

Climate, lactation, and treatment factors influence faecal shedding of *Escherichia coli* O157 pathotypes in dairy cows

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Received 6 March 2016; Final revision 2 August 2016; Accepted 5 August 2016;
first published online 16 September 2016

SUMMARY

Among pathogens shed by cattle, *Escherichia coli* O157 ranks highest in those causing human illness. To date, prevalence and risk factors for O157 shedding have been assessed in feedlot, but not dairy cattle. The study aimed to determine prevalence levels and risk factors for O157 atypical enteropathogenic *E. coli* (aEPEC) and enterohaemorrhagic *E. coli* (EHEC) shedding in dairy cattle. Dairy cattle ($n = 899$) within the first 21 days of lactation were sampled monthly over the course of 1 year, on three dry lot dairies surrounding Fort Collins, CO. During visits multiple factors were measured (disease history, pharmaceutical use, climate measures, etc.), and cattle faeces were collected and assessed for presence of O157 and virulence genes. Logistic regression analysis was performed using O157 outcomes and measured factors. Prevalence of O157 aEPEC was 3·7%, while EHEC was 3·0%. Many potential risk factors were highly correlated, and used to build separate multivariable models. An increase in humidity was positively associated with aEPEC, while fluid faeces and history of disease showed a negative association. Meanwhile, an increase in temperature and antibiotic treatment was positively associated with EHEC, while more days in milk, higher hygiene score and cow contact were negatively associated. These results may guide mitigation strategies that reduce O157 shedding, and contamination of the human food chain.

Key words: Antibiotics, *Escherichia coli* (*E. coli*).

INTRODUCTION

Escherichia coli O157:H7 infection continues to be one of the most common food animal-borne human diseases. National Centers for Disease Control surveillance estimates that upwards of 96 000 human cases occur annually, causing symptoms of bloody

diarrhoea, abdominal cramps, and haemolytic uraemic syndrome [1, 2]. Historically, outbreaks have occurred after the pathogen is introduced to the human food chain through bovine products or contaminated produce, or through direct human contact with cows [3]. Understanding and reducing the presence and dissemination of *E. coli* O157:H7 (O157) from bovine hosts remains paramount to ultimately mitigating human infection with this organism.

The pathogenicity of O157 strains shed by cattle is defined by the presence of virulence genes retained on mobile genetic elements [4]. These genes include *stxI*

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and *stx2*, which produce shiga toxins and exist on phage DNA [3]. The virulence gene *eae* lies in a pathogenicity island and encodes the protein intimin, which allows bacterial adherence to epithelial cells and subsequent attaching-and-effacing lesions in humans and cattle [5]. Because of the mobile nature of these elements, serotyping alone is an inferior method to use when determining the pathogenicity of *E. coli* strains [6]. Indeed, cattle have been shown to harbour O157 pathotypes with severe human infectivity, containing both *stx* and *eae* genes (enterohaemorrhagic *E. coli*; EHEC), yet they may also harbour the O157 pathotype containing only *eae*, or *eae* with a bundle-forming pilus (*bfp*) gene (atypical enteropathogenic *E. coli*; aEPEC, and enteropathogenic *E. coli*) [7]. O157 aEPEC is an *E. coli* variant from which EHEC likely descended, and although less studied may have a high prevalence on dairy farms and an ability to cause serious diarrhoeic infection and calf diarrhoeal disease [8, 9]. Not all studies to date have characterized the nature of the O157 pathogen beyond serotype, yet this is a critical step in truly understanding bovine transmission dynamics and human health consequences [10, 11].

Dairy cattle are unique in their ability to contaminate diverse food products including milk, meat, produce, and manure-fertilized crops. Even though O157 is ubiquitous in dairy herds across the United States, risk factors for colonization and shedding dynamics have more commonly been studied in beef herds [12, 13]. Dairy studies are especially relevant due to the increasing popularity of drinking raw milk, and the fact that high prevalence levels of O157 have been noted in the few published studies [14–17]. Previous work has shown several environmental risk factors for dairy O157 shedding, including season, temperature, humidity, rainfall and solar exposure [17]. Herd-level variables of importance appear to be dairy production methods, the size of herd, use of total mixed ration or pasture growth, methods for manure storage and ventilation, and number of birds present [10, 15]. To our knowledge, O157 shedding studies that include dairy cow health and treatment data have yet to be performed, but one study that assessed lactation characteristics determined that the parity, number of days in milk, somatic cell count, and milk content of individuals were linked with shedding status [18]. Given the factors that may influence shedding, early lactation cows exposed to nutritional, environmental and social changes, along with high levels of metabolic and hormonal stress due to calving and lactation, are thought to present a high-risk group [19].

In the United States, the intermittent nature and daily variation of bovine colonization with O157 indicates that implementation of a longitudinal sampling scheme is necessary to fully appreciate and define shedding dynamics [11, 18, 20]. Importantly, those animals shedding high quantities of the pathogen ('high-shedders'; $>10^3$ – 10^4 c.f.u./g faeces) may account for the majority of O157 contamination within a herd, increasing animal-to-animal transmission and risk of food chain contamination [18, 21, 22]. When classifying cows based on O157 shedding magnitude vs. positive shedding alone, outcomes for risk factor analyses can become altered [17].

Determining the risk factors for individual dairy cow O157 shedding may provide a framework within which to develop targeted prevention or treatment strategies to quell shedding and the subsequent introduction of pathogenic O157 to the human food chain. In an attempt to gain a comprehensive picture of dairy O157 shedding dynamics, the present study aimed to determine O157 shedding status, magnitude, and virulence in early lactation dairy cattle from three northern Colorado dairy herds sampled monthly over the course of 1 year. Concurrently, environmental, individual animal, and herd-level data were collected for inclusion in multivariable risk factor analyses.

METHODS

Samples and data collection

Three dry-lot dairies located within a 20-mile radius of Fort Collins, Colorado and representing a combined population of 2750 lactating cattle were utilized for the study. Herds were sampled monthly (every 2–4 weeks) for a period of 1 year (July 2013–June 2014, $n = 939$, representing 899 individuals). Although a few cows were sampled in duplicate, each sample was considered independent due to the daily variation in shedding, change of season between sample acquisition, and variation in lactation characteristics (days in milk, disease and treatment status, contact with other cows, etc.) between collections. Herd 3 could not be sampled during December due to barn construction. During sampling, 10 g faeces were collected from cows within the first 21 days of lactation via rectal palpation. As the herds studied calve on a year-round basis, cattle sampled tended to be evenly dispersed within this 21-day lactation window. At collection the rectal temperature, faecal consistency, hygiene and body condition scores for each animal

were recorded, as well as pen stocking number (cow contact). Management of the herds studied included removal of calves from the dam immediately postpartum. As youngstock are reared in geographically separated individual hutches, it is of note that the variable ‘cow contact’ measures only the influence of other adult cows. The level of precipitation, ambient average temperature and humidity were determined each week sampling was completed using National Oceanic and Atmospheric Administration values (www.noaa.gov). Animal life history features including lactation number, calving ease, days in milk, prior disease, and disease treatments were obtained from on-farm computer record systems (Dairy Comp 305™, Valley Agricultural Software, USA). Diseases and injuries catalogued in Dairy Comp and collected for analysis included history of bloat, diarrhoea, dystocia, haemorrhagic bowel syndrome (HBS), Johne’s disease, ketosis, hoof lameness, lameness due to injury, mastitis, metritis, pneumonia, retained placenta, udder injury, metabolic symptoms, oedema, abomasal ulceration, displaced abomasum, and fever. All recorded treatments were analysed, and included ceftiofur, ampicillin, oxytetracycline, pirlimycin, cephalixin, flunixin meglumine, dexamethasone, and aspirin administration. Disease and treatment data were only collected for the given lactation cycle. Specific definitions for variable measures can be found in Supplementary Appendix 1.

Given the data collected, treatment types were further collapsed and assessed as broad categories by type (antibiotic treatment, other drug treatment, never treated) and administration and type (intramammary antibiotic, systemic antibiotic, systemic anti-inflammatory, never treated). Categories were also created for cows receiving multiple treatment types (Supplementary Appendix 1). The covariate ‘disease’ was also assessed using a binary (disease/no disease) method. Since many cows were listed as having a disease but not being treated, a binary variable of ‘disease or treatment’ vs. ‘no disease or treatment’ was created. While over-assessment of recorded risk factors may result in an ultimate loss of model information, all classifications herein were evaluated in an attempt to better understand if disease or treatment may have influenced shedding status, as this is a novelty of the present study. Other variables were collapsed based on biological plausibility and historical relevance. For example, parity comparisons in the dairy setting are often reduced to compare first to second, or \geq third lactation cycles, as cattle commonly

remain in the system only to lactation cycles 2–5. Faecal consistency was broken down to compare a level of 3 (normal consistency) to levels 1 + 2 (more liquid) and levels 4 + 5 (more solid). All data collected for use as variables in regression modelling are described in Table 1, and collapsed variables are further described within Tables 2–6.

Isolation and characterization of O157 pathotypes

O157 isolation was performed following selective enrichment and detection ‘gold standard’ procedures. O157 presence and enumeration was assessed initially through direct plating. Samples were mixed 1:10 in buffered peptone water (BPW) for both enrichment and direct plating. One hundred μ l was spread-plated on sorbitol MacConkey agar with BCIG (Oxoid Diagnostic Reagents, UK) containing 1.25 mg potassium tellurite and 0.025 mg cefixime (CT-SMAC-BCIG; HiMedia Laboratories, India). Plates were incubated at 37 °C for 24 h [23]. As pathogenic O157 has been known to adapt a sorbitol-fermenting phenotype within 24 h, ‘suspect’ O157 colonies seen on plates throughout experiments were deemed as those with straw, grey, pink-grey, or too small/difficult to characterize colony coloration [24, 25].

After direct plate incubation, plates containing \geq 100 suspect colonies were chosen for latex agglutination. Three to 15 colonies per plate, depending on how many colonies were determined ‘suspect’, were tested for O157 by agglutination using an *E. coli* O157 latex kit, following the manufacturer’s instructions (Oxoid Diagnostic Reagents, UK). Positive colonies were enriched in BPW for 6 h and stored at –80 °C in 10% sterile glycerol. For PCR experiments, 10 μ l of thawed isolates were centrifuged at 5000 *g* for 5 min and re-suspended in 30 μ l molecular grade water. Once re-suspended, 5 μ l culture template was placed into Qiagen Multiplex PCR Plus kit reaction master mixes, according to the manufacturer’s instructions (Qiagen, The Netherlands). Briefly, each 25 μ l PCR reaction consisted of 12.5 μ l master mix, 2.5 μ l primer mix containing 0.2 μ M of each primer, 5 μ l molecular grade water, and 5 μ l culture template. The thermal cycling conditions consisted of an initial incubation at 95 °C for 5 min to activate the polymerase, followed by 40 cycles of amplification with denaturation at 95 °C for 30 s, annealing at 57 °C for 1 min and 30 s, and extension at 72 °C for 30 s, ending with a final extension at 68 °C for 10 min. Thermocycling was performed using a Bio-Rad S1000

Table 1. Variables used in regression analysis

Variable name	Type	Levels
Dairy farm	Categorical (GEE for clustering)	Herd 1, herd 2, herd 3
Faecal score	Categorical	≤2, 3 (ref.), ≥4
Hygiene score	Categorical	≤2 (ref.), >2
Body condition score	Categorical	<2, 3 (ref.), >3
Cow contact	Continuous	n.a.
Parity	Categorical	1, 2, ≥3
Calving ease	Categorical	≤2 (ref.), >2
Days in milk	Continuous	n.a.
Vaccine use (SRP, HBS)	Dichotomous	0, 1
Ionophore use	Dichotomous	0, 1
Disease	Categorical based on disease type; dichotomous	1–18 vs. 0; 0, 1
Treatment type	Categorical based on treatment type; Categorical based on treatment class	1–8 vs. 0; Antibiotic treatment, other treatment, vs. never treated
Any disease or treatment	Dichotomous	0, 1
Average temperature (°F)	Continuous	n.a.
Average humidity (%)	Continuous	n.a.
Amount of precipitation (inches)	Continuous	n.a.

GEE, Generalized estimating equations; SRP, *Salmonella* siderophore receptor and porin; HBS, haemorrhagic bowel syndrome; n.a., not applicable.

Table 2. Herd, climate and individual cow parameters pre-selected ($P < 0.25$) for inclusion in aEPEC modelling

Parameter	Level	<i>n</i>	OR*	95% CI	<i>P</i> value
Faecal score	(1,2)	3	0.937	0.218–4.035	0.931
	(4,5)	2	0.466	0.140–1.545	0.212
	3†	30			
Hygiene score	>2	7	0.609	0.263–1.412	0.248
	1 or 2†	28			
Cow contact	Each unit increase	n.a.	1.010	1.000–1.020	0.080
Any disease or treatment	Any	9	0.550	0.190–1.570	0.260
	None listed†	26			
Average weekly humidity (%)	Each unit increase	n.a.	1.067	1.024–1.111	0.002
Weekly precipitation (inches)	Each unit increase	n.a.	0.740	0.520–1.070	0.113

aEPEC, Atypical enteropathogenic *E. coli*; OR, Odds ratio; CI, confidence interval; n.a., not applicable.

* Comparison odds of shedding aEPEC.

† Reference category.

Table 3. Pre-selected parameters for aEPEC modelling found to be correlated at $P < 0.05$

Parameter	Avg. weekly humidity (%)	Cow contact	Weekly precip. (inches)	Any disease or treatment	Faecal score	Hygiene score
Avg. weekly humidity (%)			×	×		×
Cow contact			×	×	×	
Weekly precip. (inches)	×	×				×
Any disease or treatment	×	×				
Faecal score		×				
Hygiene score	×		×			

aEPEC, Atypical enteropathogenic *E. coli*.

Table 4. Herd, climate and individual cow parameters pre-selected ($P < 0.25$) for inclusion in EHEC modelling

Parameter	Level	<i>n</i>	OR*	95% CI	<i>P</i> value
Hygiene score	>2	5	0.53	0.199–1.409	0.203
	1 or 2†	23			
Cow contact	Each unit increase	n.a.	0.971	0.958–0.984	<0.0001
Parity	≥3	7	0.512	0.206–1.272	0.149
	2	6	0.572	0.219–1.495	0.255
Days in milk	1†	15			
	Each day increase	n.a.	0.884	0.819–0.953	0.0014
Any disease or treatment	Any	10	1.758	0.761–4.06	0.187
	None listed†	18			
Antibiotic or other treatment	Ever antibiotic	8	1.66	0.709–3.886	0.243
	Ever other	2	0.467	0.107–2.035	0.311
	None listed†	18			
Average weekly temperature (°F)	Each unit increase	n.a.	1.06	1.027–1.093	0.0002
Average weekly humidity (%)	Each unit increase	n.a.	1.031	0.987–1.076	0.167
Weekly precipitation (inches)	Each unit increase	n.a.	1.19	0.91–1.54	0.21

EHEC, Enterohaemorrhagic *E. coli*; OR, Odds ratio; CI, confidence interval; n.a., not applicable.

* Comparison odds of shedding EHEC.

† Reference category.

Table 5. Pre-selected parameters for EHEC modelling found to be correlated at $P < 0.05$

Parameter	Hygiene score	Avg. weekly humidity (%)	Cow contact	Parity	Days in milk	Avg. weekly temp. (°F)	Any treatment	Antibiotic or other treatment	Weekly precip. (inches)
Hygiene score	■	×		×	×	×			×
Avg. weekly humidity (%)	×	■	×		×	×	×		
Cow contact			■	×	×	×	×		×
Parity	×		×	■		×	×	×	×
Days in milk	×	×	×		■		×	×	×
Avg weekly temp (°F)	×		×	×		■		×	×
Any disease or treatment		×	×	×	×		■		
Antibiotic or other treatment		×		×	×	×		■	×
Weekly precip. (inches)	×	×	×	×	×	×	×		■

EHEC, Enterohaemorrhagic *E. coli*.

Thermal Cycler (Bio-Rad, Australia). PCR products were analysed by agarose gel electrophoresis using a 2% agarose gel (Lonza Group Ltd, Switzerland).

The faecal dilution remaining after direct plating was enriched for 6 h at 37 °C, and stored overnight at 4 °C. Enriched samples not confirmed as O157 positive through direct plating (those not 'high-shedding'; having either none or $<10^3$ – 10^4 c.f.u./g faeces) were subjected to immunomagnetic separation (IMS) using Dynabeads anti-*E. coli* O157 and a BeadRetriever System (Life Technologies, Norway). IMS samples were subsequently plated onto CT-SMAC-BCIG and incubated for 24 h at 37 °C. Suspect colonies were confirmed by latex agglutination and PCR targeting

the O157 *rfb* gene [26]. All *rfb* (and thus O157) positive isolates were subsequently PCR tested for *stx1*, *stx2*, and *eaeA* (a variant of the *eae* intimin gene) using the same PCR protocol outlined above [27].

In addition to isolates that contained *rfb*, *eaeA*, and any *stx* genes (EHEC), or *rfb* and *eaeA* genes alone (aEPEC), many were seen to only harbour the O157 *rfb* gene. To further characterize these allegedly non-virulent isolates, complete typing (O, H, toxin and fimbriae) was performed on a subset of samples. Briefly, an '*rfb* only' isolate was selected from each herd, at three staggered time points throughout the year-long sample period (nine isolates in total). Typing was performed by the American Association

Table 6. Final multivariable models determined for aEPEC and EHEC shedding, using 'dairy herd' as a clustering constraint

Parameter	Comparison	OR*	95% CI	P value	QIC	QICu	
Final models aEPEC							
1	Any disease or treatment	Any vs. none†	0.55	0.33–0.90	0.018	297.9	299.9
2	Average weekly humidity (%)	Each unit increase	1.067	1.034–1.10	<0.0001	290.3	294.6
	Faecal score	(1, 2) vs. 3†	0.45	0.21–0.95	0.037		
		(4, 5) vs. 3†	0.94	0.28–3.19	0.92		
Final models EHEC							
1	Hygiene score	>2 vs. (1, 2) †	0.4	0.25–0.65	0.0002	237.9	235.3
	Cow contact	Each unit increase	0.97	0.96–0.99	0.0002		
	Antibiotic or other treatment	Ever antibiotic vs. none listed†	1.32	1.017–1.72	0.0373		
		Ever other vs. none listed†	0.49	0.17–1.44	0.196		
2	Average weekly temperature (°F)	Each unit increase	1.06	1.02–1.10	0.003	238.9	229.9
	Days in milk	Each day increase	0.88	0.79–0.99	0.028		

aEPEC, Atypical enteropathogenic *E. coli*; EHEC, Enterohaemorrhagic *E. coli*; OR, Odds ratio; CI, confidence interval; QIC, quasi-likelihood independent model criterion.

* Comparison odds of shedding.

† Reference category.

of Veterinary Laboratory Diagnosticians (AAVLD)-accredited *E. coli* reference centre at Pennsylvania State University, in the College of Agricultural Sciences.

Statistical analyses

Monthly and annual prevalence of pathogenic variants of O157 were estimated within and between herds. Confidence intervals of shedding prevalence were calculated using the Wilson procedure with a correction for continuity. Shedding outcomes with respect to presence of 'O157 pathotype' were evaluated for association with potential risk factors. Pathotypes were assessed in separate models because of their different indications for human disease and food safety, and were either those bacteria with the presence of both *rfb* and *eaeA* genes (low pathogenicity aEPEC), or those with presence of *rfb*, *eaeA*, and any *stx* genes (high pathogenicity EHEC). Potential risk factors associated with pathotype outcome were pre-selected using a threshold of $P < 0.25$ in a univariate logistic regression analysis. As a main project goal was to identify the specific factors most significantly associated with the risk of shedding, factors that showed significant correlations or associations with one another were then included in separate multivariable models. This strategy was also chosen because the adoption of future cattle management methods, aimed at reducing O157 in the food chain, may ultimately be

more conceivable through targeting one significant collinear factor and not the other. For each model, a backward stepwise selection process was implemented using a P -to-retain criteria of 0.1 at each step. Final covariate selection was done using a P value of < 0.05 . Interaction terms between all significant variables were evaluated for significance at $P < 0.05$. All multivariable analyses were adjusted for the effect of 'dairy herd' using a generalized estimating equation (GEE) approach. Models created for the same outcome were assessed for biological plausibility and compared using QIC (quasi-likelihood independent model criterion) values based on GEE, to determine models with the best working correlation structure for the data and to identify the most robust shedding predictors based on model fit [28]. All statistical analyses were performed using SAS v. 9.4 (SAS Institute Inc., USA).

RESULTS

Only one sample was confirmed as O157 positive after direct plating, and deemed to originate from a 'high-shedder' ($>10^3$ – 10^4 c.f.u./g faeces when collected). The sample was collected mid-September from herd 2, and contained all tested virulence genes.

Univariate and multivariable statistical analysis was not performed using stratified shedding outcomes of 'high' or 'low' given the lack of high-shedder

detection. Samples were simply classified as positive ($\geq 10^2$ c.f.u./g faeces) or negative for O157 pathotypes after IMS, latex agglutination, and PCR. Level of detection is based on IMS manufacturer indication, which claims the ability to detect as low as 100 c.f.u./g faeces in enriched samples with high amounts of background flora.

Prevalence of *E. coli* O157

During the year-long study period, 939 faecal samples from early lactation cows were collected during 38 separate visits. One hundred and eighty-nine [21%, 95% confidence interval (CI) 17.6–22.9] isolates contained the *rfb* gene alone, 35 (3.9%, 95% CI 2.7–5.2) contained both *rfb* and *eaeA* (aEPEC), 28 (3.1%, 95% CI 2.0–4.3) contained *rfb*, *eaeA*, and any or both *stx* genes (EHEC), and five (0.55%, 95% CI 0.19–1.3%) contained *rfb* and any *stx* genes. For EHEC isolates, a total of 22 contained the *stx2* gene (79%, 95% CI 59.0–91.0), 26 contained *stx1* (93%, 95% CI 75.1–98.8), and 20 contained both *stx1* and *stx2* (71%, 95% CI 51.1–86.1).

The number of aEPEC and EHEC isolates varied by dairy herd and season, with a majority of O157 pathotypes being isolated between June and October (Fig. 1). Herd 1 had the greatest proportion of aEPEC isolates (20/35) and a low proportion of EHEC (5/28). Herd 2 had the greatest proportion of EHEC isolates (23/28) and a moderate number of aEPEC (8/35). In comparison, herd 3 showed relatively few isolates of aEPEC (7/35) and no EHEC isolates (Fig. 1).

A subset of isolates possessing only the *rfb* gene were typed by the Pennsylvania State *E. coli* reference centre, where all were confirmed to contain the *rfb* gene and lack the *stx1*, *stx2*, *eae*, *STa*, *cnf1*, *cnf2*, *CS31A*, *F41*, and *K99* virulence genes. Isolates were all sorbitol fermenting and H+, being either O157:H12, O157:H45 or O157:H+ (untypable).

Significant factors

Risk-factor categories were collapsed based on the power of study data and biological plausibility for comparing measured levels (Table 1). For aEPEC shedding, several variables univariately influenced status (Table 2). Many pre-selected aEPEC parameters were significantly ($P < 0.05$) associated with one another (Table 3). Each set of variables without correlations was included in a multivariable model with

‘dairy herd’ as a clustering constraint. Backward stepwise regression was used to determine multiple significant models for aEPEC (Supplementary Appendix 2).

Many factors were also univariately pre-selected ($P \leq 0.25$) for association with EHEC shedding (Table 4). Significant associations ($P < 0.05$) between univariately significant EHEC parameters can be found in Table 5. All EHEC multivariable models determined using combinations of non-collinear factors can be found in Supplementary Appendix 3.

Biological significance and QIC correlation structure values were used to determine two final ‘best’ multivariate models for aEPEC and EHEC (Table 6). In a final model, history of any disease or treatment, as a single factor, significantly reduced the odds of shedding aEPEC. In a second model, an increase in the average weekly humidity significantly increased, and a more watery faecal score significantly decreased, the odds of shedding aEPEC. For EHEC, an increase in hygiene score and cow contact were shown to reduce the odds of shedding, while recent treatment with any antibiotic significantly increased the odds. In a second model, an increase in the average weekly temperature increased the odds of EHEC shedding, while an increase in the number of days in milk reduced the odds of shedding.

DISCUSSION

E. coli O157 has been shown to adapt a sorbitol-fermenting phenotype within 24 h, and sorbitol-fermenting strains have historically caused human disease outbreaks and harboured a high number of virulence genes [29]. Many O157 strains were isolated in the current study in an attempt to detect all bacteria, sorbitol fermenting and non-sorbitol fermenting, of public health importance. Some studies to date have not PCR-confirmed IMS-isolated O157 strains, resulting in unknown O157 pathogenicity. The multitude of non-virulent O157 isolates found in the current study further validates that PCR confirmation of virulence genes during O157 studies is an absolute necessity.

Strains not confirmed as having *eaeA* or *stx* genes in the current study were confirmed to have O157 and H12 or H45 flagellar antigens. H12 subtypes have previously been isolated from watersheds and cattle at slaughter, and non-pathogenic strains have been used as control strains in EHEC studies [30, 31]. Although H12 strains can become infected with similar phages to O157:H7, it is unknown whether strains isolated in the current study retain an ability to

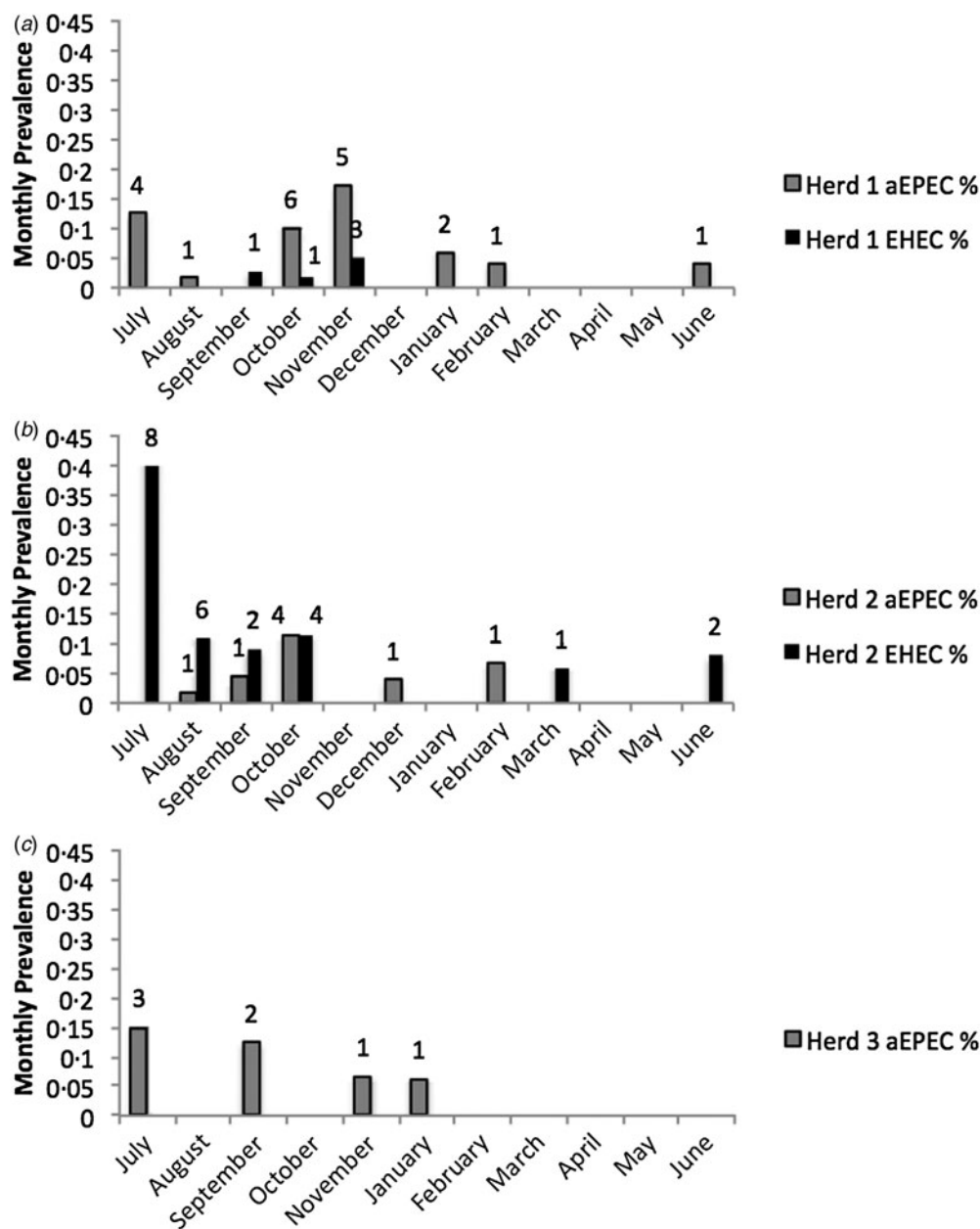


Fig. 1. Monthly prevalence of atypical enteropathogenic *E. coli* (aEPEC) and enterohaemorrhagic *E. coli* (EHEC) measured for (a) herd 1, (b) herd 2 and (c) herd 3, respectively. No isolates collected from herd 3 were positive for EHEC.

become pathogenic. When isolated, O157:H45 strains commonly have contained EPEC virulence genes (*bfp*, *eae*, *tir*) [32, 33]. These strains have been detected in human prevalence and gastroenteritis outbreak studies [34, 35]. Strains in the current study appeared avirulent based on AAVLD typing, and future studies are needed to determine if they have capacity to acquire mobile virulence elements.

Of the dairies studied, shedding prevalence of aEPEC and EHEC were both relatively low (3.7% and 3%, respectively). Although studies to date have

not assessed aEPEC prevalence, other studies of *stx* and O157:H7 dairy prevalence have shown both higher (72.7%, 11.1–32.3%) [15, 18] and lower (1%) levels [10]. Importantly, high-shedding cattle have been hypothesized to influence herd EHEC propagation, and only one was observed in the current study [21, 22]. To date, this is the first assessment of EHEC prevalence on dairies in the Rocky Mountain biome, so it is possible that lower shedding prevalence and measured outcomes may be inherent to the particular environment studied.

aEPEC was isolated more commonly than EHEC on the farms studied. To our knowledge this shedding outcome has not been modelled previously, but may pose a public health risk. Although not associated with haemolytic uremic syndrome like EHEC, the intimin gene conserved between aEPEC and EHEC strains imparts the ability for aEPEC to cause attaching-and-effacing lesions, watery diarrhoea, and paediatric infection [35]. A high infective dose is required for human EPEC infection [7], which is probably due to its lower virulence as a human pathogen. However, strong evidence exists for EHEC strain evolution from EPEC, and a close homology among these strains [3]. This homology and the mobile nature of phage-transmitted *stx* genes indicates that modelling aEPEC may be advantageous for both the prediction of future foodborne EPEC infections, and potentially understanding the emergence of new *stx* strains.

Final multivariable models for aEPEC revealed positive associations between shedding and increased humidity, while watery faeces and a recent history of any disease or treatment showed reduced odds of shedding. In prior studies climatic factors and faecal consistencies have been shown to influence virulent O157 shedding in dairy cattle [17]. The negative association of disease and treatment with aEPEC shedding is difficult to interpret. The stress that accompanies disease processes and administration of treatments may influence intrinsic immune or physiological bovine processes that permit less ideal colonization conditions of the lower gastrointestinal tract to ingested aEPEC strains. However, future studies are required to confirm this assumption.

Multivariable models of EHEC revealed a negative association between shedding and a high hygiene score, greater cow contact, and a higher number of days in milk. An increased hygiene score and greater cow contact appear counterintuitive with historical factors thought to influence shedding. Although a subjective measure, hygiene scores are meant to reflect animal cleanliness, and cleanliness of the farm environment. Dirtier cows may reflect a combination of changes in environmental parameters (rainfall, humidity) more discretely than those parameters do when measured directly and individually. The exact mechanism by which this influences shedding remains to be determined. Cows housed with a greater number of other cows would presumably have an increased risk of shedding pathogenic O157, both through an increase in exposure and stress. The current study revealed an opposite trend, and this may be due to

the daily employee modifications of pen size depending on cow number, which was especially evident based on the management of herd 2 (having the highest prevalence of EHEC). A more meaningful measure of animal contact would have taken into account both the number of cows in a pen and the size of the enclosure, which was not possible due to daily fluctuations. With changes in pen size it is possible that increasing animal numbers actually reflected a less confined environment for the animals studied. Previous studies have shown that cow age, a higher parity, and greater number of days in milk may be associated with reduced EHEC shedding [14, 18]. The current study showed that both a higher parity and number of days in milk were associated with reduced shedding, with a significant interaction of these factors (Supplementary Appendix 3 and Table 6). Cattle were studied within the first 21 days of milk, so it is of note that the findings associated with this initial time from calving may become more or less apparent, should studies of shedding be carried further into the lactation period. Regardless, these results continue to support a notion that early lactation heifers, experiencing their initial calving and metabolic challenge of milk production, are a group more likely to shed EHEC. These individuals may therefore be targeted during future EHEC intervention studies.

Recent treatment with an antibiotic and a higher environmental temperature were significantly associated with EHEC shedding. Temperature has been previously implicated in O157 shedding in both beef and dairy settings, especially as it pertains to season [17, 18]. To date, this is the first study that has assessed medical treatments given during the current lactation, and their influence on the risk of a cow to shed EHEC. Results showed that cows treated with any antibiotic (both systemic and intramammary administration) had an increased risk of shedding compared to those individuals receiving no treatments. This indicates that antibiotic administration may modulate the bovine flora in a manner that facilitates future colonization with EHEC. Although prevalence levels had low power to detect bovine disease parameters that influenced EHEC, the collapsed variable that investigated cows with any disease or treatment history indicated that these animals are at increased risk of shedding, compared to non-diseased and non-treated cows, respectively (Supplementary Appendix 3). Taken together, these EHEC results support an idea that early lactation heifers with a reported status of disease, and history of receiving any antibiotic during the

current lactation, may be the individuals most likely to contribute to EHEC shedding and contamination of the farm.

The differences in aEPEC and EHEC shedding prevalence between farms are likely due to variation in management factors, specifically those related to nutrition and modulation of the gastrointestinal tract. These factors may include feeding ionophores, and using haemorrhagic bowel syndrome (HBS) and *Salmonella* siderophore receptor and porin (SRP) vaccines. Herds 1 and 3 were both fed ionophores during the study period. Herd 1 also employed use of a *Salmonella* Newport SRP vaccine (Epitopix, Willmar, USA) while herd 2 utilized an autogenous *Clostridium perfringens* type A, HBS vaccine. Parallels between management variability and O157 pathotype prevalence indicates a need to look at gastrointestinal bacterial communities under the influence of these managerial factors, and determine the specific intestinal environment that facilitates or impedes O157 growth. To truly understand the ecology of O157 shedding and farm prevalence, future studies should include an assessment of calves and cattle beyond the early lactation period, and a comparison of cows in ill-health with treatment history (specifically antibiotic use for EHEC) to those with no prior disease or history of treatment administration.

Given its ubiquity in a multitude of unprocessed food products and ability to cause outbreaks and isolated incidents of human illness worldwide, *E. coli* O157 remains a pertinent public health concern. Many strains and pathotypes of the O157 pathogen exist, having been isolated and characterized during instances of disease. Both the current and previous studies have shown that shedding of this pathogen from bovine hosts is complex, and may vary due to season, environment, specific host factors, and bacterial pathotype. The current study aimed to comprehensively model the shedding of O157 pathotypic variants from reservoir dairy hosts. Our findings related to risk factors and shedding dynamics of pathogenic strains can guide future studies aimed to detect dairy hosts colonized with O157, and influence the development and assessment of prevention or treatment techniques directed at reducing O157 shedding and food chain contamination.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit <http://dx.doi.org/10.1017/S0950268816001928>.

ACKNOWLEDGEMENTS

The authors acknowledge Dave Hicks and Michael Russell in the CSU Veterinary Diagnostic Laboratory for their expertise in identifying and screening *E. coli* O157 isolates of varying pathotype. The authors also thank several students who aided in faecal culture and isolation of samples: Paige Tenneson, Meagan Chriswell, Clarissa Freemyer, and Sean Montgomery. The authors are sincerely indebted to the owners and personnel of the three dairies for their invaluable collaboration during the study.

This research was funded, in part, by the High Plains Intermountain Center for Agricultural Health and Safety, the Colorado State University Infectious Disease Supercluster, and USDA Animal Health and Disease Formula Funds.

DECLARATION OF INTEREST

None.

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