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HEALTH & SAFETY LABORATORY

Wild Rabbits as potential carriers of *E. coli* VTEC – Final Report

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EXECUTIVE SUMMARY.

Background

In summer 2001, cases of infection with verocytotoxigenic (VTEC) *E. coli* 0157 occurred in members of the public visiting a wildlife park in Norfolk. Infection with *E. coli* 0157 was ultimately determined to be associated with visiting the park, however contact with the animals was minimal. Members of Health & Safety Executive (HSE) and the Health Protection Agency (HPA) were subsequently informed. Studies commissioned by an Outbreak Control Team (OCT) found that a herd of cattle on an adjacent farm excreted *E. coli* 0157 with an identical phage type to the *E. coli* 0157 isolated from all the human cases. The most plausible connection between the two was a colony of wild rabbits that inhabited the farm but also strayed on to the wildlife park. In further investigations rabbit faecal matter collected from the picnic area of the wildlife park also yielded *E. coli* 0157 with identical phage type. What is unclear is whether this was a unique set of circumstances leading to infection, or whether wild rabbits are carriers of *E. coli* 0157. HSE funded this pilot study with the aim of establishing factors affecting influencing links between farm animals known to be *E. coli* 0157 positive and wild rabbits. The following steps were taken:

1. Dairy and beef farms within a 30 mile radius of York were contacted. Those which had a resident rabbit population were invited to participate in the project.
2. Cattle faecal samples from sixteen farms were collected and analysed for the presence of *E.coli* 0157 by culture on selective agar and by polymerase chain reaction (PCR).
3. The baiting and subsequent trapping of rabbits at six farms with *E.coli* 0157 positive cattle was undertaken by staff from the Central Science Laboratories (CSL), York. Two farms were initially visited in late winter, a time of low rabbit activity, and all the farms were visited in summer when there is a far greater interaction between cattle and rabbits. Droppings from individual rabbits were analysed for the presence of *E.coli* 0157 and non-0157 VTEC.

Main Findings

Of the sixteen farms initially visited, five were found to be positive for *E.coli* 0157 by both culture on selective agar and PCR and a further two farms were found to be positive by PCR alone. Rabbits were trapped from six farms, the rabbit population at the seventh farm being too small at the time of trapping.

None of the 32 samples of rabbit faeces collected in late winter were found to be *E. coli* 0157 positive. Further sampling in summer revealed rabbits at both farms were excreting *E. coli* 0157 and two of the further four farms had *E. coli* 0157 positive rabbits. This suggests there may be a seasonal effect on the ability of rabbits to excrete *E. coli* 0157. Of the 97 samples collected in the summer, eight (8.25%) were positive for *E. coli* 0157 and twenty (20.6%) were positive for VTEC including non-0157 VTEC. All the positive samples were from female rabbits and although not statistically significant, the body condition index of infected rabbits were slightly lower than uninfected rabbits. It cannot be determined from this study whether rabbits become colonized by *E. coli* 0157, however the results have given an early indication that when rabbits are in contact with *E. coli* 0157 positive cattle, it is likely that their faecal pellets will contain *E. coli* 0157.

Recommendations

In areas where wild rabbit populations are resident and in close proximity to cattle, their faeces should be treated as being potentially infected with *E. coli* 0157 or other VTEC. Agricultural workers and those involved in the rural leisure industry should be made aware of the risks of aquiring VTEC infection by the faecal –oral route from rabbit faeces. Where land in close proximity to cattle is used for recreational purposes such as camping and caravanning or picnicking, preventive measures to deter rabbits entering the land should be considered. Precautions should also be taken to prevent locally resident rabbits entering land which has been cleared of cattle for subsequent recreation use. Although a three week time period between cattle grazing and public admission to the land has been stipulated by HSE, if a local rabbit population is present, there is a risk these animals will excrete *E. coli* 0157 and continue contaminating the land. Overall, workers which may come into contact with rabbit faeces should be made aware of the potential risk of infection and the requirement of good personal hygiene in particular hand washing.

1 INTRODUCTION.

1.1 BACKGROUND.

Vero-cytotoxigenic (VTEC) *Escherichia coli*, in particular *E. coli* 0157, are capable of causing severe human infection following ingestion. Symptoms potentially range from diarrhoea, through haemolytic uremic syndrome (HUS) to life threatening kidney failure (Karmali, 1989; Bell *et al.*, 1994, Karch *et al.*, 1996, Weir, 2000). The infectious dose is small, estimated to be less than 10 organisms (Willshaw *et al.*, 1994). A particular risk factor for infection is contact with infected animals, which can carry the bacteria in their digestive tract while remaining asymptomatic or with little discernible illness (Louie *et al.*, 1999; Synge, 2000; O'Brien *et al.*, 2001).

Cattle are a major reservoir of infection; in a study on randomly selected farms in England and Wales, *E. coli* 0157 was isolated from 4.7% of individual animals and 44.0% of herds. In infected herds, 10.2% of individual cattle were excreting *E. coli* 0157 (Paiba, 2000). Excretion was greatest in cattle under 24 months. By comparison, prevalence of faecal carriage in sheep was 1.8% and in pigs 0.16%. These figures are comparable to those reported in other countries. Other farm animals which are known carriers of *E. coli* 0157 include goats. Experimental studies have also demonstrated that it is possible to infect rats and pigeons, which shed the bacteria for up to nine and 20 days respectively. If infected rat faeces were stored in a moist environment, *E. coli* 0157 survived for 34 weeks (Cizek *et al.*, 2000). The bacteria are particularly capable of surviving outside the mammalian host, e.g., for several days in soils (Maule, 2000; Ogden *et al*., 2001) and in cattle slurry (McGee *et al.*, 2001).

Members of the public are most likely to come into contact with infected animals when visiting open farms. Some high profile cases of outbreaks have occurred following contamination acquired during such visits (Parry *et al.*, 1995; Shukla *et al.*, 1995; Trevena *et al.*, 1996; Parry *et al.*, 1998; Milne *et al.*, 1999; Pritchard *et al.*, 2000). However, indirect exposure to contamination as well as direct contact with infected animals can lead to infection. Twenty people were infected at a scout camp in Scotland held on land previously used for grazing and which was heavily contaminated by sheep faeces, with *E. coli* 0157 isolated from soil, lying water, wellington boots and a climbing frame on the site (Ogden *et al.*, 2002).

In a period between mid July and September 2001, Verocytotoxigenic (VTEC) *E. coli* 0157 infection was identified in ten children and two adults from unrelated families and different parts of Britain. Symptoms varied from mild to acute haemorrhagic diarrhoea. Four people were hospitalized, one developing haemolytic uraemic syndrome (HUS) but all made a complete recovery. The common factor between these people was they had all visited a wildlife park in Norfolk. At the park, direct contact (touching) was only possible with two exotic pigs and two goats housed in pens; these animals were quickly diagnosed as VTEC negative and ruled out as possible sources of infection. An Outbreak Control Team (OCT) was convened under the chairmanship of the local Consultant in Communicable Disease Control (CCDC) to investigate the outbreak of infection (Bailey, 2002). Using pulse-field gel electrophoresis, these workers found that a herd of cattle on an adjacent farm excreted *E. coli* 0157 phage type (PT)2 with genes for Vero cytotoxin (VT)2, identical to the VTEC isolated in all the human cases. The only plausible connection between the two was a colony of wild rabbits which inhabited the neighbouring farmland but also strayed on to the wildlife park. The rabbits were particularly noted to be present on the picnic / play area. Further investigations by the team found rabbit faecal matter collected from the picnic area of the wildlife park to contain *E. coli* 0157 with identical phage and VT gene. It was concluded by the OCT that the outbreak arose following visits to the wildlife park, due to contact with contaminated wild rabbit faeces.

What is unclear is whether this was a unique set of circumstances leading to infection, or whether wild rabbits are a more general vector for VTEC. Despite several laboratory studies which have utilized rabbits as models for investigating *E. coli* 0157 (Farmer *et al*, 1983, Sherman *et a*l., 1988, Ashkenazi *et al*., 1992, Li *et al*., 1993,), Bailey *et al.*, (2002) are the first and only to report wild rabbits as a potential vector of the bacteria. The implications may be far-reaching and it is thus important to establish as early as possible whether members of the public coming into contact with wild rabbits near farmland, and agricultural workers who may also be exposed, need to be alerted to possible risk.

The aim of this pilot study was to establish factors influencing links between farm animals known to be *E. coli* 0157 positive and wild rabbits. This report describes the selection of farms, sampling of cow and rabbit faeces, analysis methods developed and the outcome of the investigation.

2 MATERIALS AND METHODS

2.1 SELECTION OF FARMS AND SAMPLING STRATEGY

The rationale behind the sampling strategy was to select farms known to have VTEC *E coli* 0157 positive cows, with a rabbit population in contact with the cows, so that there would be the greatest opportunity for transmission of *E. coli* 0157 from cows to rabbits.

The assumption was that samples of fresh cow faeces taken at random from a farm would give a reasonable representation of the bacterial microflora prevalent in the herd. It was agreed that at least three farms known to have cattle shedding *E. coli* 0157 would be included in the study. Working on the data from previous studies, it was assumed that a random selection of around 15 farms should produce at least the required number of *E. coli* 0157 positive herds. Each farm selected for study was required to have the combination of an *E. coli* 0157 positive herd and an established population of wild rabbits, which had access to cattle pasture. Sampling points were selected to provide summary data of the herd bacterial excretion, e.g., fresh yard scrapings from dairy herds following milking, or fresh cow manure from pasture. In the original project plan, it was intended that collection of droppings from locations close to burrows would be undertaken. However, following discussion with Dr David Cowan, a small mammal ecologist from DEFRA Central Science Laboratory (CSL), Sand Hutton, York, it was agreed that a more rigorous method of sampling would be adopted. This involved the live capture of individual rabbits overnight using cage traps on selected farms with the animals being released the following morning. Collection of faecal deposits from beneath the cages would ensure that recent faecal material was collected and minimize the potential for loss of viability of any bacteria present. This work was carried out by Jason Finney of CSL.

Advice was sought from FOD Agriculture Sector about making contact with farms. It was agreed that to minimize travelling time farms in the North East England and ideally within the York area would be used, assuming that there is unlikely to be any regional difference in *E. coli* 0157 infection in cattle herds or any factors which would preclude transmission of *E. coli* 0157 to rabbits should it occur. Acting on FOD's advice, contacts were made initially with a Veterinary Investigation Centre in the area. However, this proved unproductive. The next approach was to contact the National Farmers Union local area office, who in turn provided details of a farming co-operative in the vicinity, but again this did not lead to the identification of suitable farms. A third approach, via DEFRA in Leeds was attempted. Following

correspondence with John Stanley (DEFRA, Leeds), a list of dairy farms was forwarded. Farmers were approached by telephone call to establish whether they still had cattle herds and whether a rabbit population inhabited the farmland. The list sent was found to be many years out of date and personal information incorrect. An outbreak of myxomatosis was also found to have affected the rabbit population in several localised areas. Finally, to supplement this list, further dairy farms were identified and approached using information listed in the Yellow Pages. A total of 16 farms had both cattle and a rabbit population in close proximity and agreed to participate in the project. All the farms were located within a thirty mile radius of CSL, Sand Hutton near York. A letter outlining the projects aims was sent to each participating farmer followed by telephone contact to arrange visiting times.

2.2 SAMPLING OF FARMS.

2.2.1 Sampling of cattle faeces.

Each farm was given a number from 1 to 16 according to when they accepted to be part of the project. The farms were visited and six samples of random cowpats were collected. On each occasion, following discussions with the farmers, the samples were taken, whenever possible from the field closest to where the rabbits were observed and were less than three days old. On two occasions the cattle had to be visited in the sheds as they had been brought in for more than three days due to bad weather. Briefly, the procedure for sampling the faeces was to collect the sample using a plastic 50 ml scoop and place the sample in a sterile screw top plastic container. One scoop was used per sample and this was disposed of into an autoclave bag along with the pair of gloves worn during the sampling. The samples were stored in a cool box during the sampling visit and logged upon arrival at HSL, stored under refrigeration and processed within 24 hours of collection.

2.2.2. Collection of rabbit faeces.

In total, seven farms were identified as having cattle infected with *E. coli* 0157 and resident wild rabbit populations, and six of these farms were selected for further study. Each farm, determined as positive for *E. coli* 0157 was visited by Jason Finney, CSL and the rabbit harbourage (warrens) observed. With the exception of Farm 1, cage traps were placed in one location at each farm. At each farm cage traps were used for the live-capture of individual rabbits. Each trap was disinfected with 10% (v/v) Trigene (Medichem,

Sevenoaks,UK) and rinsed with water prior to use to prevent cross contamination. Traps baited with carrots but not set to catch animals were placed at equal intervals along pasture field margins, adjacent to rabbit harbourage, such as hedgerows and woodland. Previous deposits of faecal pellets were removed prior to placing traps to prevent cross-contamination. Once the rabbits were familiar with the traps, a trapping session of four nights was undertaken. Traps were visited early in the morning and the faecal pellets, deposited during the night by each captured rabbit, collected into individual sterile screw top containers and labelled. Gloves were changed between traps to reduce cross contamination. Samples collected during the trapping session were stored at 4^{o}C and couriered to HSL on the morning of the fifth day for analysis. A total of 129 samples were analysed. This comprised 32 samples obtained from two of the farms in trapping sessions carried out between 28 January and 12 March 2003 (subsequently referred to as late winter) and 97 samples obtained from all six farms during trapping sessions carried out between 30 May and 5 August 2003 (subsequently referred to as summer).

2.3 RABBIT DATA COLLECTION.

The sex, weight and hind leg length of each trapped rabbit was recorded and the rabbits were grouped according to whether they were juvenile or adult. Rabbits over 1200g were considered to be adults. Each individual was given a uniquely numbered ear-tag prior to release. An index of body condition was derived for each animal using the formula:

Body Condition Index = Body weight/ (hind leg length)^{3} (Personal Communication, J. Finney, CSL)

The larger the index the higher the body condition. Statistical analyses were performed on the body condition index of the total number of rabbits in each group which tested positive and those which were negative using the t-test and the Mann Whitney test.

2.4 ENRICHMENT FOR *E. COLI* 0157.

Samples (1g +/-5%) were weighed into 30ml sterile bottles using one disposable faeces spoon per sample. A volume (9ml) of Buffered Peptone Water (BPW) was added and the samples vortexed to disperse the faeces. A positive control sample was set up consisting of a 1ml of cultured *E. coli* 0157 at *ca*. 10^{5} organisms in 10ml of BPW and a negative control of 10ml of BPW. The samples were enriched for 5h at 37^{o}C. Following this period each sample was further

enriched for *E. coli* 0157 using immunomagnetic separation (IMS) following the manufacturer's procedures (Dynal, Wirral, UK). Briefly, magnetic beads coated with anti- *E. coli* 0157 antibody were added to enriched culture and *E. coli* 0157 bacteria allowed to adhere to the beads. The beads were concentrated using magnets and washed with PBS/ Tween. The bacteria bound to the beads was placed in a volume (10ml) of BPW and incubated at 37^{o}C overnight.

2.5 DETECTION METHODS FOR *E. COLI* 0157.

2.5.1 Detection by plating.

Cefixime tellurite sorbitol MacConkey (CT-SMAC) agar (Oxoid, Paisley, UK) and Rainbow agar (M-tech Diagnostics, Warrington, UK) are recognised as selective agars for *E. coli* 0157 (Karch *et al*., 1993; Bettelheim, 1998; Novicki *et al.*, 2000, Karch & Bielaszewska, 2001). CT-SMAC utilises the fact that *E. coli* 0157 can be distinguished from other *E. coli* serotypes in that it ferments sorbitol at a greatly reduced rate compared to other *E. coli* serotypes (Clifton-Hadley, 2000). Rainbow agar contains chromogenic substrates that are specific for two *E. coli*-associated enzymes: β-galactosidase (a blue-black chromogenic substrate and β-glucuronidase (a red chromogenic substrate). Consequently *E. coli* strains grow, yielding colonies ranging in colour through various shades of red, magenta, purple, violet, blue and black. Strain 0157:H7 is typically β-glucuronidase negative so it forms charcoal grey or black colonies on this medium. Most *E. coli* strains are β-glucuronidase positive, and they appear as red or magenta colonies whereas many other non-0157 VTEC strains overproduce β-galactosidase relative to β-glucuronidase and therefore appear as purple, violet or blue colonies (Bettelheim, 1998).

Initially, samples were diluted and spread onto duplicate agar plates of CT-SMAC and Rainbow agar following the five hours enrichment in BPW. Following overnight incubation, it was found that presence of bile salts and the wide variety of coliforms made it difficult to analyse the plates for *E. coli* 0157. Therefore, isolation on CT-SMAC and Rainbow agar was performed following IMS and overnight culture in BPW. Each sample was streaked out for single colonies on *E. coli* 0157 selective agar CT-SMAC and Rainbow agar in duplicate. The plates were incubated for 24 hours before examination. The plates were compared to the positive controls and the colour and colony morphology recorded. CT-SMAC agar plates were examined as to whether the colonies had fermented sorbitol (red colour) or not (colourless). Manufacturer's colour charts were used to determine the nature of the colonies on Rainbow agar and whether the culture was positive for *E. coli* 0157. Colonies which appeared black or grey were streaked

on fresh plates and serial ten-fold dilutions of the culture plated out onto further Rainbow agar plates in an attempt to isolate single colonies of *E. coli* 0157.

2.5.2 Detection by PCR.

Following IMS and overnight enrichment, DNA was extracted from a 100µl volume of each sample using the Qiagen DNAeasy extraction kit (Qiagen, Crawley, UK). The presence of *E. coli* 0157 was examined by PCR using primers which are specific to *E. coli* 0157 lipopolysaccharide (Paton & Paton, 1998). The presence of VTEC *E. coli* other than 0157 in the rabbit faeces was assessed using a commercial PCR kit (Takahara, Japan). The kit used primers for the verocytotoxigenic genes, VT1 and VT2 the presence of which determines the bacteria's toxigenicity. Following PCR the samples were analysed on a 0.9% agarose gel. Bands were observed using ethidium bromide and UV light.

To determine whether any of the coloured colonies isolated on Rainbow agar represented the presence of non-0157 VTEC, colonies were removed and DNA extracted. Single colonies were resuspended in Tris EDTA (TE) buffer and incubated at 95^{o}C for 10 mins. To determine the presence of the verocytotoxigenic genes VT1 and VT2, a commercial PCR kit (Takahara, Japan) was employed.

2.6 VALIDATION EXPERIMENTS.

Prior to sample collection from farms, initial laboratory experiments were undertaken to validate the detection methods. A sample of cow faeces was collected from a local farm known to be *E. coli* 0157 free. A pure culture of *E. coli* 0157 grown in the laboratory was used to seed the cow faeces. The concentration of bacteria was determined using a Thoma cell counter and the faeces seeded with *ca.* 10^4, 10^3, 10^2, 10^1 bacteria per gram. These concentrations were confirmed by plating the dilutions on CT-SMAC and Rainbow agar. Enrichment, extraction and plating out or PCR experiments as described above were then performed on the seeded faeces. This was used to verify the colony morphology of *E. coli* 0157 on CT-SMAC and Rainbow agars and to ensure that the DNA extraction and PCR analyses gave positive results. The limit of detection by PCR was determined to be 100 bacteria per gram of faeces.

3 RESULTS.

3.1 SELECTION OF FARMS AND FARM VISITS.

A total of 16 farms within a 30 mile radius of CSL, Sand Hutton participated in the project. The group of farms included a variety of cattle breeds and were situated in a variety of landscapes, e.g., lowlands and hill farms. Each farm was visited, to discuss further with the farmer the purpose of the study and their potential involvement, and for initial collection of cattle faeces. On average three farms were visited per day. FOD operational guidance on biosafety precautions (e.g., washing footwear and vehicle tyres with disinfectant) was adhered to; in order to minimise possible transmission of infections from one farm to the next.

It was agreed that feedback from the results would be given to each farmer on the *E. coli* 0157 infection status of their herd as determined from the samples tested, with the understanding that a negative result did not necessarily imply that the herd was completely free from the disease.

3.2 ANALYSIS OF CATTLE FAECES.

3.2.1 Detection on CT-SMAC and Rainbow agar.

Faeces contain a variety of organisms, including *E. coli* serotypes and other coliforms. Despite enrichment of *E. coli* 0157 and selective concentration by IMS, a wide variety of colonies were observed on Rainbow agar and a high proportion of sorbitol positive coliforms were observed on CT-SMAC agar. This overgrowth tended to mask the much smaller populations of *E. coli* 0157. There have been several cases of atypical sorbitol fermenting *E. coli* 0157 reported. Gunzer *et al.,* (1992) reported sorbitol fermenting isolates of *E. coli* 0157 isolated from cases of HUS using genetic techniques. In another study, two isolates of sorbitol-fermenting *E. coli* 0157 were unable to grow on CT-SMAC agar (Karch *et al.,* 1996). This emphasises the importance of using both CT-SMAC agar and Rainbow agar. Bettelheim (1998) reported that the presence of *E. coli* 0157 in deliberately mixed cultures of various *E. coli* serotypes was not easily distinguishable on Rainbow agar. The use of Rainbow agar in this study led to the isolation of colonies with a variety of colours. Novicki *et al.,* (2000) and the manufacturers of Rainbow agar, Biolog, have published a list of *E. coli* serotypes associated with the various colony colours on Rainbow agar. The manufacturer reported that *E. coli* 0157 form black compact colonies yet Novicki *et al.,* (2000) found only 25% of their *E. coli* 0157 isolates formed such colonies. The majority formed grey, mucoid colonies. In this study, the majority of the colonies

isolated from both the cattle and rabbit faeces were pink, blue and cream. PCR analysis of colonies of a variety of colours using primers specific for the *E. coli* 0157 lipopolysaccharide revealed that only black colonies were *E. coli* 0157. Analysis with primers for verocytotoxin genes VT1 and VT2 also showed that only the black colonies were VTEC (Figure1**).**

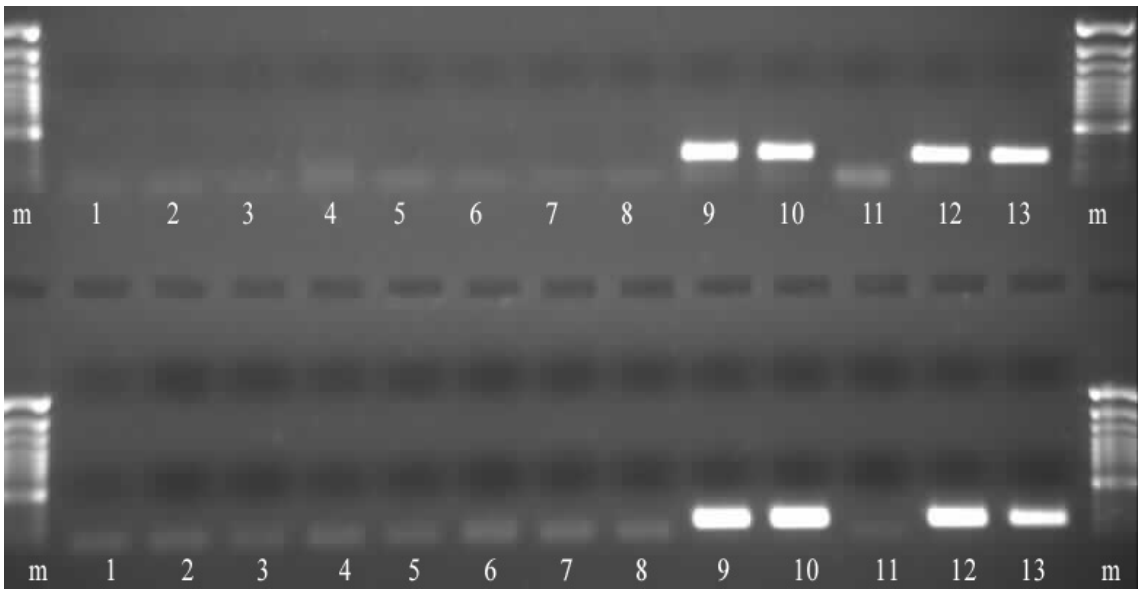

Figure 1. Gel image following PCR of coloured colonies isolated from rabbit faeces on Rainbow Agar. Row 1; primers for lipopolysaccharide. Row 2; primers for VT1 and VT2 genes. Lanes 1,2 = cream colonies, Lanes 3,4=pink colonies, Lanes 5,6=purple colonies, Lanes 7,8=blue colonies, Lanes 9, 10 = black colonies, Lane 11 *E. coli* 0157 DNA positive, Lane 12 VTEC positive DNA

It is interesting to note that the manufacturer of Rainbow agar report blue or purple colonies to be due to typical non-0157 VTEC. The lack of VTEC in the pink and cream colonies was consistent with the manufacturers data. Any suspected *E. coli* 0157 colonies were streaked out onto fresh agar and ten-fold dilutions of culture were also plated. The plates were examined after overnight incubation at 37^{o}C. The number of samples containing *E. coli* 0157, as determined by culture on CT-SMAC and / or Rainbow agars are shown in Table 1.

Table 1. Summary of results of culture based analysis for *E coli* 0157 on 16 farms.

Farm No.	No. of positive samples CT-SMAC agar (max 6)	Percentage positive (%)	No. of positive samples Rainbow agar (max 6)	Percentage positive (%)	Outcome*
1	2	33	6	100	Positive
2	0	0	0	0	Negative
3	1	16	0	0	Negative
4	0	0	0	0	Negative
5	0	0	1	16	Negative
6	3	50	0	0	Negative
7	0	0	1	16	Negative
8	2	33	1	16	Negative
9	0	0	0	0	Negative
10	0	0	1	16	Negative
11	1	16	1	16	Negative
12	2	33	1	16	Negative
13	6	100	3	50	Positive
14	6	100	3	50	Positive
15	6	100	4	66	Positive
16	3	50	4	66	Positive

*** Criteria for positive result = presumptive positive colonies on both types of isolation medium and at least 50% positive on at least one medium.**

Some difficulties were experienced in determining with absolute certainty the presence of VTEC *E. coli* 0157 in cow faecal samples by enrichment and selective agar culture alone, because of the presence in the fresh faeces of large numbers of other bacteria. This emphasised the value of supplementing culture-based tests with molecular tests.

3.2.2 Detection by PCR.

Figure 2 shows a typical agarose gel. The gel shows a clear band of 259bp which is consistent with the positive control.

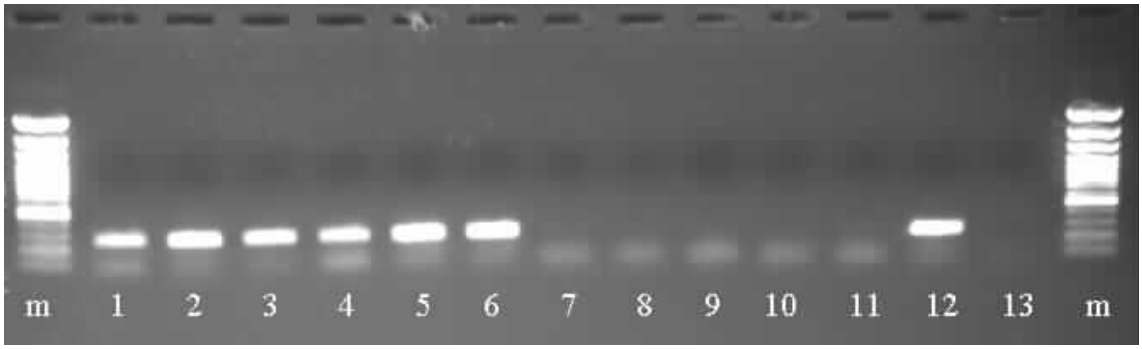

Figure 2. Typical gel image following PCR of bovine faecal DNA samples. **M= Standard marker, Lanes 1-11; cattle DNA, Lane 12; positive experimental control, Lanes 13; negative control.**

The results of the PCR investigation are summarised in Table 2.

Table 2. Summary of results of molecular based analysis for *E coli* 0157 on 16 farms.

Farm No.	No of positive sample (max. 6)	Percentage of positive samples	Outcome*
1	6	100	Positive
2	4 strong & 2 weak	100	Positive
3	2	33	Negative
4	1	16	Negative
5	0	0	Negative
6	2	33	Negative
7	0	0	Negative
8	1	16	Negative
9	0	0	Negative
10	6	100	Positive
11	0	0	Negative
12	0	0	Negative
13	6	100	Positive
14	6	100	Positive
15	5	80	Positive
16	6	100	Positive

* Criteria for positive result = at least 50% PCR positive samples

3.3 ANALYSIS OF RABBIT FAECES.

Of the sixteen farms, seven were found to have cattle faeces positive for *E. coli* 0157 by PCR, five of which were positive by both plating on CT-SMAC and Rainbow agar. Permission to revisit these farms was obtained and the collection of rabbit faeces conducted by staff from CSL. The baiting and then trapping of wild rabbits occurred at two locations (Farm No. 1 & 10) in February, a time of low rabbit activity, and at six locations (Farm No. 1,2,10,13, 15,16) in June, July and August when there is far greater interaction between cattle and rabbits. Both the locations visited in February were visited again in summer. One site was dropped from the project as no rabbits were observed (Farm No. 14).

3.3.1 Detection on CT-SMAC and Rainbow agar.

Analysis of rabbit faeces by culture on CT-SMAC and Rainbow agar incurred similar problems to those found with the cow faeces. Despite enrichment and concentration by IMS, any *E. coli* 0157 were out competed by other coliforms / *E. coli* strains on Rainbow agar and other sorbitol positive coliforms on CT-SMAC agar. Any suspected *E. coli* 0157 colonies were streaked onto fresh agar and ten-fold dilutions of culture plated out. Some eight samples from Farm 1 (summer samples), one sample from Farm 2, one sample from Farm 10, three samples from Farm 13 and two samples from farm 15 were suspected of containing *E. coli* 0157 by culture on Rainbow agar but further culturing failed to yield confirmed positive samples.

3.3.2 Detection of *E. coli* 0157 by PCR.

Figure 3 shows a typical agarose gel image following PCR of DNA extracted from rabbit faecal samples. The gel shows a clear band of 259bp which is consistent with the positive control.

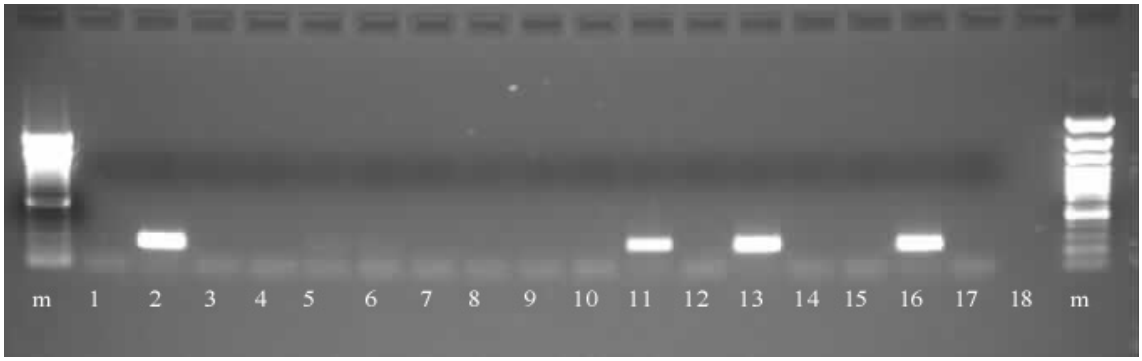

Figure 3. A typical gel image following PCR of rabbit faecal DNA samples. M= Standard marker, Lanes 1-12; rabbit DNA, Lane 13; positive experimental control, Lane 16; positive control DNA, Lanes 14, 15, 17, negative controls.

The results of the PCR investigation of rabbit faeces are shown in Table 3.

Table 3. Summary of results of molecular based analysis for *E coli* 0157 in rabbit faeces.

Farm No.	Sampling time	Total No. of samples	No of positive samples	Percentage of positive samples
1	Late Winter	24	0	0
1	Summer	21	2	9.5%
1	Summer (2^{nd} location)	7	0	0
2	Summer	12	2	16.7%
10	Late Winter	8	0	0
10	Summer	5	2	40%
13	Summer	6	0	0
15	Summer	17	2	11.8%
16	Summer	29	0	0

E. coli 0157 was not detected in any of the 32 samples collected from two farms in late winter. However, further collection of rabbit faeces at these farms during the summer months led to two positive samples at each farm. The rabbits were tagged in late winter so any further capture could be noted. In particular, one rabbit was found to be negative for *E. coli* 0157 in late winter but was positive when trapped in summer. A further two positive samples were detected at Farm 2 and two positive samples at Farm 15 in summer. A total of 97 samples were collected in the summer months of which eight (8.25%) were positive for *E. coli* 0157.

3.3.3 Detection of non-0157 VTEC by PCR.

Although *E coli* 0157 is the most common verocytotoxic producing *E. coli* (VTEC) isolated from cases of human illness, verocytotoxic-producing bacteria other than *E. coli* 0157 also occur. These organisms are increasingly causing human illness and can remain undetected using standard cultural methods for *E. coli* 0157 (Law, 1997). Rabbit faeces were analysed for the presence of non-*E. coli* 0157 VTEC and of the 97 samples collected in the summer, 20 (20.6%) contained verocytotoxin-producing bacteria. A typical gel following PCR analysis for VTEC is shown in Figure 4.

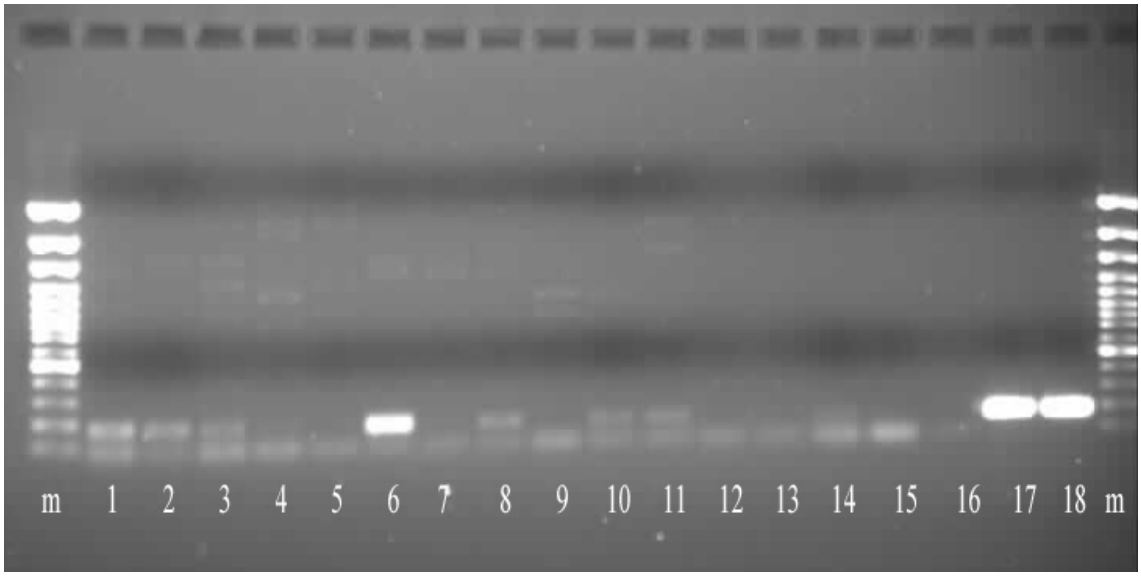

Figure 4 Typical gel image following PCR analysis for VTEC in rabbit faecal DNA samples. M= Standard marker, Lanes 1-15; rabbit DNA, Lane 16: negative control, Lane 17; positive experimental control, Lane 18 positive control DNA.

All the VTEC positive DNA samples were from farms which had been found to have rabbits excreting *E. coli* 0157. The data is summarised in Table 4.

Table 4. Summary of results of molecular based analysis for VTEC in rabbit faeces

Farm No.	Sampling time	Total No. of samples	No. of samples positive for VTEC	Percentage VTEC (%)	No. of samples positive for *E. coli* 0157	Percentage *E. coli* 0157 (%)
1	Late Winter	24	2	8.33	0	0
1	Summer	21	7	33.33	2	9.5
1	Summer (2^{nd} location)	7	0	0	0	0
2	Summer	12	0	0	2	16.7
10	Late Winter	8	0	0	0	0
10	Summer	5	5	100	2	40
13	Summer	6	0	0	0	0
15	Summer	17	7	41.2	2	11.8
16	Summer	29	0	0	0	0

3.3.4 Rabbit data collection.

Analyses of rabbit parameters that might influence the presence of *E. coli* 0157 were carried out for the samples obtained during summer trapping sessions at the four farms where positive samples were obtained. Analysis of the body condition index data of female rabbits grouped according to the presence of *E. coli* 0157 and age (adult or juvenile) of the rabbit is summarised in Table 5. Results for the individual rabbit data collection are shown in Appendix 1.

Table 5. Summary of female body condition index statistical analysis.

Rabbit	Status	No. of rabbits	Mean condition index	Mean condition index (std dev.)	T-test	d.f	Significance	Mann Whitney test	Significance
Juvenile	Negative	17	1.609	0.2235	0.157	18	Not	0.265	Not
	Positive	3	1.587	0.1671			significant		significant
Adult	Negative	18	2.335	0.2235	0.613	21	Not	0.895	Not
	Positive	5	2.169	0.6509			significant		significant

Of 43 female samples, eight (19%) were positive for *E. coli* 0157 while all 20 samples from males were negative. This difference is statistically significant (Fishers exact test P=0.047). Out of 20 juvenile females three (15%) were positive while five (22%) out of 23 adult female samples were positive. The mean body condition index of the five adult females positive for *E. coli* 0157 was 2.169$\pm$SD 0.609 compared to 2.335$\pm$SD 0.2235 for the 18 adult females who were negative. This difference is not statistically significant (Mann Whitney U=0.895). However, the body condition for each rabbit positive for *E. coli* 0157 was slightly lower than rabbits found to be negative.

4 DISCUSSION.

In the summer of 2001, there was an outbreak of *E. coli* 0157 infection associated with visiting a wildlife park in Norfolk. The subsequent investigation determined that the infectious organism had entered the wildlife park via wild rabbits. In order to establish the link between the human infection and rabbit faeces the investigation team collected rabbit faeces from both the neighbouring farm and wildlife park. The samples were pooled to create 12 samples from the farm and 29 from the picnic area of the wildlife park. *E. coli* 0157 with the same phage type and VT genes was found to be excreted by the cattle and rabbits of the adjacent farm and was isolated from the infected visitors. These results cannot be disputed but the fact the test samples consisted of pooled faeces gives little indication of the proportion of the rabbit population excreting *E. coli* 0157. The question as to whether this was a unique set of circumstances leading to infection, or whether wild rabbits are capable of being vectors for *E. coli* 0157 transmission was therefore asked.

The pilot study aimed to establish factors affecting the likelihood of cross contamination between farm animals known to be *E. coli* 0157 positive and wild rabbits. Seven out of sixteen dairy and beef farms in the York area with resident rabbit populations were found to have cattle excreting *E. coli* 0157 and following assessment by staff from CSL, six farms were chosen for the trapping of rabbits. In each case, except Farm 1, the rabbits were observed to have direct contact with cattle and their faeces.

In order to achieve a greater insight into the proportion of rabbits excreting *E. coli* 0157, rabbits were trapped individually and their droppings collected as individual test samples. Every effort was made to prevent cross contamination between samples. The results of the project show that four (67%) out of six farms were inhabited by rabbits which excreted *E. coli* 0157. The number of samples obtained from each farm differed greatly but reflected the rabbit population size. These were either small and discrete such as those in Farm No. 10 and Farm No. 13 which led to five or six samples or much larger populations such as at Farm No. 15 which co-inhabited with a high density of cattle and led to 28 samples. The percentage of infected rabbit faecal samples collected from each farm were between 9.5% and 40% as determined by PCR. Bailey *et al.,* (2002) reported the presence of *E. coli* 0157 in three (25%) of twelve pooled samples of rabbit faeces collected from a field adjacent to the wildlife park, housing cattle from the infected neighbouring farm and 17 (59%) of 29 pooled samples from the picnic area inside the wildlife park. As these samples were pooled the true proportion of rabbits infected is impossible to

conclude due to the potential to introduce faeces from a single infected rabbit into each pooled sample. However, as far as such comparisons can be made, our results were consistent with those of the wildlife park investigation.

The original investigation at the wildlife park was conducted in late summer with pooled samples of rabbit faeces being reportedly collected in late September. The report also suggested that the local rabbit population had increased significantly in 2001. The results of this project suggest there may be a seasonal variation in the excretion of *E. coli* 0157 by rabbits. Two of the six farms were sampled in late winter and again in summer. During the late winter, all samples collected were negative for *E. coli* 0157 while both farms were found to have rabbits excreting *E. coli* 0157 in the summer. One rabbit in particular, found to be negative in the late winter had acquired an infection of *E. coli* 0157 and was excreting the organism at the time of the summer sampling. In late winter the cattle were still indoors and thus there would be no direct contact with rabbits. Furthermore, rabbit densities are at their lowest at this time before breeding begins whilst they are at their highest in early summer (Cowan, 1987). In this study only female rabbits were found to carry and excrete *E. coli* 0157. The reason for this is unclear although it seems to be unrelated to pregnancy and/or lactation as the trend was also apparent amongst juveniles. There was thus no indication of age-specific variation in *E. coli* 0157 excretion with juvenile females as likely to be positive as adults.

Analysis of rabbit faecal samples for the presence of non-0157 VTEC by PCR revealed that 20 of 97 summer samples (20.6%) contained VTEC, which is greater than the proportion of rabbits found to excrete *E. coli* 0157. This is comparable to studies that have shown that non-0157 VTEC can be isolated from cattle faeces at a higher frequency than *E. coli* 0157 (Montenegro *et al*., 1990, Wells *et al.;* 1991, Hull *et al.*, 1993 and Law; 1997). In a German study, 21.1% of cattle, 66.6% of sheep and 56.1% of goats harboured VTEC (Beutin *et al.*, 1993). The proportion of rabbits found to excrete VTEC in this study is similar to that reported for cattle by Beutin *et al.*, (1993). It can therefore be suggested that wild rabbits are a potential source of non-0157 infections. Several non-0157 serogroups have been isolated from cases of human infection, of which serogroups 026, 0103 and 0111 are relatively common. These serogroups have been responsible for outbreaks in many countries including a community wide outbreak involving nine children in Italy (Caprioli *et al.*, 1994) and an outbreak of bloody diarrhoea and HUS in France (Mariani-Kurkdjian *et al.*, 1993).

Garcia and Fox (2003) have recently reported that laboratory and pet rabbits are a new reservoir host of enterohaemorrhagic *E. coli* (EHEC) which have also been implicated in outbreaks of human disease. These workers examined the faeces of groups of rabbits reared in a facility for laboratory use, from a pet store and from a petting farm. Culture on Rainbow agar was used to isolate *E. coli* colonies and the authors reported that the colonies ranged from pink to purple in colour. This is consistent with the findings of this pilot study and suggests the possibility that wild rabbits may also be reservoirs of EHEC. Garcia & Fox (2003) found none of the rabbits tested was a reservoir of *E. coli* 0157 as determined by culture on Rainbow agar.

Bettelheim (1998) reported that the presence of *E. coli* 0157 in deliberately mixed cultures of various *E. coli* serotypes was not easily distinguishable on Rainbow agar. The difficulties encountered with the culture based detection, caused by the presence of other coliforms, meant it was not possible to quantify the *E. coli* 0157 present. If this had been possible, it would have given information regarding the colonisation status of the rabbits. It is not clear whether the alimentary tracts of the infected rabbits are heavily colonised, or whether the bacteria are transient residents. Garcia *et al.,* (2002) reported an outbreak of haemorrhagic diarrhoea and HUS in rabbits bred by a commercial vendor that were naturally infected with EHEC. This suggests rabbits are capable of showing symptoms of *E. coli* infection. Although differences were not significant, the body condition was estimated to be lower in *E. coli* 0157 positive rabbits. This may be an indication that presence of *E. coli* 0157 in the alimentary tract is having an adverse effect on the health of the rabbits. Numbers were too few in the pilot study to evaluate this fully.

It is unknown how rabbits acquire *E. coli* 0157 but as the natural route of transmission is faecal-oral, it could be suggested that rabbits may become infected when washing their fur if contaminated with infected cattle faeces or by the consumption of contaminated vegetation. Leclercq & Mahillon (2003) suggested the mode of transmission could be the consumption of contaminated grass. These authors reported a case of a single rabbit carcass at a slaughterhouse in Belgium being contaminated with *E. coli* 0157. The rabbit concerned was from a farm that bred rabbits and therefore direct contact with cattle was not possible. Garcia & Fox (2003) found an apparent clonal nature of their isolates by using repetitive-element-sequence PCR which suggests transmission of *E. coli* between rabbits. To establish whether a direct link between cattle and rabbits is necessary for infection to occur, rabbits which have had no contact with cattle would have to be tested. Pulse –field gel electrophoresis (PFGE) could be employed

to established exact matches between *E. coli* 0157 organisms isolated from the cattle faeces and resident rabbit population.

In summary, the results have shown that wild rabbits residing in close proximity to cattle excreting *E. coli* 0157 can also act as vectors for the disease. There is therefore the potential risk of farm workers and members of the public accessing the farmland to acquire *E. coli* 0157 from infected rabbit faeces via the faecal oral route. However, unless the requisite for close proximity to infected cattle can be established, it is not possible to conclude whether there is a risk associated with wild rabbits and their faeces *per se*.

5. RECOMMENDATIONS

Based on the evidence from the pilot study; in areas where wild rabbit populations are resident and in close proximity to cattle, their faeces should be treated as being potentially infected with *E .coli* 0157 or other VTEC. Agricultural workers and those involved in the rural leisure industry should be made aware of the risks of VTEC infection by the faecal –oral route from rabbit faeces. Where land in close proximity to cattle is used for recreational purposes such camping and caravanning or picnicking, prevention measures to deter rabbits entering the land should be considered. Precautions should also be taken to prevent locally resident rabbits entering land which has been cleared of cattle for subsequent recreation use. There is a stipulation that farm pasture intended for recreational use should be cleared of animals for three weeks prior to use (HSE Press Release). This was a result of previous outbreaks of infection e.g. in scouts camping on farmland (Ogden *et al.*, 2002). It is recommended that farms offering pasture for recreational use should be made aware of the potential hazard of cross contamination if a large population of rabbits exists in or near that area as these animals may excrete *E. coli* 0157 and continue contaminating the land. Overall, workers who may come into contact with rabbit faeces should be made aware of the potential risk of infection and the requirement of good personal hygiene in particular hand washing.

The results of the pilot study demonstrated the potential for rabbits to excrete VTEC when living closely with VTEC positive cattle. It is recommended that, in any subsequent studies of the potential for rabbits to carry VTEC, rabbit populations living away from cattle should be studied.

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APPENDIX 1.

Appendix 1. Rabbit data collection

Farm No.	Sample No.	Sex	Weight	Hind Leg Length	Body Condition	Age	*E. coli* 0157 positive
1	2W105	F	1580	9.2	2.0	1	
	2W106	M	760	7.9	1.5	2	
	2W112	F	1760	8.9	2.5	1	
	2W113	F	460	6.9	1.4	2	
	2W114	F	450	6.7	1.5	2	
	2W115	F	1580	8.5	2.6	1	
	2W116	F	1560	8.2	2.8	1	
	2W117	M	460	6.7	1.5	2	
	2W202	M	1620	9.0	2.2	1	
	2W208	F	820	7.9	1.7	2	
	2W210	F	1000	8.9	1.4	1	
	2W211	F	2000	9.2	2.6	1	
	2W212	F	400	6.9	1.2	2	
	2W213	F	460	6.6	1.6	2	
	2W214	F	550	6.6	1.9	2	
	2W215	F	500	6.7	1.7	2	
	2W217	F	1450	8.9	2.1	1	
	2W218	M	1040	8.5	1.7	1	
	2W219	F	520	7.1	1.5	2	
	2W301	F	1820	8.9	2.6	1	
	2W304	F	1920	8.0	3.8	1	
	2W315	F	1880	9.0	2.6	1	
	2W317	M	1550	8.9	2.2	1	
	2W401	F	1020	8.1	1.9	2	
	2W406	F	1040	8.3	1.8	2	
	2W412	F	800	8.3	1.4	2	Yes
	2W413	F	920	8.1	1.7	2	
	2W418	F	1200	9.1	1.6	1	Yes
1	2W105	F	1580	9.2	2.0		
(2nd Site)	2W106	M	760	7.9	1.5		
	2W202	M	1620	9.0	2.2		
	2W208	F	820	7.9	1.7		
	2W210	F	1000	8.9	1.4		
	2W301	F	1880	8.9	2.7		
	2W304	F	1550	8.0	3.0		
	2W401	F	1020	8.1	1.9		
	2W406	F	1040	8.3	1.8		
2	M108	M	430	6.4	1.6	2	
	M109	F	870	8.2	1.6	2	

Farm No.	Sample No.	Sex	Weight	Hind Leg Length	Body Condition	Age	*E. coli* 0157 positive
	M202	F	1960	9.5	2.3	1	
	M203	F	850	7.7	1.9	2	
	M204	M	880	7.8	1.9	2	
	M205	F	920	8.9	1.3	2	
	M215	F	1940	8.4	3.3	1	Yes
	M302	M	400	5.6	2.3	2	
	M305	F	450	6.2	1.9	2	
	M306	M	1550	8.4	2.6	1	
	M402	F	880	8.0	1.7	2	Yes
	M409	M	980	8.4	1.7	2	
10	2E106	F	1580	9.1	2.1	1	Yes
	2E201	F	780	7.8	1.6	2	Yes
	2E204	M	780	7.8	1.6	2	
	2E205	M	820	8.0	1.6	2	
	2E405	M	1650	9.2	2.1	1	
13	D102	F	1250	8.8	1.8		
	D107	M	1360	9.1	1.8		
	D109	M	1100	8.7	1.7		
	D110	F	1140	8.9	1.6		
	D203	F	1200	9.0	1.6		
	D204	M	1050	8.5	1.7		
15	B101	F	1650	9.1	2.2	1	
	B103	M	320	6.2	1.3	2	
	B104	F	1380	8.8	2.0	1	
	B105	F	1460	8.9	2.1	1	Yes
	B108	F	1650	8.9	2.3	1	
	B114	F	450	6.9	1.4	2	
	B201	F	1650	8.9	2.3	1	
	B202	M	390	6.4	1.5	2	
	B203	F	350	6.2	1.5	2	
	B211	F	1300	9.0	1.8	1	
	B213	M	550	7.4	1.4	2	
	B214	F	1140	8.7	1.7	1	
	B302	M	1730	9.0	2.4	1	
	B304	F	1450	8.4	2.4	1	
	B309	M	1170	8.9	1.7	1	
	B314	M	1250	8.8	1.8	1	
	B407	F	1320	9.0	1.8	1	Yes
	B408	M	1630	9.4	2.0	1	

Farm No.	Sample No.	Sex	Weight	Hind Leg Length	Body Condition	Age	*E. coli* 0157 positive
16	1G103	F	1400	9.2	1.8		
	1G104	M	1100	9.2	1.4		
	1G109	F	1560	8.9	2.2		
	1G110	M	1000	8.5	1.6		
	1G111	M	1320	8.9	1.9		
	1G112	M	900	8.2	1.6		
	1G113	M	1320	9.2	1.7		
	1G116	F	1160	8.9	1.6		
	1G117	M	980	8.3	1.7		
	1G118	M	920	8.2	1.7		
	1G202	M	1480	9.5	1.7		
	1G204	M	1300	8.4	2.2		
	1G206	F	720	7.8	1.5		
	1G207	F	1720	9.6	1.9		
	1G208	F	350	5.7	1.9		
	1G209	F	1270	9.1	1.7		
	1G213	F	940	8.6	1.5		
	1G214	M	1700	8.9	2.4		
	1G216	F	980	8.8	1.4		
	1G218	F	1400	8.8	2.1		
	1G220	M	1030	8.7	1.6		
	1G301	F	2080	8.6	3.3		
	1G308	F	920	8.7	1.4		
	1G309	F	700	7.3	1.8		
	1G401	M	1630	9.5	1.9		
	1G402	F	1080	8.9	1.5		
	1G404	F	1260	8.7	1.9		
	1G405	F	1050	8.7	1.6		
	1G417	M	1250	9.0	1.7		