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Heterogeneous distributions of *Escherichia coli* O157 within naturally infected bovine faecal pats

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Abstract

Escherichia coli O157 is an important human pathogen for which cattle are considered a reservoir. This paper describes and models the variation in counts of *E. coli* O157 that exists within individual bovine faecal pats. The presence and concentration of *E. coli* O157 in faecal samples was determined using a combination of direct spiral plating followed by a more sensitive isolation procedure. The data were modelled using multilevel random effect models, in which the random effects were allowed to be correlated to allow for the fact that pooled and individual samples come from the same pat. Up to a two log difference in the concentration of *E. coli* O157 was demonstrated in samples from different areas within a faecal pat. Pooling of individual samples from throughout the faecal pat and processing it as one composite sample allows this heterogeneity to be overcome.
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1. Introduction

Escherichia coli O157 is a significant public health issue in the UK, causing an estimated 995 cases of intestinal disease in 2000 [1]. The pathogen is excreted in the faeces of healthy cattle and is therefore often regarded as a part of the normal gut flora. It has been suggested that reducing the prevalence and magnitude of shedding by cattle may result in a reduction in human cases of infection with *E. coli* O157 [2]. Before devising and implementing control strategies, accurate and representative data on the prevalence and magnitude of the infection in cattle are required.

Many epidemiological studies estimate the prevalence of *E. coli* O157 in large populations of cattle by sampling freshly voided faecal pats [3–5]. This method of faecal sampling has several advantages in that it is quicker, less intrusive for both cow and farmer and does not induce the same degree of stress on animals as other alternative methods, (e.g., digital rectal retrieval). Also, as environmental sources of infection are now regarded as important pathways in the transfer of *E. coli* O157 to humans [6], the sampling of faeces within the environment gives a more representative estimate of the pool of contamination that exists in the environment.

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The status of *E. coli* O157 infection is often required at the group or herd level. Pooling of samples from faecal pats within a group or herd is often carried out as an economical and simpler alternative to other sampling methods, particularly when group level prevalence is required. Pooled sampling, combined with mathematical and statistical modelling, has also been used to estimate prevalence within groups of animals [7].

Another important feature of the epidemiology of *E. coli* O157 is the concentration at which cattle shed the pathogen in their faeces. Some recent studies have examined the concentration of *E. coli* O157 in naturally infected faeces [8,9]. Current reports, regarding counts, are mainly qualitative with point estimates of shedding for individual cattle, with limited discussion of the distribution and variation in counts. In order to design efficient studies and correctly interpret count or concentration data, a greater understanding of the sources of variation within the sampling hierarchy is required. One such source of variation is the degree of homogeneity of *E. coli* O157 within faecal pats.

Despite numerous studies having been conducted on the ecology of *E. coli* O157 in the bovine GI tract, many of the factors important for colonisation or adherence of *E. coli* O157 to the gut mucosa are still unclear. Although *E. coli* O157 has been isolated from several areas throughout the GI tract some principal sites of colonisation have been suggested [10,11]. Naylor et al. [11] showed that in persistently colonised animals, *E. coli* O157 was found associated with tissue within 5 cm proximal to the recto–anal junction (RAJ). If this is indeed the site of colonisation then *E. coli* O157 is unlikely to be homogenously distributed within faecal pats as very little mixing of the faecal matter will occur after this site before voiding of the faeces into the environment.

Here, we report a study that was conducted to ascertain whether a faecal pat represents a homogenous mixture of *E. coli* O157 or whether certain areas within the faecal pat are more likely to contain *E. coli* O157, for example areas of the pat in closer proximity to the RAJ mucosa during passage through the colon may contain significantly more *E. coli* O157. The concentration of *E. coli* O157 present in faecal samples was determined using a previously validated method [12].

2. Materials and methods

2.1. Sample collection

A group of calves aged 6–11 months were sampled at two visits conducted over 1 week. When animals previously identified as shedding *E. coli* O157 were observed defecating, samples were taken from within the faecal pat. Only when an entire pat was voided at one site was it sampled. The faecal pat was divided up into an approximate grid consisting of three rows, three columns and two layers. Random samples from within the grid were taken, and each site within each pat was recorded. The pat was split using a palette knife that was sterilised after the removal of each layer. The location of each individual sample was also recorded as being from either the inner or outer areas (often identified by crusting) of the faecal pat. Ten individual samples of <10 g were taken from each of six pats at each visit. Sampling was carried out over two visits to reduce the time to processing. In the laboratory the 10 individual samples per pat were tested separately and three pooled samples per pat were generated and tested. Therefore, from each faecal pat 10 individual samples and three pooled samples were tested.

Each pooled sample was generated by selecting four individual samples to form one composite pooled sample (but with the individual information on which individual samples made up a pooled sample retained). Therefore, two of the pooled samples were formed using different individual samples but the third pooled samples contained two unique samples and one sample from each of the other pooled samples.

2.2. Enumeration and isolation of E. coli O157

The maximum time from sampling to processing was 2 h. Isolation and enumeration of *E. coli* O157 was carried out as previously described [13], with some minor modifications. Briefly, after mixing, 2 g faeces from each individual sample and each of the pooled samples were added to 18 ml buffered peptone water (Lab M) containing vancomycin ((8 mg/l, Sigma–Aldrich, UK) BPW + V) at room temperature. Samples were homogenised and 2 ml removed and added to 3 ml maximum recovery diluent (MRD, Lab M, Bury, UK) to give a 1 in 25 dilution of original faecal sample for further spiral plating. The remaining faecal broths were cultured at 37 °C for 18 h. Sub-samples taken for spiral plating were vortexed and 100 µl plated in duplicate onto Harlequin™ SMAC BCIG agar (Lab M) supplemented with cefixime (0.05 mg/l) and potassium tellurite (2.5 mg/l) (Lab M) using a WASP 2 spiral plater (Don Whitely Scientific, Shipley, UK) set in logarithmic mode. Plates were incubated at 37 °C for 18 h and presumptive *E. coli* O157 colonies counted. The identity of the plates was blinded before counting.

If no presumptive *E. coli* O157 colonies were identified on direct plates, cultured samples were subjected to IMS. After the enrichment period 1 ml samples of the enriched faecal broth were added to 20 µl of magnetic beads coated with antibody to *E. coli* O157 antigen (Dynal, Bromborough, UK) and immunomagnetic separation (IMS) was performed according to the

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manufacturer's instructions. The bead suspension obtained was split and inoculated onto CT-SMAC (Lab M) and CHROMagar™ O157 (M-Tech Diagnostics). Samples were only subjected to IMS if there were no presumptive isolates on the Harlequin count plates.

2.3. Isolate confirmation

Plates with presumptive positive colonies from either IMS plates or count plates were then randomly selected. For count plates, all colonies contributing to the count for that plate (with a maximum of 10) were then screened by polymerase chain reaction (PCR) for the presence of the *rfb* (O-antigen-encoding) regions of *E. coli* O157 [14] and for the presence of genes encoding *eae* A, *vt1* and *vt* 2 as previously described [12]. Random sub-samples of isolates, positive by PCR for the *rfb* gene, were also confirmed using the latex agglutination test (Oxoid, Basingstoke, UK). Isolates from IMS plates were confirmed as *E. coli* O157 by latex agglutination. Isolates were then frozen on beads and stored at −80 °C for further isolate characterisation.

Twenty seven *E. coli* O157 isolates were randomly selected and sub-typed by pulsed-field gel electrophoresis (PFGE) according to the Centers for Diseases Control (CDC) manual standard 1-day protocol [15].

2.4. Statistical analysis

A statistical model was devised for analysing the variation in counts of *E. coli* O157, assessing the relative contributions to the variability by different sampling methods. The subscripts i, j, k, l, m, are used to denote the various samples collected. Each faecal pat, i, resulted in 10 individual samples, j, taken from different areas of the faecal pat, and three pooled samples, l, a composite from different areas of the faecal pat. All samples were plated in duplicate, k, m.

The number of *E. coli* O157 colonies counted on individual agar plates ijk, is denoted as W_{ijk} and the count from pooled sample, ilm as Y_{ilm}. For a description of spiral plate method see Robinson et al. [13]. We assume the counts are Poisson distributed, with

$$W_{ijk} \sim \text{Poisson}(S_{ijk}\alpha_{ijk}), \text{ and}$$

$$Y_{ilm} \sim \text{Poisson}(R_{ilm}\lambda_{ilm}).$$

Here, α_{ijk} and λ_{ilm} are the intensities of *E. coli* O157 in the individual and pooled samples, respectively, measured in cfu/g, and S_{ijk} and $R_{ilm}$are the scaling factors used to calculate intensities from raw colony counts from agar plates using spiral plating techniques (i.e., the dilution factor of the sample from the raw faecal sample and the sector of the agar plate on which the bacteria were counted). The Poisson distribution is widely regarded as appropriate for these kind of count data. Assuming the bacteria are mixed homogenously throughout the faecal material, samples taken from the faecal pats should follow a Poisson distribution.

The logged intensities are modelled as Gaussian random variables, with random effects modelling the variation and covariation between different faecal pats, between samples in a faecal pat and from different agar plates of the same sample. The intensities are modelled as

$$\log(\alpha_{ijk}) = \mu + X_{ij}\beta + A_i + B_{ij} + D_{ijk},$$

$$\log(\lambda_{ilm}) = \mu + A_i + C_{il} + D_{ilm},$$

where A_i, B_{ij}, C_{il}, D_{ilm} and D_{ijk} are normally distributed random effects with mean zero and variances $\sigma_A^2, \sigma_B^2, \sigma_C^2$ and σ_D^2, respectively. Notice that the faecal pat effect A_i is the same for both the pooled samples and the individual samples, but the sample effects B_{ij} and C_{il} are different because of the different sampling methods. The plate effect D is different for the individual samples and pooled samples, but has the same variance, as the effect of plating is assumed to be the same regardless of the sampling method. The covariate X_{ij} indicates whether sample ij was taken from faeces that appeared to have been the outer or inner surface during passage through the intestines, with β being the effect on the intensity from the sample being taken from the inner region of the faecal pat.

Inference on the model was preformed in a Bayesian framework and the posterior distributions sampled using Markov chain Monte-Carlo methods in the software package Winbugs. Various uninformative priors were used and the choice of prior proved to have little influence on the results. Convergence of Markov chains was assessed by examining time traces of the simulations and using routines in the Coda package (www-fis.iarc.fr/coda).

3. Results

3.1. Descriptive summary

A total of 120 faecal samples were taken directly from 12 faecal pats over the study period, a further 36 samples were generated by pooling of these individual samples, yielding a total of 156 samples tested. From four of the faecal pats no *E. coli* O157 was isolated from individual or pooled samples by direct spiral plating or the more sensitive IMS procedure (Fig. 1). The concentration of *E. coli* O157 from four of the faecal pats was consistently high in all 10 individual samples, and the three-pooled samples. Up to a 100-fold difference was observed between different samples from the same faecal pat. As can be seen from Fig. 1 there appeared to be less

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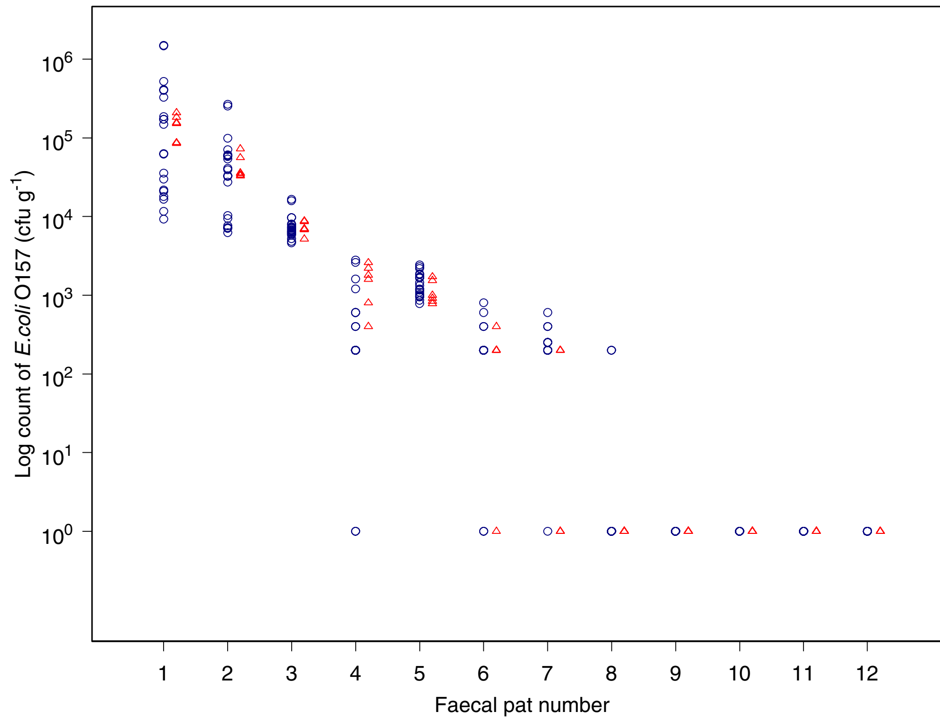

Fig. 1. Variation in the concentration (cfu/g) of *E. coli* O157 present within samples taken from 12 naturally infected faecal pats. Ten individual faecal samples (○) and three pooled samples (△) were taken from each pat and plated in duplicate yielding 20 and six data points, respectively. Samples in which *E. coli* O157 was isolated by IMS methods are represented as 200 cfu/g and those in which no *E. coli* O157 was detected by either method are represent as 10^0 [se1].

variation in the counts of *E. coli* O157 recovered from the pooled samples in these four pats. These counts were within the range of counts observed from the individual samples.

In the remaining four faecal pats, the variation in counts of *E. coli* O157 included zero for some areas of the pat, with counts ranging from 0 (negative by both IMS and direct spiral plating) up to 10^3 cfu/g. Although the limit of detection by direct plating was approximately 250 cfu/g, the more sensitive method of isolation (selective enrichment followed by IMS) would have detected *E. coli* O157 if present at concentrations above a threshold well below this level. All of the *E. coli* O157 isolated were *eaeA* and *vt1*, *vt2* positive and were indistinguishable by PFGE.

3.2. Statistical analysis

The results from fitting the model are shown in Table 1. Faeces from the inside of the faecal pat contained significantly lower concentrations of *E. coli* O157 than faeces taken from the outside of the faecal pat (the 95% credible intervals do not include 0).

Most of the variation in counts of *E. coli* O157 was attributable to different faecal pats (denoted in Table 1 by σ_A^2) and the variation within faecal pats (denoted in Table 1 by σ_B^2). The point estimates of the variance of these random effects were similar, suggesting that variation within a faecal pat contributes a similar amount to the residual variation as the variation between different faecal pats. However, these estimates were associated

Table 1
Multilevel model of the variation in counts of *E. coli* O157 in faecal pats

		Posterior mean	Posterior standard deviation	95% Credible intervals
Fixed effects				
μ (Mean of logged counts from outside of pat)		8.08	1.57	4.87, 11.23
β (Effect of sampling from inside of pat)		−2.12	0.66	−3.47, −0.85
Random effects				
Between animal	σ_A^2	18.84	16.69	5.32, 49.42
Within faecal pat (individual samples)	σ_B^2	21.08	21.00	9.42, 60.80
Within faecal pat (pooled samples)	σ_C^2	0.31	0.19	0.11, 0.64
Laboratory	σ_D^2	0.006	0.003	0.0021, 0.01

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with a high degree of uncertainty and the credible intervals were wide owing to there being a relatively small number of pats in this study. The corresponding variance estimate for the pooled samples from each pat was low in comparison, 0.31, (denoted in Table 1 by σ_C^2). Therefore, pooling samples from throughout the faecal pat appears to be an effective means of overcoming the heterogeneous distribution of *E. coli* O157 and providing representative estimates of the average intensity within a faecal pat. The variation between counts of *E. coli* O157 from duplicate plates was very low (variance estimate = 0.006).

The fact that the third pooled sample contains some faeces in common with the other two pooled samples suggests that this sample should be correlated with the other two. The model was modified to include correlation between the third sample and the first two, though the results changed very little and the correlation parameter was poorly identified. It was therefore thought that the simpler model without correlation was the preferable model and its results are the ones presented.

4. Discussion

The major finding of this study was that *E. coli* O157 is not homogenously distributed throughout bovine faecal pats. The greatest variation in the distribution of *E. coli* O157 observed within a single faecal pat was 100-fold. Four of the faecal pats sampled yielded no *E. coli* O157 even though many of the faecal pats within this pen were found to contain *E. coli* O157. This indicated that even with extensive sampling, some faecal pats remain free, or have undetectable levels of *E. coli* O157. Although the degree of heterogeneity of *E. coli* O157 within faecal pats was evident on initial examination of the data, statistical modelling of the data was required to account for other sources of variation that may have attributed to the variation in counts. Taking a multilevel modelling approach allowed us to ascertain the relative importance of different sources of variation within the sampling hierarchy whilst allowing for the fact that samples from the same faecal pat were not independent. The majority of the variation within the sampling hierarchy was between faecal pats from different animals and within faecal pats, with an insignificant source of variation introduced by the laboratory methods employed. The variation in counts of *E. coli* O157 within the faecal pat could not be attributed to between strain variation of *E. coli* O157 as the PFGE patterns from 27 of the isolates indicated the presence of indistinguishable strains in the faeces of these animals.

It has been proposed that faeces become contaminated with *E. coli* O157 as they pass through the distal colon or recto–anal junction [11]. Therefore, a possible explanation for the observed variation in counts is that as faeces pass through the distal part of the GI tract, *E. coli* O157 contaminates the area of the faeces in close contact with the gut wall. Faeces were voided at approximately hourly intervals by cattle in this study suggesting a relatively rapid gut transit through the lower GI tract. It is likely that mixing of formed faeces containing *E. coli* O157 occurs primarily when they impact with the pen floor. This is unlikely to be sufficient to distribute populations of *E. coli* O157 throughout faecal pats. This hypothesis is further supported by the marked increase in the concentrations of *E. coli* O157 recovered from outer areas of the faecal pat, more likely to have been in contact with the gut wall. These findings are in agreement with a similar study in which the distribution of *E. coli* O57 within faeces of cattle inoculated with strains of *E. coli* O157 was examined [11]. Although there may not be a direct relationship, crusting appeared to be largely confined to outer areas of the faecal pat which would indicate that faecal material that has been on the outside during passage through the rectum, remains on the outer areas of the voided faecal pat.

The non-homogeneous distribution of *E. coli* O157 within faecal pats has important implications for the design and interpretation of future studies. We recommend that studies aiming to gain accurate and reliable estimates of the intensity of *E. coli* O157 in faecal pats, should consider pooling faecal material from several sites (as was the case in this study) throughout the faecal pat to overcome the heterogeneity within faecal pats. The findings in this study and those presented in the study by Naylor et al. [11] suggest that if researchers are only concerned with the presence/absence of *E. coli* O157 in faecal pats, rather than the intensity, sampling from the outer regions of faecal pats would maximise the probability of detection. However, the ability to identify areas of the faecal pat that have been on the outside during passage through the intestines may vary with faecal consistency.

There has been considerable variation in prevalence estimates of *E. coli* O157 carriage in cattle between studies (for a review see [16]). This variation has been attributed to isolation methods used, the study population, region and time of study. From the findings presented here, it also evident that faecal sampling method may also explain some of this variation. It has previously been shown that different prevalence estimates of the pathogens *Campylobacter* spp., *Cryptosporidium parvum* and *Giardia duodenalis* are obtained from samples taken from voided faecal samples and those collected from the rectum of cattle [17]. Although, the authors suggest that this may be due to inactivation of pathogens in faeces after defecation, other factors such as the length of time between defecation and sampling of the faecal pat and the consistency of digital rectal retrieval for sampling animals of different ages may also affect the probability of detecting *E. coli* O157 in faecal samples. Sampling of

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the recto–anal mucosa has been reported to be more sensitive for the direct culture of *E. coli* O157 compared to direct faecal culture [18]. The authors propose that sampling of the recto–anal mucosa may also distinguish between those cattle colonised and those transiently shedding the bacteria. Although, the relationship between *E. coli* O157 at this intestinal site and the concentrations of *E. coli* O157 shed in faeces is not yet known, this would suggest that studies reporting estimates of bovine faecal carriage of *E. coli* O157 from rectal samples are not directly comparable to those taking faecal pat samples. Therefore, in the design of future studies, the use of different methods of faecal sampling for different groups of cattle in the same study should be avoided.

Faecal pat sampling provides a quick, non-intrusive way of sampling large numbers of animals. Representative and comparable estimates of the prevalence of *E. coli* O157 within a population of cattle can be gained, provided that multiple samples are taken from throughout the faecal pat. As human exposure to *E. coli* O157 occurs via various routes, including environmental pathways, gaining estimates of prevalence based on environmental samples, including faecal samples from the farm environment, may give a truer reflection of the burden posed by *E. coli* O157 within this important pathway for transmission.

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