RESEARCH LETTER

Persistence and metabolic activity of *Escherichia coli* O157:H7 in farm animal faeces

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Received 17 April 2008; accepted 14 July 2008.
First published online 19 August 2008.

DOI:10.1111/j.1574-6968.2008.01310.x

Editor: Rob Delahay

Keywords
bovine; ovine; pathogen; ruminants; swine; VTEC.

Abstract

Ruminants, and to a lesser extent monogastric farm animals, are known to be natural reservoirs of *Escherichia coli* O157:H7, and contact with contaminated faeces has been linked to human infection. This study used a nontoxigenic, chromosomally marked, *lux* reporter strain to compare the persistence and activity (bioluminescence) of *E. coli* O157:H7 over 21 days in the faecal liquor of five farm animals: horse, sheep, cow, pig and piglet. Samples were inoculated with the *lux E. coli* O157:H7 ($7.82 \log \mathrm{CFU\,mL^{-1}}$) and stored at $20 \pm 1\,^{\circ}\mathrm{C}$. The organism was recovered from all samples throughout the experimental period, although lower numbers were recovered from horse faecal liquor relative to all other types ($P < 0.001$). The organisms' activity declined in all samples over time and no luminescence could be detected in any sample 21 days postinoculation. However, activity did increase greatly within pig and piglet faeces during initial stages of monitoring and overall luminescence was greater in piglet samples compared with all other samples ($P < 0.001$). This is the first study to demonstrate how both the persistence and metabolic activity of *E. coli* O157:H7 notably varies within a range of ruminant and nonruminant animal faeces. Further research is needed to elucidate the factors that govern differential persistence and metabolic activity of *E. coli* O157:H7 within such matrices.

Introduction

Escherichia coli O157:H7 is a pathogen which may cause serious illness in humans (Chart, 2000). Ruminants are known to be the main natural reservoir of the bacterium; however, it has been traced to domestic monogastric farm animals, including pigs and horses (Chapman, 2000). Infected animals shed the organism within their faeces, and many cases of human infection have been related back to direct or indirect contact with faeces, for example following faecal contamination of water supplies (Jones, 1999).

Over recent decades, petting farms and horse riding stables have become increasingly popular with visitors of urban background. This might therefore increase the occurrence of contact between humans and farm animal faeces, and thus the possibility of *E. coli* O157:H7 infections. While existing work depicts the ability of the organism to survive in ruminants faeces (e.g. Kudva *et al.*, 1995; Avery *et al.*, 2005), its survival in nonruminant farm animals such as pigs and horses is much less understood. Furthermore, no studies have compared both survival and activity of the bacterium within the faecal material of such animals, which is required for comparative risk assessment purposes. Such a comparison ideally requires a segregationally stable, reporter construct of *E. coli* O157:H7 where reporter gene expression provides a measure of metabolic activity. The chromosomal *lux* reporter constructed by Ritchie *et al.* (2003) meets these requirements. The aim of this work was to use this bioluminescent construct to investigate the persistence and metabolic activity of *E. coli* O157:H7 in horse, sheep, cow, pig and piglet faecal matter.

Materials and methods

Faeces collection and characterization

Replicate faecal samples ($n = 4$ for each faeces type) were collected in sterile plastic bags (1 day before commencement of experimental work) from a commercial sheep and beef farm, a commercial pig farm, and horse riding stables in North Wales. All samples were of approximately the same age (< 2 h since excretion). None of the animals had been administered antimicrobial agents or feed additives that could have an effect on bacterial growth within 2 months before sampling. Samples were transported to the laboratory within 40 min and stored at 4.0 ± 0.1 °C before use. A portion of each faeces was retained to perform chemical and microbiological characterization by the same methods as detailed for manures in Williams *et al.* (2006). In addition to those methods, organic matter content was determined after 'ashing' at 450 °C; and non-sorbitol-fermenting colonies were confirmed as *E. coli* O157:H7 by latex agglutination (Oxoid DR620).

Preparation of *E. coli* O157:H7 inoculum

An inoculum was prepared from a fresh overnight culture [Luria–Bertani (LB) broth; Difco Ltd, Teddington, Surrey, UK; 150 r.p.m., 18 h, 37 °C] of a bioluminescent strain of *E. coli* O157:H7 (strain 3704 Tn*5 luxCDABE*; Ritchie *et al.*, 2003) in stationary growth phase. Although the strain has been proven to be nontoxigenic, it still accurately reflects survival patterns of toxigenic *E. coli* O157:H7 strains (Kudva *et al.*, 1998; Bolton *et al.*, 1999; Ritchie *et al.*, 2003). Cells were washed and concentrated by centrifugation as described in Avery *et al.* (2005). The concentration of *E. coli* O157:H7 in the inoculum was *c.* 7.82 log CFU mL^{-1}, determined by plating out in duplicate onto sorbitol MacConkey agar plates supplemented with 0.05 mg L^{-1} cefixime and 2.5 mg L^{-1} potassium tellurite (CT-SMAC; Oxoid, Basingstoke, UK) and enumeration of colonies with the characteristic appearance of *E. coli* O157:H7 following incubation (18 h, 37 °C). Non-sorbitol-fermenting colonies were examined via latex agglutination, as above.

Preparation and inoculation of faecal liquor

Ten gram samples of each faecal type were placed in each of three sterile centrifuge tubes containing 30 mL of deionized water, in a series of eight replicates (to correspond with the number of harvests, as described below). These were subsequently shaken (60 min, 200 r.p.m.) and centrifuged (10 min, 10 000 ***g***), then filtered (Whatman # 42); resulting in triplicate 20-mL samples from each faeces type, for every harvest event (i.e. 3×20 mL $\times$ 8).

All liquor samples were then spiked with the same volume of inoculum, to give a starting concentration of 6.67 log CFU *E. coli* 0157:H7 mL^{-1}. Tubes were sealed and mixed gently, then loosely capped and maintained at 20 ± 1 °C for the remainder of the experimental period. This temperature was chosen to reflect late spring/early summer UK air temperatures, when most animals are out on pasture and manure is most frequently spread on land.

Harvests

Destructive harvests were performed from randomly selected samples at 1, 3, 6, 9, 12, 15, 18 and 21 days postinoculation. At each harvest, 5 mL was taken from each of the triplicate samples, and placed into individual 31-mL sterile, plastic bottles. Bottles were subsequently shaken (15 min, 200 r.p.m., 20 °C) in 15 mL of sterile, 1/4-strength Ringer's solution before plating, incubation and enumeration onto CT-SMAC agar, followed by latex agglutination, as above.

Bioluminescence of *E. coli* O157:H7

A parallel experiment was designed to assess how the activity of *E. coli* O157:H7 varies between the above faeces types. Bioluminescence of bacteria in faeces was measured at 0 h (immediately after inoculation), 2, 5, 10, 20, 24, 48, 72, 168, 240 and 480 h postinoculation. At each time-point, a 1-mL aliquot from samples used for the enumeration study detailed above was placed into a plastic luminometer cuvette and its luminescence [relative light units (RLU)] determined using a SystemSURE 18172 luminometer (Hygiena Int., Watford, UK).

Data analysis

The means of triplicate plate count data for *E. coli* O157:H7 concentrations in faecal liquor samples (CFU mL^{-1}) were $\log_{10}(y+1)$ transformed to meet the assumptions of ANOVA before statistical analyses. All data (log CFU mL^{-1} for persistence and RLU for bioluminescence) were analysed by a multifactorial ANOVA using GENSTAT 8.1 (Rothamsted Experimental Station, Hertfordshire, UK), incorporating faecal liquor type and time as factors. Significant differences between treatments were identified using Fisher's least significant difference (LSD) test within GENSTAT. Regression analysis was also performed with the same package to identify correlations between mean log CFU mL^{-1} or RLU values and faeces characteristics, employing Pearson's correlation coefficient.

Results

Characterization of faeces

A summary of chemical and microbiological characteristics of the faeces is presented in Table 1. The pH of faeces ranged

Table 1. The intrinsic chemical and microbiological properties of farm animal faeces used in the study

	Faeces type				
Parameter	Horse	Sheep	Cow	Pig	Piglet
pH	6.50 ± 0.03	8.06 ± 0.01	8.60 ± 0.02	7.93 ± 0.09	6.86 ± 0.05
Electrical conductivity (mS cm^{-1})	1.65 ± 0.10	2.43 ± 0.03	7.43 ± 0.10	5.38 ± 0.53	4.02 ± 0.18
Moisture content (g kg^{-1})	781 ± 2	620 ± 5	884 ± 1	712 ± 3	742 ± 2
Organic matter (g kg^{-1} dry weight)	933 ± 2	789 ± 4	792 ± 3	845 ± 1	830 ± 1
DOC (mg L^{-1})	2113 ± 132	4558 ± 146	2447 ± 50	2480 ± 210	4272 ± 1179
TDN (mg L^{-1})	196 ± 22	612 ± 7	1601 ± 47	1289 ± 281	1033 ± 216
NO_3^- (mg L^{-1})	2.2 ± 0.3	2.4 ± 0.4	3.1 ± 0.3	1.9 ± 0.4	1.2 ± 0.3
NH_4^+ (mg L^{-1})	21 ± 3	40 ± 6	147 ± 1	145 ± 3	140 ± 2
Na (mg L^{-1})	172 ± 12	380 ± 65	424 ± 7	747 ± 56	431 ± 37
K (mg L^{-1})	1758 ± 38	2520 ± 920	5091 ± 187	2324 ± 88	2578 ± 206
Ca (mg L^{-1})	530 ± 21	2422 ± 66	903 ± 19	1169 ± 96	667 ± 13
PO_4^{3-} (mg L^{-1})	649 ± 35	21 ± 11	37 ± 3	59 ± 15	1548 ± 170
Background bacteria (log CFU g^{-1})	8.31 ± 0.05	9.09 ± 0.11	7.16 ± 0.05	8.53 ± 0.05	9.44 ± 0.05
E. coli O157:H7 (log CFU g^{-1})	ND	ND	ND	ND	ND

All values represent means $\pm$ SEM ($n = 4$).
mS cm^{-1}, milli Siemens per centimetre; DOC, dissolved organic carbon; TDN, total dissolved nitrogen; ND, nondetectable (< 5 CFU g^{-1}).

from slightly acidic (horse, 6.50 ± 0.03) to slightly basic (cow, 8.60 ± 0.02). Although piglet faeces contained high levels of sodium and phosphate, cow faeces possessed the overall greatest levels of cations and hence electrical conductivity value. Cow faeces were the wettest of all faeces types and sheep the driest. Organic matter content was similar in all faeces. Cow, pig and piglet faeces were notably nitrogen-rich and possessed considerably greater levels of ammonium in comparison with horse and sheep faeces. All faeces types supported high populations of background bacteria; however, no *E. coli* O157:H7 were isolated from any sample following enrichment.

Survival of *E. coli* O157:H7

Overall, significantly lower numbers of *E. coli* O157:H7 ($P < 0.001$) were recovered from horse faecal liquor relative to all other faeces types (mean log CFU mL^{-1} faecal liquor $\pm$ SEM: horse, 5.68 ± 0.24; other faecal liquors, 7.75 ± 0.12). Although overall counts were greatest in sheep faecal liquor, these were the same ($P > 0.05$) as in cow, pig and piglet faecal liquor. Counts of *E. coli* O157:H7 followed similar trends in all types of faecal liquor, increasing in all samples (except horse) after inoculation, followed by a peak on day 3 (Fig. 1). Numbers declined thereafter (overall mean log CFU mL^{-1} faecal liquor $\pm$ SEM: day 3, 8.46 ± 0.38; day 21, 6.45 ± 0.33; $P < 0.001$), albeit at significantly different rates, depending on faeces origin (time $\times$ faecal liquor type interaction, $P = 0.004$); and greater divergence was evident between samples towards latter stages of the experiment (Fig. 1). Only within horse faecal liquor had populations increased slightly (day 15), then stabilized by day 21. Nevertheless, *E. coli* O157:H7 counts were still less ($P < 0.001$) in horse faecal liquor than in samples with the highest numbers

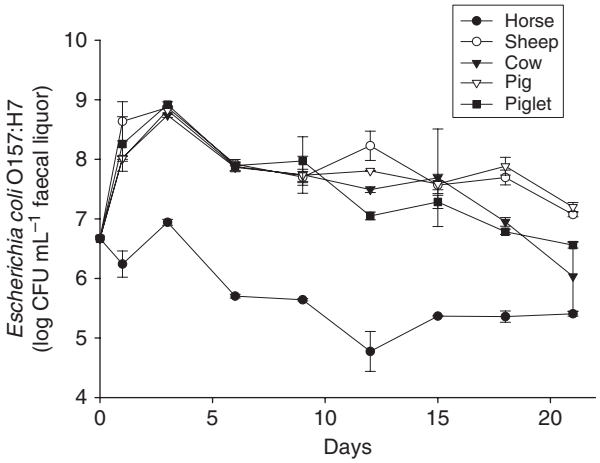

Fig. 1. Survival of *Escherichia coli* O157:H7 (log CFU g^{-1}) in faecal liquor samples from different farm animals over 21 days. Values represent means $\pm$ SEM ($n = 3$). The initial *E. coli* O157:H7 inoculation level was 6.67 log CFU mL^{-1} liquor. Counts of *E. coli* O157:H7 were significantly less ($P < 0.001$) in horse faecal liquor than in all other faecal liquors at every sampling date except on day 21.

(pig and sheep faecal liquor) at the last sampling date. Overall mean counts of *E. coli* O157:H7 were significantly negatively correlated ($P = 0.039$; $r^2 = 0.804$) with organic matter content but not with any of the other measured faeces characteristics.

Bioluminescence of *E. coli* O157:H7

Overall, bioluminescence values decreased over the course of the experiment in all faecal liquor samples (Fig. 2); although at significantly different rates with the type of faecal liquor (time $\times$ faecal liquor type interaction, $P < 0.001$). Initial

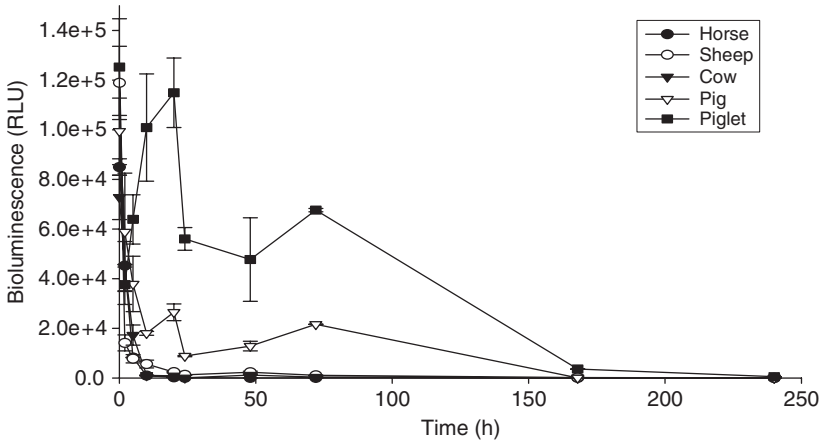

Fig. 2. Bioluminescence (RLU) of *Escherichia coli* O157:H7 over time in faecal liquor samples from different farm animals. Values represent means ± SEM ($n = 3$).

readings showed greatest bioluminescence in pig and sheep faecal liquor; yet, these were also the samples that displayed the greatest loss in luminescence between 0 and 2 h. However, overall luminescence was significantly greater ($P < 0.001$) in piglet samples compared with all other samples, and luminescence in pig samples was significantly greater ($P < 0.001$) than in cow and horse samples (but not sheep). Furthermore, whereas luminescence in cow, horse and sheep faecal liquor all showed a similar downward trend over time, notable increases were observed within the first 72 h post-inoculation in liquor obtained from pig and piglet faeces (Fig. 2). Between 168 and 240 h, no significant differences ($P > 0.05$) were found between types of faeces. By 240 h, no luminescence was detected in cow or horse faecal liquor, and only low luminescence was detected in sheep and pig faecal liquor (mean RLU ± SEM: sheep, 5.67 ± 2.91; pig, 12.67 ± 0.33); however, notable luminescence was detected within piglet faecal liquor (mean RLU ± SEM: 527 ± 22). No luminescence was detected in any sample taken 480 h postinoculation (data not presented). Regression analysis showed no correlation ($P > 0.05$) between bioluminescence values and any of the measured faeces characteristics.

Discussion

It is not entirely clear why ruminants (particularly cattle) have been found to be the foremost animal reserve of *E. coli* O157:H7; however, it is known that the ruminant gut structure favours primary colonization, and subsequent persistence and proliferation (Grauke *et al.*, 2002; Moxley, 2004) in comparison with monogastric species such as pigs and horses. Nevertheless, some studies (Booher *et al.*, 2002; Cornick & Helgerson, 2004) have found that pigs inoculated with *E. coli* O157:H7 shed the bacterium for a similar duration to that of sheep (Cornick *et al.*, 2000). In this study, *E. coli* O157:H7 were still recovered after 21 days from both sheep and cow faeces; supporting previous work showing survival of the organism in ruminant faeces (e.g. Kudva *et al.*, 1998; Scott *et al.*, 2006). While there is also some evidence that pigs (Johnsen *et al.*, 2001; Booher *et al.*, 2002) and horses (Bauwens *et al.*, 2000; Chapman *et al.*, 2000) occasionally harbour the organism, its subsequent survival within their shed faeces is unknown. However, this study showed that *E. coli* O157:H7 persisted within swine faeces for over 21 days and at statistically equal numbers to that within cow and sheep samples. Furthermore, the greater bioluminescence within pig and piglet samples ($P < 0.001$) indicates that the organism may display enhanced metabolic activity within swine faecal matter. With regards to horse faeces, although we found that both organism numbers and activity were lowest in such samples, it was still recovered throughout the experimental period. Our findings highlight the capacity for *E. coli* O157:H7 to survive in the faecal matter of a range of common farm animals.

There are many variables which may affect both populations (e.g. competition and predation) and activity (e.g. O_2 and nutrient availability) of *E. coli* O157:H7 in matrices such as faeces (Avery *et al.*, 2005, 2008; Williams *et al.*, 2008). However, in this study, only one of the measured characteristics (organic matter content) significantly correlated with numbers, while none statistically correlated with luminescence. A lack of correlation between matrix properties and survival of *E. coli* O157:H7 within the matrix has occurred in previous work (e.g. Avery *et al.*, 2005, 2008), and in the current study restricts our ability to explain the different patterns of persistence and activity that occurred within different animal faeces. That increasing organic matter was negatively correlated with counts of *E. coli* O157:H7 was somewhat surprising; however, there was a tentative (but statistically insignificant) relationship where the concentration of all nutrients (except carbon) decreased as organic matter increased (data not shown). Greater faecal organic matter may thus have induced a nutrient-limited environment for the inoculated bacteria, with a resulting decrease in survival rates. Alternatively, increased organic matter may have stimulated greater competition.

Diet has been proven on numerous occasions to affect the carriage and excretion of the normal gut microflora, hence influence competition and/or predation of organisms such as *E. coli* O157:H7 in animals (Kudva *et al.*, 1997; Hovde *et al.*, 1999). For this study, all of the faecal samples were collected over winter when animals were housed indoors. Horses were fed a mixture of *c.* 80% high-fibre feedstuffs (hay and haylage), supplemented with *c.* 20% concentrated feed; while cattle and sheep were fed a similar mixture comprising mostly of silage, supplemented with a concentrate. Conversely, pigs were fed a higher protein, lower fibre diet, and young piglets were fed milk that is high in fat and proteins. While studies have noted the relevance of dietary fibre to persistence of *E. coli* O157:H7 within ruminant faeces (Kudva *et al.*, 1997; Hovde *et al.*, 1999), its effects on the organisms' activity is unknown. As highlighted above, the activity of *E. coli* O157:H7 in pig and piglet faeces showed a markedly different trend to other samples; while, interestingly, activity within horse faecal matter followed a similar pattern to ruminants. This may suggest that animal diet influences bacterial activity (as well as numbers) within animal faecal matter. The persistence of the organism in horse faecal matter supports previous studies that suggest that fibre is related to long-term low-level persistence (Kudva *et al.*, 1997; Hovde *et al.*, 1999).

The persistence of *E. coli* O157:H7 in horse faeces is of concern, as their manure is commonly used by both farmers and gardeners as a fertilizer, and humans are also more likely to come into contact with horses and their faeces through recreation. It is also common for horses to share grazing land with ruminants such as sheep and cattle, which are known natural reservoirs of *E. coli* O157:H7. The spread of the bacterium within groups of animals (e.g. cattle herds) is acknowledged (Turner *et al.*, 2006); however, little is known about the ability of the bacterium to spread between different species. A study into the prevalence of the organism in horses on grazing shared with colonized ruminants would elucidate this, and hence indicate whether the practice of shared grazing may increase the risk of animal infection. Studies have shown that young cattle show the highest prevalence of *E. coli* O157:H7 (Johnsen *et al.*, 2001; Kang *et al.*, 2004) and shed higher numbers of the organism in their faeces compared with adult cattle (Fukushima & Seki, 2004). To our knowledge, no such work has investigated survival of the organism in adult and young pigs; although results from this study (greater activity and similar numbers in piglet) show that this may warrant such investigation.

The bioluminescence measurements provide an insight into the metabolic state of the pathogen cells in the different samples. Luminescence could not be detected in any sample 240 h postinoculation, whereas viable cells were still recovered throughout the experimental period. This is a similar pattern to a previous study we conducted in a different environment (Williams *et al.*, 2007), and again illustrates the pathogens' ability to persist for extended periods in a low-metabolic state. We cannot currently ascertain whether viable cells displaying low activity are as capable of causing infection as highly active cells, or if infection is more dependent on the number of cells ingested. However, in future work we aim to correlate activity with virulence attributes, which will provide a powerful tool for estimating the threat posed by *E. coli* O157:H7 in matrices such as animal faeces. Performing further trials on the existing format but with additional constructed *lux*-marked strains of *E. coli* O157:H7 would also be worthwhile to determine whether interstrain variation exists with regards to activity.

In conclusion, the use of stable *lux* reporter strain demonstrated that *E. coli* O157:H7 persisted in a range of ruminant and nonruminant farm animal faecal types over 21 days. Further, this study has illustrated for the first time, the notable differences in the pathogens' metabolic activity within the faeces of different animal species. Greater work is needed to investigate how animal species, their diet and age affect the behaviour of *E. coli* O157:H7 in their respective faeces.

Acknowledgements

We are grateful to the BBSRC Agri-Food for funding this work. We also wish to acknowledge all farmers that supplied animal faeces.

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