END OF PROJECT REPORT

Horizontal transmission of *Escherichia coli* O157:H7 during cattle housing, survival kinetics in feces and water of *Escherichia coli* O157:H7 and characterisation of *E. coli* O157:H7 isolates from cattle faeces and a feedlot environment

December 2005

Grange Beef Research Centre and the National Food Centre
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1. Overall summary

Escherichia coli O157:H7 can cause severe illness and in some cases leading to death. Cattle are the main reservoir with transmission to humans occurring through contamination of food or the environment. Improved understanding of the survival and transmission and survival of *E. coli* O157:H7 on the farm is essential for developing future controls of this pathogen. This study showed that transmission of *E. coli* O157:H7 can occur rapidly in groups of housed cattle, with contamination of the pens and hides occurring in 24 hrs. The inoculation dose for cattle is lower than previously reported. Ingestion of bacteria from the hide through social grooming is important for pathogen transmission in housed cattle along with faecal contamination of the environment. Sampling hide will improve the estimation of prevalence of *E. coli* O157:H7 in pens.

The survival of *E. coli* O157:H7 in water and faeces was assessed using laboratory prepared bacteria and bacteria, which had survived passage through the digestive tract. *E. coli* O157:H7 survival in low nutrient conditions, such as water, is enhanced after passage through the GI tract. The effect of different diets on the survival and transmission of the bacteria was examined and not found to be of significance. The findings of this these studies suggest that many factors affect the spread of *E. coli* O157:H7 on the farm. Attention to hygiene, particularly in feedlot or housed cattle may reduce the risk of transmission. Caution must be taken when extrapolating laboratory results to on-farm situation and it is recommended to use naturally occurring bacteria for future experiments. This research also detected numerous multiply antibiotic resistant non-O157 VTEC in cattle in 2 geographical locations. These rare serotypes have been previously implicated in human disease and have never been previously identified in Ireland.

2. Study 1

Running title: Transmission of *E. coli* O157:H7 in cattle

Horizontal transmission of *Escherichia coli* O157:H7 during cattle housing

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Short Title: Transmission of *E. coli* O157:H7 in cattle

Keywords: *E. coli* O157:H7, EHEC, cattle, feces, transmission, hide, animal grooming

2.1. Abstract

Ruminant livestock, particularly cattle, are considered the primary reservoir of *E. coli* O157:H7. This study examined the transmission of *E. coli* O157:H7 within groups of cattle during winter housing. Holstein Friesian steers were grouped in 6 pens of 5 animals. An animal inoculated with, and proven to be shedding a marked strain of *E. coli* O157:H7 was introduced into each pen. Fecal (rectal swabs) and hide samples (900cm^2 right rump) were taken from the thirty six animals throughout the study. Water, feed and gate/partition samples from each pen were also examined. Within 24h of introducing the inoculated animals into the pens, samples collected from the drinking water, pen barriers and animal hides were positive for the pathogen. Within 48h, the hides of 20 of 30 (66%) cohort animals from the 6 pens were contaminated with *E. coli* O157:H7. The first positive fecal samples from the non-inoculated cohort animals were detected three days after the introduction of the inoculated steers. Over the 23 days of the study 15 of 30 cohort animals shed the marked *E. coli* O157:H7 strain in their feces on at least one occasion. Animal behavior in the pens was monitored over a 12h period using CCTV cameras. The camera footage showed an average of 13 instances of animal grooming in each pen per hour. The study suggests that transmission of *E. coli* O157:H7 between animals may occur following ingestion of the pathogen at low levels, and that animal hide may be an important source of transmission.

2.2. Introduction

The foodborne pathogen, *E. coli* O157:H7 poses a significant health risk to consumers and consequently, a considerable economic threat to the beef industry. Serious human illness including bloody diarrhea, hemolytic uremic syndrome and in some cases death, have been attributed to this organism since it was first reported in 1982 (17). Consumption of beef and beef products contaminated with *E. coli* O157:H7 is a major cause of human infection worldwide (15, 17). However, more recently, numerous outbreaks have been reported due to direct contact with animals and animal feces (11, 12, 19, 21, 23). Cattle are regarded as the primary reservoir of *E. coli* O157:H7, with fecal prevalence rates of 2 to 24% being reported (16, 29). Colonization of the gastrointestinal tract appears to be transient, with typical shedding patterns involving long periods of low level shedding, punctuated by short periods of high level shedding (2, 28).

It is generally accepted that the greatest capacity for pathogen control is through the implementation of an integrated farm to fork approach. Several strategies aiding the reduction of *E. coli* O157:H7 carcass contamination have been introduced during the slaughter process, such as steam pasteurization and organic acid washes (15). In contrast, control strategies are not so advanced at farm level, where the primary sources of *E. coli* O157:H7 are found. This is mainly due to an insufficient understanding of the ecology of the pathogen on the farm, despite over twenty years of research. Although an abundance of studies have examined the prevalence of *E. coli* O157:H7 in the feces of cattle of different type (beef/dairy), age and from different regions, few studies specifically examine the modes of transmission of the organism between cattle under natural conditions. Several studies have looked at the transmission of verotoxigenic *E. coli* between small groups of calves or sheep under artificially controlled conditions (3, 7, 30, 36), however this may not adequately represent cattle to cattle transmission on the farm. The objective of this study was to define the principal means of transmission of *E. coli* O157:H7 between adult cattle and to elucidate the role of the environment in its spread.

2.3 Materials and Methods

Organism. A bovine fecal *E. coli* O157:H7 (VC 047) strain, isolated during a survey of an Irish cattle feedlot, was used throughout the study (29). The strain was negative for both *vt*1 and *vt*2 genes but possessed the genes encoding enterohaemolysin *A* and *eae*, as determined by PCR. To aid detection, the strain was made resistant to streptomycin sulphate (1000µg ml^{-1}) and nalidixic acid (50 µg ml^{-1}) as described by Park (31), and stored on cryoprotective beads (Technical Services Consultants, Lancashire, UK) at –20°C.

Description of the animals and housing facilities. Thirty six Holstein Friesian steers, aged 6 – 9 months, were selected by weight (289 ± 21 kg) and randomly assigned to one of two treatment groups, A and B. Group A was divided into 3 pens, each containing 5 animals and 1 pen of 3 animals (to be used for inoculation). The animals were adapted to an ad-lib barley based concentrate diet for 3 weeks. Group B was divided into a further 3 pens, each with 5 animals and 1 pen of 3 animals (to be used for inoculation). These animals were adapted to an ad-lib grass silage diet for 3 weeks. A plastic tag on the right ear individually identified all animals. One week prior to the start of the study, feces from each animal was screened for *E. coli* O157:H7 to ensure that they were not shedding the organism and that they did not contain enteric bacteria possessing the same antibiotic resistance profile as the marked pathogen. The animals were housed indoors in pens with concrete slatted flooring, enclosed by a 2-meter high solid timber partition on three sides of each pen. The feed barrier was to the front of each pen with feed offered on the concrete solid passage. The timber partitions extended either side of the feed area, ensuring that no direct contact was possible between pens. Each pen had an individual water supply. Personnel did not enter the pens except during sampling, when disposable protective clothing was used as described below. On day 16, closed circuit television cameras (Eneo, Germany) were fitted over 2 pens from each diet group, to continuously monitor animal behavior over a 24-hour period. The number of times grooming occurred was recorded each hour during this period. The impact of fasting on the shedding of *E. coli* O157:H7 was examined by withdrawing feed from all animals on day 21 for a 24-hour period.

Throughout the experiment, the ambient temperature (°C) in the shed was recorded on an hourly basis using dataloggers (Testostor 175, UK).

Preparation of Inoculum. One bead containing *E. coli* O157:H7 was aseptically transferred into 9ml Brain Heart Infusion broth (BHI, Oxoid) and incubated at 37°C for 24 h. From this culture 1 ml was transferred into 100ml BHI, and incubated at 37°C for 18 hours to achieve a stationary phase culture. The culture was centrifuged at 3000g for 10 min (Eppendorf Centrifuge 5403, Eppendorf, Hamburg, Germany), washed three times in Maximum Recovery Diluent (MRD, Oxoid) and the resultant pellet resuspended in 10 ml MRD. A 1ml aliquot of this suspension, containing approximately 10^{10} cfu of *E. coli* O157:H7, was added to 50ml of sterile distilled water (SDW).

Inoculation Procedure. Six animals (3 concentrate diet, 3 silage diet) were inoculated orally by syringe (Plastitek 60ml, Becon and Davidson, Dublin) with 50 ml SDW containing approximately 10^{10} cfu of *E. coli* O157:H7. Immediately post inoculation (p.i.) each animal was dosed with 1 liter SDW to wash the culture down. Fecal samples from the 6 inoculated animals were examined 24h p.i. for the presence of the marked strain, using the methods described below. All 6 animals were shedding the marked organism. One animal shedding *E. coli* O157:H7 was then placed into each of the 6 pens of five uninoculated steers consuming the same diet. The inoculated animal (876), from pen 2 in the concentrate group (Table 1) escaped from its pen on day 20 of the experiment. It was omitted from the experiment for the final three days

Enumeration of *E. coli* O157:H7. Different collection and initial processing procedures were used for each sample type, and are described in detail below. However, a common procedure for direct plating and enrichment of *E. coli* O157:H7 was used for all samples. To determine direct counts of *E. coli* O157:H7 present in each sample, aliquots were plated in duplicate onto Sorbitol MacConkey agar (SMAC, Oxoid) containing nalidixic acid (50µg/ml) and streptomycin sulphate (1000µg/ml) (SMAC-nas) and incubated at 37°C for 48h. Aliquots were also plated onto Tryptone Soy Agar (TSA, Oxoid), incubated at 37°C for 2 hrs, over-poured with SMAC-nas and reincubated for a further 48 hrs to allow for the recovery of injured cells. The direct counts reported throughout the paper were derived using the latter method. Samples for enrichment were incubated for 24h at 37°C without agitation, and then plated onto SMAC-nas, and reincubated at 37°C for 48h
For all samples, identification of suspect positive colonies was confirmed by latex agglutination (Wellcolex *E. coli* O157:H7, Murex, UK). In addition, colonies from positive samples selected at random were confirmed by polymerase chain reaction (PCR), as previously described (32).
Sampling procedure. To avoid cross contamination between pens during sampling,

separate disposable overalls and shoe covers were worn in each pen. Latex gloves were worn and changed following collection of each sample. All samples were placed on ice immediately after collection, and transported to the laboratory within 2 hours.

Fecal samples. Fecal samples (approximately 0.1g) were obtained from the rectum of each animal using 2 cotton tipped swabs (Bibby Sterilin, Staffordshire, UK) inserted simultaneously while the animal was eating at the feed barrier. Following withdrawal, individual swabs were immediately placed into 2 sterile 30ml tubes (Sterilin) containing 5ml volumes of Modified Tryptone Soya Broth (mTSB, Oxoid), plus nalidixic acid (50µg/ml) and streptomycin sulphate (1000µg/ml), (mTSB-nas).

On return to the laboratory, each tube was vortexed for 1 min. A dilution series in 9ml volumes of MRD was performed, from one sample prior to direct plating. The duplicate swab was enriched at 37°C for 24h. The detection limit for direct counts and enriched fecal samples was, $\log_{10}$ 1.4 cfu g^{-1} and $\log_{10}$ 0.95 cfu g^{-1} respectively. This was calculated by spiking feces in the laboratory with known numbers of *E. coli* O157:H7 - VC 047. Fecal samples were taken on days 0, 1, 2, 3, 4, 7, 9, 10, 11, 14, 15, 16, 21, 22, 23.

Hide samples. Hide samples, approximately 900cm^2, were taken from the right hand rump area of each animal, using individual sterile cellulose sponges (Sydney Heath & Son Ltd., Staffordshire, UK), moistened with 10 ml MRD and placed into sterile stomacher bags (Seward Laboratory, London, UK). On return to the laboratory, sponges were placed in 90ml of mTSB-nas and stomached in a Colworth Stomacher (Model BA 6024, A. J. Steward & Co.Ltd. London, UK) for 30 sec. Samples were direct plated on days 0 and 2 and enriched on days 1, 4, 7, 10, 15 and 23. The limit of detection for recovering direct numbers of *E. coli* O157:H7 from the hide was calculated to be -1.25 $\log_{10}$ cfu cm^2.

Water troughs. Sterile 150ml containers (Sterilin, Staffordshire, UK) were used to remove 25ml samples from the water trough of each pen. On return to the laboratory, samples were filtered through a 0.2µm-pore size filter (Sartorius, Goettingen, Germany). The filter was placed in 20ml of mTSB-nas and vortexed for 1min. All samples were plated directly and enriched on days 1, 2, 3, 4, 7, 9, 14 and 23. The limit of detection for direct counts was –0.4 $\log_{10}$ ml^{-1}. Sediment samples, approximately 10g, were taken from each water trough on day 7, and added to 90ml mTSB-nas, prior to enrichment.

Feed samples. Samples of feed, approximately 50g of both silage and concentrates, were collected from each pen on day 3, and placed in sterile 150ml containers (Sterilin Staffordshire, UK). Samples were added to 200ml volumes of mTSB-nas, stomached for 1 min. and enriched.

Pen barrier. An unlimited area of wooden partition and metal feed barriers from each pen were swabbed using the sponge method described above. The sponge was added to 90ml mTSB-nas and stomached for 1 min. prior to enrichment. Samples were taken on days 1, 2, 4, 7 and 23.

Overshoes. On exiting the pens post sampling, disposable overshoes were removed and placed in a sterile stomacher bag. On return to the laboratory, 90ml mTSB-nas was added to each bag, stomached for 1 min. and then enriched. Overshoes were sampled on day 9.

2.4 Results

All animals remained healthy for the duration of the experiment. The fecal shedding patterns of the six inoculated animals are presented in Table 1. At 24h p.i., the three inoculated animals consuming the concentrate and silage diets shed the marked *E. coli* O157:H7, at concentrations of 5.3 - 6.2 $\log_{10}$ g^{-1} and 4.5 - 5.1 $\log_{10}$ g^{-1}, respectively. When these inoculated animals were introduced to the 6 pens (48h p.i.), each containing 5 uninoculated cohorts, *E. coli* O157:H7 was detected in the inoculated animals' feces at concentrations of 2.7 - 4.2 and 3.8 - 4.0 $\log_{10}$ g^{-1} for the concentrate and silage fed, animals respectively. Over the following 23 days, positive *E. coli* O157:H7 fecal samples were detected sporadically from the 6 inoculated animals (Table 1).

Table 2 presents the fecal shedding pattern of the cohort animals from the 6 pens. Three days after the introduction of the inoculated animals, two of five cohort animals from one of the concentrate pens had fecal samples positive for the marked organism. Within 4 days at least one cohort animal tested positive in each of the six treatments and 6 of 15 from the silage treatments tested positive at least once for the *E. coli* O157:H7 isolate. Generally the cohorts did not shed the organism on consecutive sampling days. Three of 9 cohort animals on the concentrate diet, tested positive on two or more sampling occasions. One cohort animal tested positive on days 3, 16, 21, and 22. Two of 6 cohort animals on the silage diet tested positive on two occasions.

E. coli O157:H7 was detected only by enrichment (0.95 - 1.4 $\log_{10}$ g^{-1}) in 13 of the 15 positive cohort animals. One cohort animal in the concentrate treatment shed the marked organism at 4.2 and 3.8 $\log_{10}$ g^{-1} on days 21 and 22 respectively, having previously tested positive by enrichment on days 3 and 16. Another cohort animal in a separate pen but on the same diet shed the organism at 3.0 $\log_{10}$ g^{-1} on one occasion.

Table 3 summarizes the results for hide, water, and pen barrier samples. On introduction to the pens (48h p.i.), the hide of 5 of 6 inoculated animals was positive for the organism, at levels ranging from 1.2 to 4.0 $\log_{10}$ cm^2. Within 24h in pen 1 of the concentrate group, all five cohort animals had positive hide samples. After 48h, 20 of 30 hides of cohort animals were positive for the marked *E. coli* O157:H7. On that day, direct counts of the organism were calculated for the 36 animals, with low levels ranging from –0.82 to –0.20 $\log_{10}$ cm^2 detected. Throughout the experiment at least one positive hide sample was detected from 14 of 15 and 12 of 15 cohorts on the concentrate and silage treatments respectively.

Two of three water troughs in pens housing the animals fed concentrates, were positive 24h after the introduction of the shedding animals to the pens. All three water troughs from animals fed silage diets had at least one positive sample by day 4. Water samples were positive following enrichment (< -0.4 $\log_{10}$ ml^{-1}), with the exception of pen 3 from the concentrate treatment, in which concentrations of 1.4 $\log_{10}$ ml^{-1} were detected on day 1.

On exiting each pen on day 9, overshoes were examined for *E. coli* O157:H7. Two of three, and one of three samples were positive from the concentrate and silage diet pens respectively, following enrichment.

Feed samples taken from each pen on day 3 were negative.

Data from the video recording of cattle behavior were collected from a 12 hour period as the video quality was too poor to analyze during the night. The average number of times per hour that an animal groomed another in any pen was 12.5 (range 0 – 27 times an hour).

The average ambient temperature recorded in the shed throughout the study period was 5.4°C (0°C – 12.4°C).

2.5 Discussion

E. coli O157:H7 has been isolated from multiple sources on the farm (16, 18, 33), with some studies citing the surface of pens, animal feed and water in particular, as important sources for transmission of the pathogen (7, 16, 33, 35). In the present study, the introduction of the animals inoculated with *E. coli* O157:H7 caused rapid contamination of the environment, with the first positive pen barrier

and water samples detected within 24 hours. However, although contamination occurred rapidly, the number of positive samples from these sites decreased as the study progressed. Feed from each pen was sampled on one occasion and was negative, although multiple sampling may have detected a positive sample . In order to investigate the potential for animal handlers to act as vectors of *E. coli* O157:H7 between groups of cattle, overshoes were sampled on exiting the pens with three positive samples detected. Other studies have implicated animal handlers in the transmission of verotoxigenic *E. coli* between groups of animals (7). In the present study this mode of transmission was prevented as access to the pens was prohibited except on sampling days, when disposable clothing and overshoes were worn.

While extensive environmental sampling has been carried out in many other studies, sampling of the animal hide is often not done or only examined at time of slaughter (1, 15, 37). The results of the current study showed that in some pens, all hides were positive for *E. coli* O157:H7 24 hours after the introduction of the inoculated animal, proving that one animal shedding *E. coli* O157:H7 has the potential to contaminate the hides of several cohorts (25). It was also observed that on seven different sampling days *E. coli* O157:H7 was detected on the hide of at least one animal in a pen even though all fecal cultures from that pen were negative on that day. This is in agreement with findings of surveys in which *E. coli* O157:H7 was present in 5.9% of cattle fecal samples and 60.6% of hide samples (1). These findings suggest that *E. coli* O157:H7 contamination of the hide cannot be accurately interpreted from fecal sample results. As animal hide is regarded as a major source of pathogens on beef carcasses (1, 15, 34), it would seem prudent to determine the prevalence of *E. coli* O157:H7 on hides in addition to feces, in order to estimate the potential risk of carcass contamination.

The fact that some cohort animals began to shed the pathogen in the latter days of the study, when water and pen barrier samples were negative, suggested that the hide may be an important source of the pathogen for the animals. The results from the camera footage suggested that the time spent on social grooming (average of 13 instances an hour through all pens) was sufficient to ingest *E. coli* O157:H7 present in small amounts of fecal matter on the hide, thus causing colonization of the cohort animals. Therefore, it is reasonable to assume, particularly in housed animals where a greater level of animal grooming would be expected, that the hide may be one of the most significant sources of animal to animal transmission.

The infective dose of *E. coli* O157:H7 for cattle has been the subject of many experiments. Generally in animal inoculation experiments cattle are inoculated with high levels of *E. coli* O157:H7 (10 $\log_{10}$ cfu) in order to achieve colonization, and it has been assumed that these levels of laboratory grown strains of *E. coli* O157:H7 are the 'infective dose' for cattle on the farm. It is reported that when experimental inoculation doses are reduced below this level, variable success rates in animal colonization are achieved. Cray and Moon (9), showed that inoculation with doses of 7 $\log_{10}$ cfu *E. coli* O157:H7 was insufficient to infect all cattle, while no shedding was recorded from 5 cattle inoculated with 4 $\log_{10}$ cfu. Shere *et al*. (36), inoculated calves at 6 $\log_{10}$ cfu but did not cause shedding in all calves despite repeated doses. In this study the cohort cattle began to shed the organism when fecal shedding from inoculated steers was detectable only by enrichment or when *E. coli* O157:H7 was found solely on the hide. Given the low numbers of the organism present on the hides, the 'inoculation dose' of *E. coli* O157:H7 ingested by the cohort animals was low, probably in

the region of 2 – 3 $\log_{10}$ cfu. Horizontal transmission of *E. coli* O157:H7 has been previously reported in sheep when the animals were shedding less than 4 $\log_{10}$ cfu/g feces or at levels only detectable by enrichment (8, 26). Elsewhere, in poultry the colonization potential of a laboratory *Campylobacter* isolate was increased 10,000 fold, following a single passage *in vivo* (6). The results from these studies suggest that passage of a pathogen through the digestive tract may enhance it's ability to colonise the species concerned, when compared to a laboratory grown strain. Consequently, significantly lower numbers of *E. coli* O157:H7 may be sufficient to colonise animals under natural conditions on the farm. This would not be unexpected given that the ingestion of 10 $\log_{10}$ cfu *E. coli* O157:H7 from the farm environment would be virtually impossible. Therefore the 'form' of the inoculum used in experimental inoculation studies, i.e. laboratory grown or naturally occurring bacteria, may warrant further investigation.

The effects of diet on the fecal shedding patterns of animals colonized with *E. coli* O157:H7 have been the subject of much debate. Some studies have suggested that hay fed cattle shed *E. coli* longer and in higher numbers than if fed on grain (20, 27) while others have suggested the opposite is true (14, 24). In this study 6 and 9 cohorts on the silage and concentrate diets respectively, shed the organism in their feces on at least one occasion. It was also observed that one inoculated animal fed the silage diet ceased shedding after day 6, while a cohort animal on the concentrate diet commenced shedding *E. coli* O157:H7 in high numbers towards the end of the study . A definitive conclusion regarding the effect of diet on fecal shedding patterns cannot be established due to the small number of animals involved in this study. Large sample sizes would be required to allow for variations in animal shedding patterns, which are influenced by factors such as microbial flora, age and immune status (38, 22, 10).

Feed withdrawal commonly occurs in beef cattle as a result of time spent during transport and in lairage prior to slaughter. The present study found no clear effect of fasting on *E. coli* O157:H7 shedding which is similar to previous studies (4, 9, 26, 29). This is in contrast to other bacteria such as *Salmonella* spp., where a fasting period leads to a marked increase in fecal shedding of this organism (5). It is therefore unclear whether fasting of cattle will lead to an increased incidence of *E. coli* O157:H7 in the feces and subsequent carcass contamination.

This study has provided further insight into the ecology of *E. coli* O157:H7 on the farm. It was observed that cohort animals began to shed *E. coli* O157:H7 in their feces when the pathogen was being shed at very low levels by their inoculated pen mate, or was present solely in the pen environment. The study suggests that the contamination of animal hide, in conjunction with animal grooming, may be an important source of *E. coli* O157:H7 for cattle, particularly if the infectious dose is lower than previously thought. Further work is necessary to establish the dose required to infect cattle with *E. coli* O157:H7 having passed through the animal, as opposed to with laboratory grown cultures.

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Table 1: Detection of *E. coli* O157:H7 in fecal samples of inoculated cattle on two different diets, over a 25 day period.

Pen	Animal ID	Time (days)																
		-1 [a]	**0**	**1**	**2**	**3**	**4**	**7**	**8**	**9**	**10**	**11**	**14**	**15**	**16**	**21**	**22**	**23**
concentrate diet																		
1	**608**	5.3 [b]	3.7	3.5	+[c]	2.0	+	_[d]	1.4	+	__	+	2.7	1.7	+	+	+	__
2	**876**	6.2	2.7	2.4	__	+	+	+	__	__	__	1.4	__	+	+	NS	NS	NS
3	**898**	5.9	4.2	__	+	3.7	4.3	2.4	+	+	+	__	__	+	__	+	__	__
grass silage diet																		
1	**533**	5.1	3.8	3.5	+	+	+	__	__	__	__	__	__	__	__	__	__	__
2	**887**	4.5	3.8	4.3	+	4.5	3.4	+	+	+	__	__	+	__	__	+	+	+
3	**463**	4.6	4.0	4.2	+	4.0	4.0	4.0	3.8	3.3	+	+	+	__	__	+	__	__

[a]: Time –1 indicates 24 hours post inoculation, Time 0 indicates 48 hours post inoculation when each inoculated animal was placed in pen of uninoculated cohorts.
[b]: Direct counts $\log_{10}$ *E. coli* O157:H7 g^{-1} (detection limit $\log_{10}$ 1.4 cfu g^{-1})
[c]: Positive samples detected by enrichment only (detection limit $\log_{10}$ 0.95 cfu g^{-1})
[d]: *E. coli* O157:H7 not isolated from the sample
NS, Not sampled

Table 2: *E. coli* O157:H7 fecal shedding patterns of cohort animals (assigned individual tag numbers) over a 23 day period, following natural infection from exposure to an experimentally inoculated pen mate

Pen	Time (days)															
	0 [a]	**1**	**2**	**3**	**4**	**7**	**8**	**9**	**10**	**11**	**14**	**15**	**16**	**21**	**22**	**23**
							concentrate diet									
1	— [b]	—	—	—	44[c]	—	—	—	26	**41**	—	—	—	—	—	—
2	—	—	—	—	**28**	**37**	—	—	—	—	—	—	—	—	—	**28**
3	—	—	—	**32, 38**	—	—	—	—	—	—	—	**38**	**32, 25**	32	32, 31	**38**
grass silage diet																
1	—	—	—	—	02	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	12	—	—	—	—	01	—	—	—	—	—	—
3	—	—	—	—	15, 17	—	11	15	—	—	—	—	—	—	—	11

[a]: Time 0 indicates when an inoculated animal was placed in a pen of uninoculated cohorts.
[b]: *E. coli* O157:H7 not isolated from any cohort animal
[c]: ID of animal when positive samples were detected by enrichment (detection limit $\log_{10}$ 0.95 cfu g^{-1}).

Table 3: Positive *E. coli* O157:H7 hide and environmental samples recovered over a 23 day period, following the introduction of 1 experimental inoculated animal into six pens of 5 uninoculated cohort animals

Pen	Time (days)								
	1 [a]	**2**	**3**	**4**	**7**	**10**	**14**	**15**	**23**
concentrate diet									
1	H (6/6)[b] W, P	H(4/6) P	__[c]	H (4/6) P[d]	W[e]	H (6/6)	__	H (3/6)	H (1/6)
2	H (2/6) P	H (3/6) P	__	**H (1/6)**	**H (4/6) W, P**	H (1/6)	__	__	__
3	W, P	H (6/6) W, P	**W**	H (4/6) W, P	H (6/6) W, P	H (5/5)	__	**H (6/6)**	**H (1/6)**
grass silage diet									
1	__	H (5/6)	W	H (1/6) W	__	H (1/6)	__	H (1/6)	__
2	__	H (2/6)	W	P	H (3/6)	H (3/6)	__	H (1/6)	__
3	H (1/6) P	H (6/6) W, P	__	H (3/6) P	H (3/6)	H (3/6)	__	H (1/6)	__

[a]: Time 1, 24 hours after an inoculated animal was placed in a pen of uninoculated cohorts.
[b]: Number of hide samples positive for *E. coli* O157:H7. Hides sampled on day 1, 2, 4, 7,10, 15 and 23
[c]: No positive samples on this day
[d]: *E. coli* O157:H7 detected on pen barrier. Samples taken on day 1, 2, 4, 7 and 23
[e]: *E. coli* O157:H7 detected in water. Samples taken on day 1, 2, 3, 4, 7, 9, 14 and 23

3. Study 2

A comparison of the survival in feces and water of *Escherichia coli* O157:H7, grown in laboratory conditions or obtained from cattle feces

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Short Title: Survival of *E. coli* O157:H7 in feces and water
Keywords: *Escherichia coli* O157:H7, survival, water, feces, cattle, animal passage

3.1 Abstract

E. coli O157:H7 is an important food borne pathogen that can cause hemorrhagic colitis and hemolytic uremic syndrome. Cattle feces and fecally contaminated water are important in the transmission of this organism on the farm. This study compared the survival of *E. coli* O157:H7 in feces and water following passage through the animal digestive tract or preparation in the laboratory. Feces were collected from steers before and after oral inoculation with a marked strain of *E. coli* O157:H7. Fecal samples collected prior to cattle inoculation were subsequently inoculated with the marked strain of *E. coli* O157:H7 prepared in the laboratory. Sub-samples were taken from both animal and laboratory-inoculated feces in order to inoculate 5-litre volumes of water. *E. coli* O157:H7 in feces survived up to 97 days and survival was not affected by the method used to prepare the inoculating strain. *E. coli* O157:H7 survived up to 109 days in water with the naturally-prepared organism detected up to 10 weeks longer than the laboratory-prepared inoculum. This study suggests that pathogen survival in low nutrient conditions may be enhanced by passage through the gastrointestinal tract.

3.2 Introduction

Escherichia coli O157:H7 is an important zoonotic pathogen that can cause a range of symptoms from bloody diarrhea to hemolytic uremic syndrome. Cattle are considered the primary source of this pathogen, although other farm animals have also been implicated in outbreaks of disease (6, 22, 28, 31). Fecal material is considered an important vehicle for transmission of *E. coli* O157:H7 and it is well documented that infection may result from contact with fecal matter on the farm and through contaminated foodstuffs or water supplies (2, 8, 9, 16, 19). One of the distinguishing characteristics of this pathogen is that it has a low infective dose, with the ingestion of as few as 10 colony-forming cells required to cause disease in humans (30). Investigation of the survival of *E. coli* O157:H7 in water and feces is therefore vital, as persistence of even small numbers of this organism is significant. Occasionally *E. coli* O157:H7 strains derived directly from animal sources have been used in survival experiments (21), however more often the strains used are adapted to laboratory conditions. A comparison of the survival of isolates derived directly from the animals or following preparation in the laboratory has not been performed. Enhanced bacterial virulence after passage through the gastrointestinal tract (GI) has been previously reported using a *Camplylobacter* isolate (5, 20), and a

recent study proposed that a lower inoculation dose of naturally occurring *E. coli* O157:H7 cells could colonize cattle (26). These findings suggest that bacteria obtained directly from the farm environment in which they have been subjected to stressful conditions, may have an enhanced ability to survive, in comparison to bacteria cultured in the laboratory under optimal conditions. The objective of this study was to determine if an *E. coli* O157:H7 isolate grown in the laboratory had a similar survival time, in feces and water, as an isolate that had been ingested by cattle and survived passage through the GI tract.

3.3 Materials and Methods

Organism. A bovine fecal, non-toxigenic strain of *E. coli* O157:H7 was used throughout the study. To aid detection, the isolate was made resistant to streptomycin sulphate (1000μg ml^{-1}) and nalidixic acid (50 μg ml^{-1}) as previously described (27). The antibiotic resistant strain used in the current study was labeled as VC 047 and stored on cryoprotective beads (Technical Services Consultants, Lancashire, UK) at -20°C. A comparison was previously performed between the survival of an *E. coli* O157:H7 strain marked as described above and the parent strain, with no differences observed (10). Under field conditions such as comparison could not be made.

Description of Animals. Eight Holstein Friesian steers were used, aged 9 – 12 months. The steers were randomly assigned to one of 2 groups of 4 and adapted over 1 month to a diet of either ad lib silage or ad lib concentrates. One week prior to the start of the study and 24 hours prior to inoculation with strain VC 047, feces from each animal were taken and tested, to ensure that they were not shedding *E. coli* O157:H7, or enteric bacteria with resistance to nalidixic acid and streptomycin sulphate.

Inoculation of Animals. Cells from strain VC 047, were washed and centrifuged as previously described (26). A 1 ml aliquot of the inoculum suspension was added to 50 ml of sterile distilled water (SDW). A total of 50 ml SDW, containing approximately 10^{10} cfu of *E. coli* O157:H7, was administered *per os* to each of the 8 animals using a syringe (Plastitek 60ml, Becon and Davidson, Dublin). Immediately post inoculation (p.i.), each animal was dosed with 1 litre SDW to ensure none of the inoculum was lost.

Collection of feces pre-inoculation. Approximately 600g of feces was collected from the rectum of each animal 24 hours prior to inoculation with *E. coli* O157:H7. The feces fecal material was stored in separate covered plastic containers in a shed for 3 days, at 7 – 10° C, after which it was inoculated with strain VC 047 prepared in the laboratory as described below.

Collection of feces post inoculation. A further 600g of feces was taken from each animal 24 hrs p.i. and the numbers of the marked strain of *E. coli* O157:H7 determined, using the methods described below. Duplicate 200g portions were then removed from each 600g sample, and placed into sterile plastic containers. These samples are referred to as 'naturally-inoculated' feces.

Inoculation of feces with a laboratory strain. Duplicate 200g samples derived from the 8 fecal samples collected pre-inoculation, were placed into sterile plastic containers. These samples were inoculated in the laboratory with strain VC 047, at a level similar to that present in the 'naturally-inoculated' feces of the corresponding animal. In brief, the organism was grown in Brain Heart Infusion broth (BHI, Oxoid) to stationary phase at 37°C, centrifuged and washed, as previously

described (26). A 10ml volume ofMaximum Recovery Diluent (MRD, Oxoid) containing a known concentration of *E. coli* O157:H7 was added to the duplicate samples from each animal, and mixed with a sterile spatula. These samples were referred to as 'laboratory inoculated' and stored with the 'naturally-inoculated' feces in a shed. Samples were taken once a week from each of the 32 fecal samples for the duration of the experiment.

Source of water. Farm water in 5 litre volumes was collected from an untreated river water source and stored in covered plastic containers in a shed for not more than 3 days prior to inoculation with strain VC 047. The water was not tested for the presence of naturally occuring *E. coli* O157:H7.

Inoculation of water samples. Feces containing *E. coli* O157:H7 (VC 047) were used to inoculate the water samples. Duplicate 100g samples of 'naturally-inoculated' feces were collected from 6 animals, 3 from the group of 4 fed a concentrate diet and 3 from the group of 4 fed a silage diet. Each sample was mixed into 12 separate 5-litre water samples (2%w/v), which were subsequently known as 'naturally-inoculated water'. Similarly, duplicate 100g samples of feces inoculated in the laboratory, taken from the 6 corresponding animals, were added to a further 12 water samples and known as 'laboratory-inoculated water'. The containers were sealed and stored in a shed for the duration of the study. Samples were taken twice weekly for 14 days and once a week thereafter.

Enumeration of *E. coli* O157:H7 in fecal samples. A sterile wooden spatula was used to mix each 200g fecal sample. A 5 g sample was removed, added to 45 ml MRD and stomached for 90 seconds in a Colworth Stomacher (Model BA 6024, A. J. Steward & Co.Ltd., London, UK). From each sample, a dilution series was performed in 10ml volumes of MRD. Aliquots were plated onto Sorbitol Maconkey agar (SMAC, Oxoid), containing nalidixic acid (50µg/ml) and streptomycin sulphate (1000µg/ml) (SMAC-nas) and incubated for 24h at 37°C. Aliquots were also plated onto Tryptone Soy Agar (TSA, Oxoid), incubated at 37° C for 2 hrs, over-poured with SMAC-nas and reincubated for a further 48 hrs to allow for the recovery of injured cells. The detection limit was 0.7 $\log_{10}$ cfu g^{-1}. VC 047 cells were identified as pale, non sorbitol-fermenting colonies on SMAC-nas. When the numbers of organisms per sample had declined to levels such that quantitative enumeration was no longer possible, samples were examined using enrichment only. Samples were enriched by removing a 5ml volume from the original 1:10 MRD solution, and adding it to 45 ml Modified Tryptone Soya Broth (mTSB, Oxoid), containing nalidixic acid (50µg/ml) and streptomycin sulphate (1000µg/ml). (mTSB-nas). Samples were incubated for 24h at 37°C. Aliquots from the enriched samples were plated in duplicate onto SMAC-nas. Enrichment was carried out until the organism could not be detected on two consecutive sampling days.

Enumeration of *E. coli* O157:H7 in water samples. The 5-litre volumes of water were agitated for approximately 30 seconds prior to sampling. Sterile 150ml containers (Sterilin, Staffs, UK) were used to remove 45 ml samples, which were taken immediately to the laboratory. To each sample, 5ml of a 5% flocculant solution (Zetag 7899, General Chemicals, Dublin) was added to enable filtering of the water samples. The water and flocculant mix was centrifuged for 1 minute at 5000g (Eppendorf Centrifuge 5403, Hamburg, Germany) causing the formation of a fecal pellet and clear supernatant. The supernatant was decanted through a 0.2 µm-pore-size filter (Sartorius, Goeftingin, Germany). The filter was then returned to the fecal pellet, to which 15ml MRD was added, and vortexed for 1

minute. From each sample a dilution series in 9ml volumes of MRD was performed and plated as described for the fecal samples. The detection limit was 0.16 cfu ml^{-1}.

For enrichment of the water samples, 30 ml of mTSB was added to the filter and fecal pellet, and incubated at 37°C for 24 hours. Aliquots from the enriched samples were plated in duplicate onto SMAC-nas and reincubated for a further 48 hrs. Samples were cultured using enrichment until the organism was undetectable on two consecutive sampling days

Both fecal and water survival experiments were conducted from February to the beginning of June. Throughout the experiment, the ambient temperature (° C) in the shed where the samples were stored was recorded on an hourly basis using a datalogger. (Testostor 175, UK).

Statistical analysis. The experiment was performed in duplicate and repeated 3 and 4 times for the water and fecal survival experiments respectively. Linear regression was used to analyze the changes in *E. coli* O157:H7 levels over time. For the fecal samples, the period before the commencement of decline was omitted from analysis, with initial decline determined as the time point from which a >0.5 log reduction in numbers occurred. The comparison of the rates of decline between treatments i.e. the slopes of the lines was carried out using Students t- test. Significant differences between treatments were determined to be those with 1% level of significance ($p<0.01$).

3.4 Results:

It was observed throughout this study that while *E. coli* O157:H7 counts determined using SMAC were generally lower than those recovered using TSA over-poured with SMAC (TSA/SMAC) the differences were not statistically significant (Figure 1). Therefore, the results presented below are for TSA/SMAC data only.

It was observed that the period of time that elapsed before numbers of *E. coli* O157:H7 in feces began to decline ranged from 27 to 55 days, depending on diet and method of inoculation (Table 1). Although counts decreased more rapidly in silage compared to concentrate, for both methods of inoculation (Figure 1), the differences were not significant, as indicated by the slopes for the different treatments (Table 1). Regression analysis indicated that there was no difference in pathogen survival between the two methods of inoculation, irrespective of diet.

When the survival of the organism in water was examined, the line of best fit to the data was linear. Decreases in counts occurred over a period of 46 days and differences between the rates of decline for the two methods of inoculation were not significant, for both diets (Figure 2).

From day 54 onwards, the organism was detected by enrichment only for a total of 63 days, but this survival time varied depending on the method of inoculation and diet (Table 2). The results are represented as individual replicates, because insufficient quantitative data was available to permit presentation as averages. During this time (day 54 – day 122) *E. coli* O157:H7 was detected for a longer period in naturally inoculated water, irrespective of diet. In the concentrate treatment this was most pronounced in replicate 1 where the pathogen survived until day 109, almost 10 weeks longer than the laboratory inoculated water. In replicate 1 of the experiment for the silage group, *E. coli* O157:H7 remained in the naturally inoculated water for 49 days longer than the pathogen in the laboratory inoculated samples. Despite the fact there was considerable variation between replicates, survival was always greater in the naturally inoculated water.

The average ambient temperature during the course of this experiment was 10° C (4.5 – 20.3 °C). The average weekly maximum and minimum temperatures are presented in Figure 3.

3.5 Discussion

The findings of this study indicate that irrespective of diet, (concentrate or silage), survival of *E. coli* O157:H7 in water was enhanced as a result of being passaged through the bovine GI tract. In bovine feces, however, survival of the pathogen was not affected as a result of being passed through the animal.

The survival times of *E. coli* O157:H7 in water under various conditions has been reported to vary from 13 to 245 days (21, 23). Research investigating the survival of *E. coli* O157:H7 in this environment is important, due to the many outbreaks associated with contaminated water (4, 14, 17, 22). In addition water troughs have been implicated in the maintenance of this organism on the farm (12). Many factors have been examined in relation to *E. coli* O157:H7 survival in water, such as temperature, inoculum level and fecal contamination (24, 33). However, the effect of passage of the pathogen through the GI tract has not been addressed previously. The results from the current study show, that in water, a naturally prepared inoculum was detected for longer than a laboratory prepared inoculum, during the last 10 weeks of the experiment. For some replicates, the natural inoculum was still present after 109 days, while the laboratory inoculum was undetectable by day 46. These results suggest that *E. coli* O157:H7 cells, which have survived transit through the testing environment of the bovine digestive system, are better prepared to endure harsh environments, such as water, than cells grown in optimum conditions in the laboratory.

The most severe stress that cells passed through the GI tract have to overcome is the acidity of the bovine abomasum (pH 2.5 – 3.0). Bearson et al (1) proposed that acid stress adaptation in *E. coli* can provide cross protection against a wide range of other stresses. It is suggested that this cross protective mechanism is caused by the induction of protective proteins on the cell membrane (7). These proteins are also essential for the maintenance of cell viability during periods of starvation, such as occurs in water (29). In the current study, it is reasonable to assume that the cross protective effect may have extended the survival in water, of cells passaged through the animal. This supports previous reports of enhanced bacterial virulence after passage through the digestive system (5, 20). In this context, additional research is required to investigate the precise bacterial changes at a cellular level. In addition, the results of the present study indicate that the survival of *E. coli* O157:H7, prepared in the laboratory, will not always mirror the survival of a naturally occurring pathogen such as would be found in fecally contaminated water on farm. This is an important consideration when conducting experiments to accurately predict the survival *E. coli* O157:H7 in the farm environment.

E. coli O157:H7 is well adapted to survival for extended storage periods in bovine feces and slurry, with detectable numbers often present over many months (3, 13, 25, 34). Bovine feces are considered a favorable nutrient environment for pathogen survival (23) compared to the low nutrient status of water. In this study a period of time elapsed (up to 55 days) prior to a reduction in *E. coli* O157:H7 numbers in feces, indicating that the cells were surviving well in ambient conditions. In contrast to water, the survival of the organism in feces was not affected by the method of inoculation. This suggests that passage through the GI tract will not affect pathogen survival in a nutrient rich

environment, such as feces. Therefore this study demonstrates that, in feces, an inoculum prepared in the laboratory can accurately predict survival under natural conditions.
Many issues affecting the survival of *E. coli* O157:H7 on the farm, such as the intestinal microbial flora and immune status of the individual animal, have been reported (13, 18). In the water experiment, there was a high level of variation in pathogen survival observed between replicates. In order to address some of this variation, animals from the same cohort, age and management conditions were used for this study. Some of the variation observed in the water experiment may have been due to the sourcing of feces from different animals. Differences in the microflora between fecal samples, may have contributed to this variation in survival (11).
The findings of the current study indicate that there was no influence on pathogen survival from feces derived from animals fed on different diets. This is in contrast to the findings of other studies which have found that *E. coli* O157:H7 can survive longer in slurry from either concentrate or silage diets (15, 25, 32). These divergent results indicate that the influence of diet on the survival of *E. coli* O157:H7 in feces is not yet clear.
In summary, the effect of passage through the bovine GI tract on *E. coli* O157:H7 is an important factor when planning survival experiments. This study demonstrates that a laboratory grown organism will not accurately represent the survival in low nutrient conditions, of a bacterium that has recently survived passage through the animal GI tract. Further research should be carried out to examine the ability of naturally occurring *E. coli* O157:H7 to survive longer under stressful conditions such as on the hide or on concrete surfaces.

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Figure Legends:

Figure 1: Survival of *E. coli* O157:H7 in feces (log_{10} cfu/g) inoculated by the natural (N) or laboratory (L) method, from cattle fed on a concentrate (■/□) or silage (▲/Δ) diet, recovered on TSA/SMAC (■/▲) or SMAC (□/Δ)

Figure 2: Survival of *E. coli* O157:H7 in water (log_{10} cfu/ml) inoculated with faeces from cattle fed on a concentrate (C) or silage (S) diet, inoculated using the natural (●) or laboratory (○) method

Figure 3: The average weekly maximum and mimimum shed temperatures during the course of the experiment (February to the beginning of June)

Table 1:Time to decline (days) and rate of decrease in counts of *E. coli* O157:H7 (log_{10} cfu g^{-1}) in fecal samples from cattle fed on a concentrate or silage diet, inoculated using the natural (N) or laboratory (L) methods.

Treatment	Slope	S.E. of slope (+/-)	R^2	Time[a] (days)
Concentrate N	0.05	0.001	0.99	34
Concentrate L	0.05	0.007	0.89	55
Silage N	0.06	0.009	0.85	27
Silage L	0.04	0.003	0.96	34

[a]: Time before a decline in counts first occurred (> 0.5 log)

Replicate	Inoculation Method	Time (days)								
		54	**68**	**74**	**81**	**97**	**102**	**109**	**117**	**122**
Concentrate diet										
1	N[a]	+[c]	+	+	+	+	+	+	-[d]	-
	L[b]	-	-	ns[e]	ns	ns	ns	ns	ns	ns
2	N	+	+	+	+	-	+	-	-	ns
	L	-	ns	ns	ns	ns	ns	ns	ns	ns
3	N	+	+	+	+	+	+	-	-	ns
	L	+	+	+	+	+	-	-	-	ns
Silage diet										
1	N	+	+	+	+	+	+	+	+	-
	L	+	+	-	-	ns	ns	ns	ns	ns
2	N	+	+	+	+	+	+	-	-	ns
	L	+	-	-	ns	ns	ns	ns	ns	ns
3	N	+	+	+	+	+	+	-	+	-
	L	+	+	+	+	+	-	-	ns	ns

Table 2: Detection of *E. coli* O157:H7 in water, inoculated with faeces from cattle fed on two different diets, using the natural or laboratory inoculation methods, after storage from day 54 to day 122.

[a]: Water inoculated with *E. coli* O157 using the natural method of inoculation

[b] : Water inoculated with *E. coli* O157 using the laboratory method of inoculation
[c] : *E. coli* O157 detected in the water sample by enrichment
[d] : *E. coli* O157 not detected in the water sample
[e] : Not sampled

Figure 1: Survival of *E. coli* O157:H7 in faeces (log_{10} cfu g^{-1}) inoculated by the natural (N) or laboratory (L) method, from cattle fed on a concentrate (■/□) or silage (▲/Δ) diet, recovered on TSA/SMAC (■/▲) or SMAC (□/Δ)

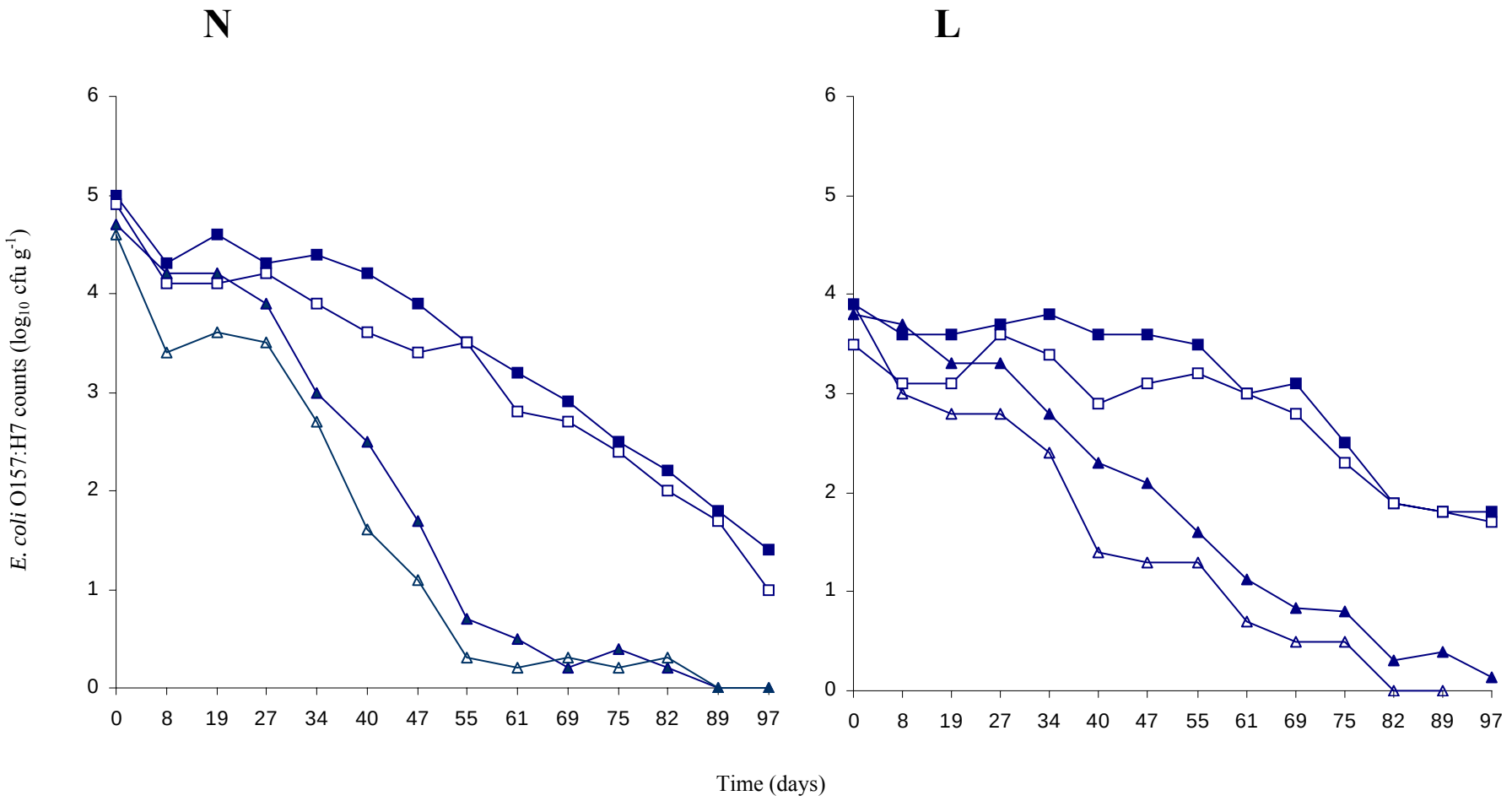


Figure 2: Survival of *E. coli* O157:H7 in water (log_{10} cfu ml^{-1}) inoculated with faeces from cattle fed on a concentrate (C) or silage (S) diet, inoculated using the natural (●) or laboratory (○) method

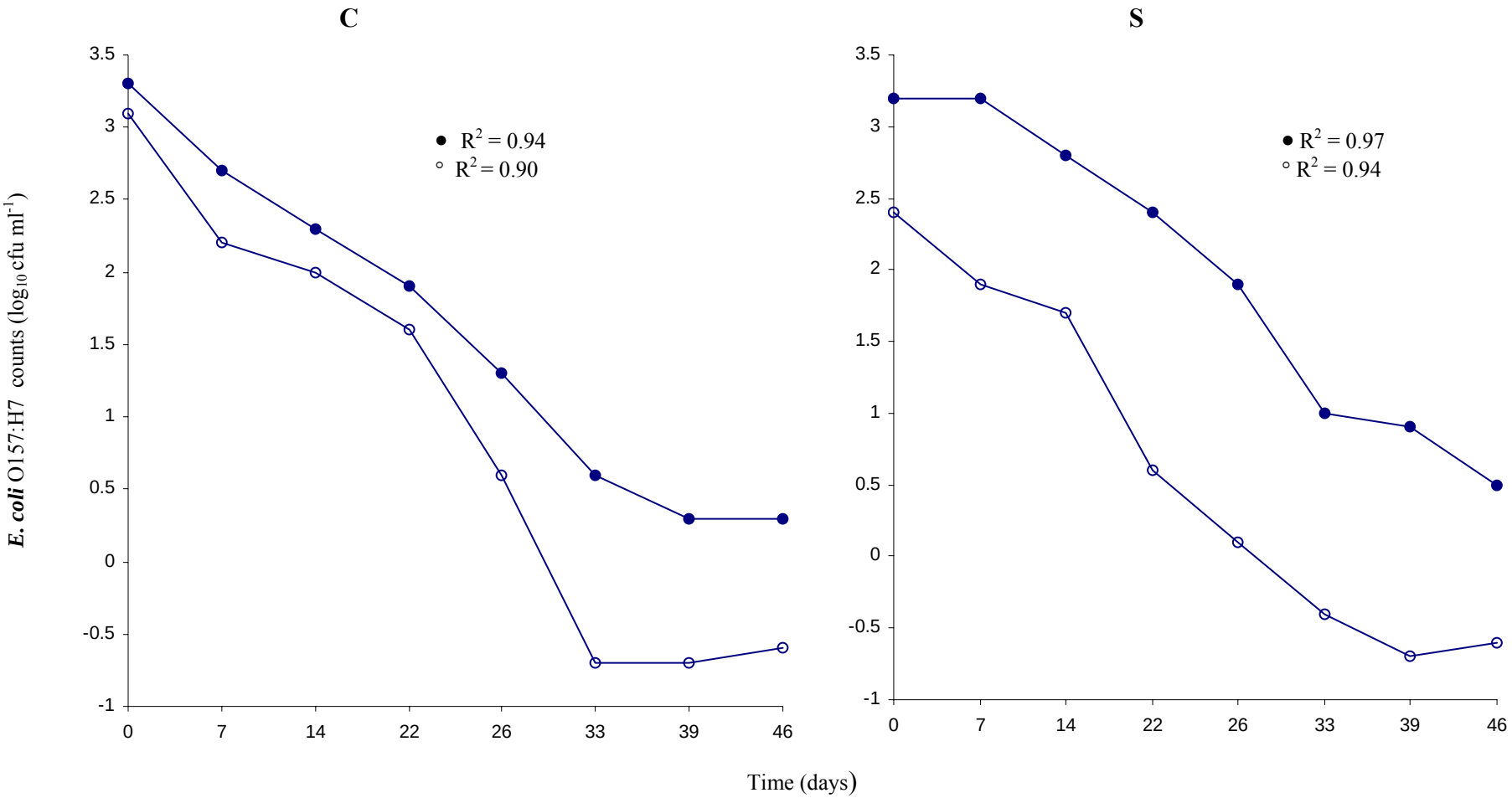
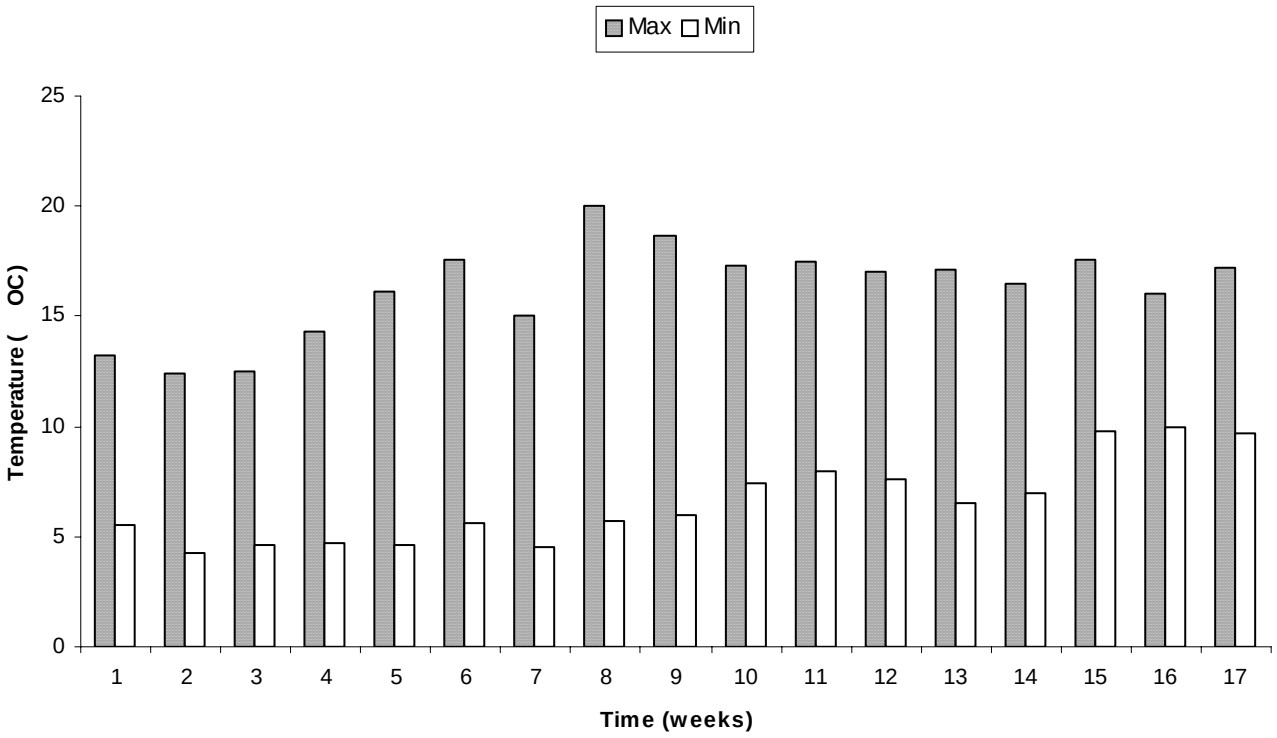


Figure 3: The average weekly maximum and mimimum shed temperatures during the course of the experiment (February to the beginning of June)



4. Study 3

The characterisation of *E. coli* O157:H7 isolates from cattle faeces and feedlot environment using PFGE

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Keywords: *Escherichia coli* O157:H7, cattle, faeces, environment, PFGE

4.1 Abstract

The objectives of this study were to investigate the diversity of *E. coli* O157:H7 isolates obtained over a 3-month period from a cattle feedlot in order to assess the relationship between environmental and faecal isolates and to determine the pattern of transmission of *E. coli* O157:H7 between groups of cattle. Faecal samples were obtained from cattle housed in 4 adjacent feedlot pens at monthly intervals, with environmental pen samples collected simultaneously. All *E. coli* O157:H7 isolates obtained were examined by pulsed field gel electrophoresis (PFGE), PCR to detect *eaeA*, *ehxA, stx*1 and *stx*2 genes and antibiotic sensitivity profiling. Ten isolates were subjected to acid shock to imitate conditions in the acidic cattle abomasum and assess the effect on PFGE profiles. *E. coli* O157:H7 was isolated from 69 faecal samples and 26 environmental samples. All isolates (n = 95) carried the genes for *eae*A, *ehxA* and *stx*2 and were sensitive to all antibiotics tested. The PFGE profiles of all isolates differed by no more than 2 bands and clustered within 80% similarity following dendrogram analysis. Acid shock had no effect on the subsequent PFGE patterns. A total of 8.7% (6/69) of cattle were shedding *E. coli* O157:H7 in the first month with faecal shedding increasing to 52% (36/69) by the third month of the study. A single isolate of *E. coli* O157:H7 may be passed rapidly through cattle pens, with the environment acting as a significant reservoir for transmission. PFGE is a useful tool for tracking the direct and indirect transmission of *E. coli* O157:H7 isolates on the farm.

4.2 Introduction:

E. coli O157:H7 is an important zoonotic pathogen, causing symptoms in humans ranging from mild self-limiting diarrhoea to more severe, haemorrhagic colitis and haemolytic uremic syndrome (Armstrong et al., 1996). Beef cattle are considered the primary reservoir of *E. coli* O157:H7 (Chapman et al., 1997), with human infection commonly occurring through the consumption of contaminated, undercooked beef products (Elder et al., 2000, Griffin and Tauxe, 1991). Increasingly, outbreaks have been reported due to direct or indirect contact with infected cattle faeces in the farm environment (Crump et al., 2003). Despite this, few control measures have been targeted to the farm, partly due to an incomplete knowledge of the ecology of the pathogen at its source. Understanding the epidemiology of *E. coli* O157:H7 in cattle, including direct and indirect transmission patterns, is essential for accurately assessing the risk factors for maintenance of the organism on farm. Indirect transmission of *E. coli* O157:H7 between animals is commonly

examined in inoculation studies, often in artificial environments and using small numbers of animals (Ohya and Ito, 1999, Besser et al., 2001, Shere et al., 2002, Cobbold and Desmarchelier, 2002). However, with recent studies establishing differences in bacterial characteristics following animal passage, (Scott et al., 2005, McGee et al., 2004, Cawthraw et al., 1996), it is critical to examine the transmission of *E. coli* O157:H7 with naturally colonised animals kept in non-experimental conditions. The aim of the current study was to assess the transmission patterns of *E. coli* O157:H7 isolates obtained from naturally infected cattle and their environment. In this study PFGE, in addition to virulence and antibiotic resistance profiling, was used to characterise isolates of *E. coli* O157:H7 obtained from cattle and the feedlot over a 3-month period and to assess transmission patterns of the pathogen. Molecular subtyping techniques such as PFGE have been widely used to investigate the ecology of *E. coli* O157:H7 at various points along the foodchain (Avery et al., 2002, Vali et al., 2005). The effect of acid shock on PFGE patterns of a limited number of strains was investigated. These data were used in interpretation of changes in PFGE patterns observed in the isolates collected during this study.

4.3 Materials and Methods:

Isolates: E. coli O157:H7 isolates were obtained during a survey of Irish feedlot cattle carried out over a 6-month period as previously reported (Minihan et al., 2003). In brief, rectal faecal samples and environmental samples (water from water troughs, faeces from the pen floor and slurry tank, pen barrier swabs, feed and dust), were taken once a month after cattle had been placed into 4 adjacent feedlot pens, each containing 20, 30, 9, and 10 steers. The current study examined 69 *E. coli* O157:H7 positive faecal samples and 26 positive environmental samples obtained over 3 months, from November 2001 – January 2002.

Molecular characterization: Isolates were screened for the presence of *eaeA*, *ehxA, stx*1 and *stx2* genes, using a multiplex polymerase chain reaction (PCR) method as previously described (Paton and Paton, 1998). Each PCR product was separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination.

Antibiotic sensitivity testing: All isolates were examined for susceptibility to 13 antibiotics using the Bauer – Kirby disc diffusion method (Bauer et al., 1966). The following discs (Oxoid, U. K.) were used: ampicillin 10µg, kanamycin 30µg, cefixime 5µg, cefachlor 30µg, streptomycin 10µg, trimethoprim 5µg, nalidixic acid 30µg, compound sulphonamides 300µg, chloramphenicol 30µg, tetracycline 30µg, minocycline 30µg, ciprofloxacin 5µg and norfloxacin 10µg. Isolates were classed as sensitive or resistant to each antibiotic according to the Clinical and Laboratories Standards Institute (formally NCCLS) guidelines (NCCLS, 2003). *E. coli* strain ATCC 25922 and *S. aureus* strain ATCC 25923 were used for quality control (Oxoid, UK).

Acid Shock: Ten faecal *E. coli* O157:H7 isolates were randomly selected to investigate the effect of acid shock on their PFGE profiles. One bead of each isolate was placed aseptically into 10 ml brain heart infusion broth pH 7.2, (BHI, Oxoid UK) and incubated at 37^{o} C for 24 hrs. After incubation, the tubes were sampled (pre-acid shock) and *E. coli* O157:H7 numbers determined as described below. The isolates were subjected to acid shock by transferring a 1 ml aliquot from the overnight culture to 9ml of pre-warmed (37^{o} C) BHI, buffered to pH 2.5 with hydrochloric acid (HCL). The tubes were immediately sampled, and *E. coli* O157:H7 numbers determined. Following incubation at 37^{o} C for 2 hrs, the inoculated acidic broth was withdrawn and *E. coli* O157:H7 numbers determined again (post-acid shock). To determine viable counts of *E. coli* O157:H7, serial dilutions were performed in 9 ml volumes of maximum recovery diluent (MRD),

with aliquots plated onto Sorbitol Macconkey Agar (SMAC, Oxoid, UK) and incubated at 37^{o} C for 48 hrs.

Molecular subtyping using PFGE: Each *E. coli* O157:H7 isolate was analysed by pulsed-field gel electrophoresis (PFGE) according to a standard protocol (CDC, 1996) with some modifications. For all strains, isolates were inoculated onto Tryptone Soy Agar (TSA, Oxoid UK) and incubated at 37^{o} C for 24 hrs. Bacteria were harvested using a sterile loop and suspended in cell suspension buffer to an optical density of 1.3-1.4 at 610nm. In addition, for the 10 isolates subjected to acid shock, one colony was taken from each of the pre-acid shock samples and 10 colonies from each of the post acid shock samples using a sterile inoculation loop (Tyco HealthCare, USA) and suspended in 200 µl cell suspension buffer (100 mmol l^{-1} Tris and 100 mmol l^{-1} EDTA). *Xba*1 digestion was performed for 4 hrs on all isolates as described previously (CDC, 1996). The resulting DNA fragments were resolved by PFGE with a CHEF DR III PFGE apparatus (Biorad) using the following parameters: separation on a 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad) in 0.5X Tris-borate-EDTA buffer, at 14°C for 22 hrs with pulse times of 0.1s to 38s (Radu et al., 2001). Lambda ladder PFG marker (Biolabs, New England) was used in two outside and one central lane for each gel to enable normalization of bands between gels. The gels were stained with ethidium bromide and photographed under UV transillumination using the Eagle Eye II image capture system (Stratagene EE 2, Germany). The presence of bands was determined visually. PFGE patterns were interpreted as recommended by Tenover et al. (1995).

4.4 Results:

The characterisation of the 95 *E. coli* O157:H7 isolates by PCR revealed that all strains possessed *ehxA*, *eaeA* and *stx*2 genes. The *stx*1 gene was not detected in any isolate and all isolates were sensitive to the 13 antibiotics tested.

The number of positive faecal samples collected from November 2001 – January 2001, (Minihan et al., 2003), are presented in Table 1. In November, *E. coli* O157:H7 was isolated from the faeces of 6 animals. The numbers of animals shedding the pathogen increased each month and in January, the pathogen was detected in the faeces of 52% of all animals (36/69).

The PFGE profiles of all isolates differed by only 1 – 2 bands from approximately 15 total *Xba*1 fragments and therefore, according to Tenover's criteria, were considered highly related (Tenover et al., 1995)**.** Based on the number of bands, the isolates exhibited 3 distinct *Xba*1 PFGE patterns, designated A, B and C. Figure 1 presents the dendrogram generated from a representative number of the isolates examined (faecal and environmental). The PFGE patterns A, B and C clustered within a similarity criterion of 80%. Overall, 72 *E. coli* O157:H7 isolates were classed as PFGE A, 29 isolates as PFGE B and 2 isolates as PFGE C. Each of the 3 PFGE patterns was observed in both the faecal and environmental isolates. PFGE pattern C was only observed in the second month of sampling from animal and tank faeces obtained from Pens 2 and 4 respectively. Isolates designated Pattern A were detected from all pens except pen 4 each month, while isolates designated PFGE Pattern B were detected each month from all pens except Pen 2.

In order to investigate the effect of low pH on *E. coli* O157:H7 fingerprints, 10 isolates were subjected to acidic conditions (pH 2.5) for 2 hrs. There was no change in the *Xba*1 PFGE patterns for 9 of these isolates after recovery from pH 2.5. A new PFGE profile was observed from one *E. coli* O157:H7 isolate, with the loss of two bands of approximately 200 kb from one of the ten colonies examined post acid shock.

The 10 *E. coli* O157:H7 isolates subjected to acid shock experienced an average reduction in numbers of 4 $\log_{10}$ cfu g^{-1} (range 2.1 – 5.4 $\log_{10}$ cfu g^{-1}).

4.5 Discussion:

In this study PFGE, anti-microbial sensitivity and virulence profiles were used to characterize *E. coli* O157:H7 isolates obtained from cattle faeces and the environment. The isolates examined had indistinguishable virulence and anti-microbial sensitivity profiles. The PFGE profiles of all isolates differed by no more than 2 bands and the 3 patterns observed shared a 80% similarity with each other. These results show that these isolates were highly related and suggest that they are likely to be the one strain. PFGE analysis using additional restriction enzymes may be used to confirm the genetic likeness of the isolates, however this was not considered necessary in the current study as the epidemiological source of the isolates was known (Gupta et al., 2004 and Davies et al., 2003). Therefore, the present data strongly support the conclusion that a single strain of *E. coli* O157:H7 can be rapidly transmitted throughout a group of cattle. The pathogen was initially isolated from 6 animals housed in 2 separate pens, while animals from all 4 pens were shedding 2 months later**.** Therefore, the presence of small numbers of animals shedding *E. coli* O157:H7 was sufficient to infect the rest of the cohort in the feedlot.

Environmental samples taken one week prior to stocking the feedlot were negative (Minihan et al., 2003). Therefore the findings of the current study suggest that cattle faecally shedding a single *E. coli* O157:H7 isolate subsequently caused contamination of the pens, resulting in highly related faecal and environmental isolates throughout the study period. This is in agreement with an experimental study where PFGE profiling confirmed that the strains of *E. coli* O157:H7 shed longest in cattle faeces were also those that persisted in the feedlot environment (Bach et al., 2005). The results from the present study suggest, that while elimination of environmental faecal contamination is not possible, regular cleaning of cattle pens and common passageways may aid in controlling the load of *E. coli* O157:H7 in the feedlot. In addition, animal contact between pens should be minimized where possible.

Differences of up to 2 bands between *E. coli* O157:H7 isolates following analysis by PFGE are reported in this study. This difference may have occurred due to the loss of a plasmid or a phage (Byrne et al., 2003). Up to 5 band losses have been reported following repeated subculturing in the laboratory (Iguchi et al., 2002), however, few studies have considered the stability of PFGE profiles following host passage or exposure to environmental stimuli. It was observed in the current study that exposure to acidic conditions, such as found in the cattle abomasum, had limited effect on the PFGE profiles, with just one colony experiencing a 2 band loss after acid shock. This is in contrast to Akiba et al. (2000), who reported a one to six band loss following PFGE analysis of *E. coli* O157:H7 isolates after calf inoculation. The data from the present study suggest that minor differences in profiles in *E. coli* O157:H7 isolates obtained from groups of cattle in close contact are unlikely to be of epidemiological significance. However, to state the significance of animal passage on PFGE profiles with more certainty, additional work is advised using more varied strains of *E. coli* O157:H7.

The *E. coli* O157:H7 isolates in the current study were sensitive to the antibiotic panel tested. Although the use of antibiotics for the treatment of *E. coli* O157:H7 infection is controversial due to the potential for verotoxin release, current research on the development of toxin absorbing drugs may lead to the increased use of antimicrobial therapy in the future (Schroeder et al., 2002). Therefore it is advisable to monitor the prevalence and persistence of resistant *E. coli* O157:H7 organisms. To the author's knowledge this is the first report of anti-microbial resistance patterns of *E. coli* O157:H7 strains isolated from an Irish farm.

Investigation of virulence markers, antibiogram and PFGE profiling of isolates strongly suggest that the transmission of a single strain of *E. coli* O157:H7 occurred between groups of

cattle housed in feedlot pens and their environment over a 3 month period. Isolates obtained directly from animals and those obtained from the environment were indistinguishable. Minor differences observed in PFGE profiles in both animal and environmental isolates and in one of 10 isolates subjected to acid shock, were considered epidemiologically insignificant.

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Figure 1 Dendrogram generated by the Gel Compar 11 software showing the relationship of 20 representative fingerprints for 95 *E. coli* O157:H7 isolates obtained from cattle faeces and the environment from 4 different pens. Isolates 2, 3, 4, 8, 11, 5, 7, 25, 16 and 18 were designated PFGE A, isolates 80, 81, 72, 76, 73, 79, 77, 74 ,and 56 PFGE and isolate 55 as PFGE profile C .

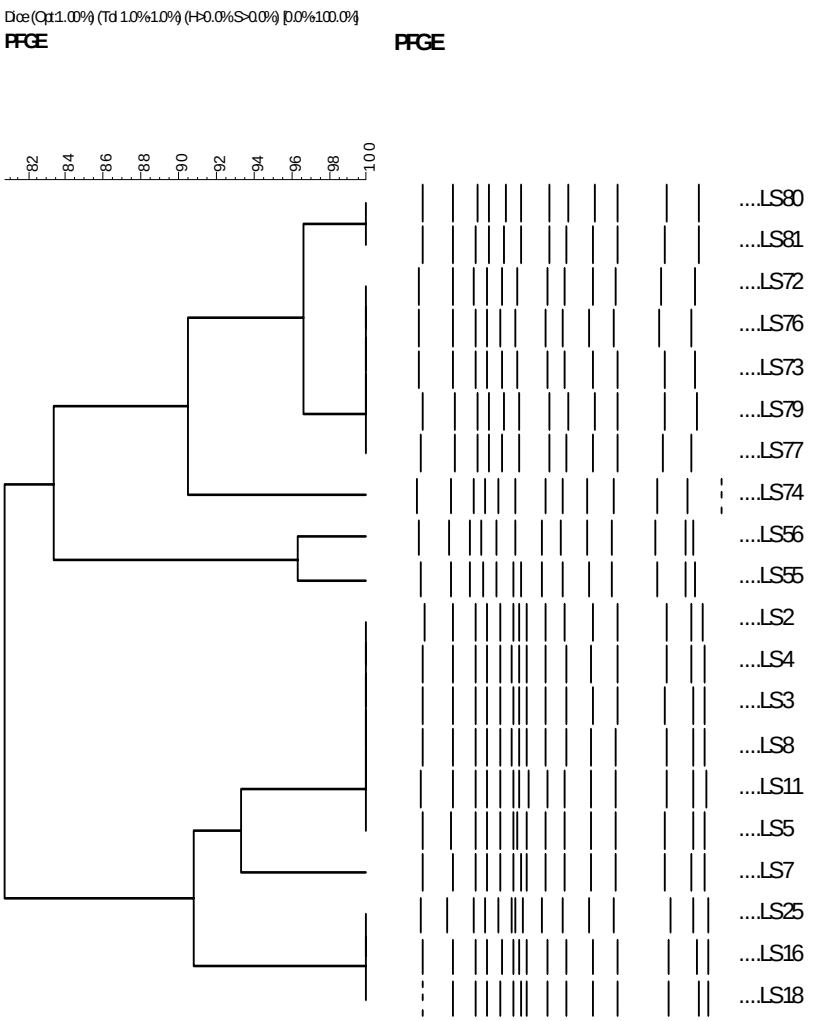

Table 1: Faecal samples positive for *E. coli* O157:H7 obtained monthly from cattle in four adjacent pens

Pen	November	December	*January*
1	0/20 *	4/20	20/20
2	4/30	28/30	4/30
3	0/9	0/9	6/9
4	2/10	3/10	6/10
Total	6/69	35/69	36/69

* Number of *E. coli* O157:H7 positive samples detected / number of samples tested