonstrated a wide range of patterns among the porcine and ovine *E. coli* O157:H7 isolates indicating a wide diversity of *E. coli* O157:H7 clones circulating within these food animals in Ireland.

Table 3 Virulence profiles and phage types of *E. coli* O157:H7 isolates from sheep and pigs

No. of Isolates	Sample Type	Source	Phage Type	Virulence Genes*																
				rfbO157	flhC _{h7}	vt1	vt2	eeA	hlyA	TIR	katP	espA	espB	espF	espP	etpD				
4	Fleece	Sheep	PT 8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1	Post CS	Sheep	PT 32																	
3	Faeces	Pig	PT 32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	Fleece	Sheep	PT 32	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Post CS	Sheep	PT 31																	
1	Pre CS	Sheep	PT 21/28																	
4	Fleece	Sheep	PT 32	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	Pre CS	Sheep	PT 32																	
1	Post CS	Sheep	PT 32																	
1	Pre CS	Sheep	PT 32	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	Pre CS	Sheep	† NT	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	Post CS	Sheep	PT 21/28																	
1	Fleece	Sheep	PT 21/28	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	Fleece	Sheep	NT	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	Post CS	Sheep	NT	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	Pre CS	Sheep	NT	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	Pre CS	Sheep	NT	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	Fleece	Sheep	NT	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	Post CS	Pig	NT	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* Gene present: + Gene absent: - † Isolate was not phage-typed

CONCLUSIONS

The highest incidence of the pathogen was found in sheep. The pathogen was not isolated from any of the 400 faecal samples examined in this study. This was considered unusual as the pathogen was present on the fleece (5.75 %) and carcass (1.25 %). This finding may be explained by differences in methodology applied in this and other studies or the location in the sheep colon from which faecal samples were removed for examination.

The incidence of *E. coli* O157:H7 was found to be much lower in pigs (0.24 %) and these animals are not considered to be a likely reservoir for *E. coli* O157:H7 infection in Ireland. Previous research has indicated that pigs reared in close contact to ruminants could become potential carriers of the pathogen due to cross contamination but the small prevalence found in pigs here suggest that it is not a common problem in Ireland.

When compared to human isolates, the virulence profiles and phage types of ovine and porcine *E. coli* O157:H7 were genetically-similar to those causing clinical infections in Ireland. PCR analysis to determine the presence or absence of important virulence markers such as the intimin gene for adhesion and both verotoxin genes suggest that most of the *E. coli* O157:H7 isolates are pathogenic to humans. In addition, phage-typing data indicate that the most common phage types found in sheep and pigs in Ireland are the same as for clinical *E. coli* O157:H7 isolates.

RECOMMENDATIONS TO INDUSTRY

The low number of *E. coli* O157:H7 isolates found on post-chill pig and lamb carcasses suggest that chilling could be used as a critical control point (CCP) to control the pathogen on chilled carcasses provided that the chilling regime was tightly regulated. Within the EU, HACCP (hazard analysis and critical control point) systems for the slaughter process do not extend to carcass-chill procedures. Other countries have successfully incorporated chilling into a HACCP plan to ensure a safe product for human consumption. Australian standards require the surface temperature of carcasses, sides, quarters or bone-

in major separated cuts to be reduced to 7°C or below within 24 hours of stunning. The legislation also provides for plants that find it difficult to operate to this standard (e.g. those boning heavy cattle) by allowing them to make an application for an alternative carcass-chilling procedure to suit individual situations, which would then be validated and approved by the relevant food safety authority. Chilling is frequently included as a CCP for slaughter plants in the United States but studies carried out at these plants suggest it cannot be relied upon to consistently reduce carcass contamination levels as the carcasses are not commercially refrigerated in a uniform manner. However, it has been observed that chilling can be an effective additional stress to kill microbial cells injured from previous decontamination treatments (e.g. thermal interventions such as hot water or steam pasteurisation).

Under commercial conditions, the use of chilling as a CCP in combination with other on-line interventions shown to kill or injure bacterial cells may be suitable to significantly reduce carcass contamination levels. For the successful implementation of chilling as a CCP to control the growth of pathogens on carcasses, critical limits would need to be established during chilling and the effectiveness of the carcass-chilling procedure validated at each plant. Carcass-chilling facilities have different capabilities and there are often variations between chillers on the same plant. Therefore, each situation would need to be assessed individually in order to meet food safety standards.

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