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The diversity of *Escherichia coli* serotypes and biotypes in cattle faeces

K.A. Bettelheim[1], A. Kuzevski[1], R.A. Gilbert[2], D.O. Krause[2] and C.S. McSweeney[2]

[1]Microbiological Diagnostic Unit, Public Health Laboratory, Department of Microbiology and Immunology, University of Melbourne, Parkville, Vic., Australia, and [2]CSIRO Livestock Industries, Queensland Bioscience Precinct, Qld, Australia

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ABSTRACT

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Aim: To study the diversity of commensal *Escherichia coli* populations shed in faeces of cattle fed on different diets.

Methods and Results: Thirty Brahman-cross steers were initially fed a high grain (80%) diet and then randomly allocated into three dietary treatment groups, fed 80% grain, roughage, or roughage + 50% molasses. Up to eight different *E. coli* isolates were selected from primary isolation plates of faecal samples from each animal. Fifty-two distinct serotypes, including nine different VTEC strains, were identified from a total of 474 *E. coli* isolates. Cattle fed a roughage + molasses diet had greater serotype diversity (30 serotypes identified) than cattle fed roughage or grain (21 and 17 serotypes identified respectively). Cluster analysis showed that serotypes isolated from cattle fed roughage and roughage + molasses diets were more closely associated than serotypes isolated from cattle fed grain. Resistance to one or more of 11 antimicrobial agents was detected among isolates from 20 different serotypes. Whilst only 2·3% of *E. coli* isolates produced enterohaemolysin, 25% were found to produce α-haemolysin.

Conclusions: Diverse non-VTEC populations of *E. coli* serotypes are shed in cattle faeces and diet may affect population diversity.

Significance and Impact of the Study: This study provides new information on the serotype diversity and phenotypic traits of predominant *E. coli* populations in cattle faeces, which could be sources of environmental contamination.

Keywords: antimicrobial resistances, cattle, *Escherichia coli*, VTEC.

INTRODUCTION

Since the emergence of verocytotoxigenic *Escherichia coli* (VTEC) as important human pathogens, contamination of food directly or indirectly by faecal mater of animal origin has been a major source of these organisms (Beutin *et al.* 1993; Sidjabat-Tambunan and Bensink 1997; Fagan *et al.* 1999; Bettelheim 2000; Kobayashi *et al.* 2001). Outbreaks of haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) have often been attributed to strains of the *E. coli* serotype O157:H7, however, VTEC strains capable of causing human infections belong to a number of different O:H serotypes (Bettelheim 2000).

There have been many studies on the distribution of VTEC in animals, particularly ruminants, with species-specific differences observed between VTEC populations found in cattle and sheep (Beutin *et al.* 1993; Wieler *et al.* 1996; Beutin *et al.* 1997; Pradel *et al.* 2000; Hornitzsky *et al.* 2002; Brett *et al.* 2003; Djordjevic *et al.* 2004). More than 400 different O:H serotypes of VTEC have been reportedly isolated from cattle (Blanco *et al.* 2004). In comparison, the commensal *E. coli* population found in the gastrointestinal tract of ruminants, has been largely uncharacterized (Jarvis *et al.* 2000; Aslam *et al.* 2003). While the study of VTEC strains is of great importance because of their direct

Correspondence to: Rosalind A. Gilbert, CSIRO Livestock Industries, Queensland Bioscience Precinct, 306 Carmody Road, St Lucia, Qld 4067, Australia (e-mail: ros.gilbert@csiro.au).

involvement in causing human gastrointestinal disease, the diversity of the non-VTEC, commensal *E. coli* within the bovine intestinal microflora, is also of interest as these organisms may potentially acquire virulence genes by mechanisms of horizontal gene transfer, via mobile genetic elements such as phage (Hacker and Kaper 2000; Schmidt 2001). In this way, commensal *E. coli* may be converted to pathogenic VTEC strains with different O:H serotypes and phenotypic characteristics.

This study is unique, in that it treats all *E. coli* as equal and is based on the collection of the greatest variety of *E. coli* types which can be selected from a primary isolation plate, without specifically selecting for VTEC strains. Faecal samples were obtained from a herd of cattle involved in a feeding trial designed to ascertain the effect of three different dietary treatments which differed in their basic source of fermentable carbohydrate (cellulosic carbohydrate, sucrose and high starch) and more than 400 *E. coli* isolates obtained from these faecal samples. In this way, the serotype diversity and some phenotypic characteristics of the predominant strains within the faecal *E. coli* population, were determined.

MATERIALS AND METHODS

Animals and diet

Thirty Brahman cross steers 329 ± 3·2 kg liveweight (mean ± sem) were housed in individual pens and initially adapted for 1 month to a high grain diet of 80% sorghum, 5% peanut shells and 5·5% cotton seed meal (Better Blend Beef Expandat; Better Blend Stockfeeds, Oakey, Australia). The 30 cattle were then randomly allocated into three groups, to form a completely randomized design, and each group provided one of three diets. Diets were fed continuously for 30 days before another faecal sample was taken. On a dry matter basis, the three diets fed *ad libitum* were (i) a high sucrose diet of 50% molasses, 28% Rhodes grass (*Chloris gayana)* hay, 15·0% whole cotton seed, 4·5% cotton seed meal, 1·5% urea and 1% mineral/vitamin premix (M); (ii) the high starch content, grain-based diet as provided during the adaptation period, described above (G); and (iii) a diet high in cellulosic carbohydrate, of Rhodes grass plus 20 g urea kg^{-1} DM (R). A total of 10 steers were fed diet 1 (M), nine steers were fed diet 2 (G) and 11 steers were fed diet 3 (R). Throughout this investigation, the care and use of experimental animals complied with all relevant local animal welfare laws, guidelines and policies and was performed with the approval of a local animal ethics committee.

Isolation of *E. coli*

Escherichia coli were isolated from faecal samples collected directly from the rectum of all animals on 1 day during the final week of the grain adaptation period to provide a baseline measurement of the *E. coli* population, and again on one occasion following 1 month of *ad libitum* feeding of the three diets. For each faecal sample, a 10-g portion was diluted (10-fold) with modified *E. coli* broth (mEC) (Okrend *et al.* 1990) and mixed by hand for 30 s. A 100-μl volume of this mixture was then serially diluted (10-fold) in a 900-μl volume of mEC before pipetting onto Chromogenic agar plates (Oxoid CM956) and incubated at 37°C for 24 h. Individual *E. coli* colonies were picked from Chromogenic agar plates at which serial dilution enabled single colonies to be distinguished (usually $\geq 10^{-4}$ dilution plate). Eight colonies were picked from each faecal sample, subcultured onto nutrient agar (Oxoid CM1) and each picked colony characterized and serotyped.

Characterization of *E. coli*

All colonies were spread on MacConkey (MAC), sorbitol MacConkey (SMAC), sheep blood (SB) and washed sheep blood (WSB) agars to confirm purity and test for their ability to produce enterohaemolysin and/or α-haemolysin (Bettelheim 1995), and ferment sorbitol and lactose. They were identified as *E. coli* on the basis of their reactions in: triple sugar iron agar (Oxoid CM277), ONPG broth (Oxoid Peptone Water, CM9 with *o*-nitro-phenyl-galactoside, BDH 400312), urea agar (Oxoid CM71 with supplement SR020 K), and Bacto-MIO (Motility, Indole, Ornithine; Difco 0735-01-1) agar. All media were made and used according to the manufacturers' instructions.

In addition the strains were O and H serotyped, using previously described methods (Chandler and Bettelheim 1974; Bettelheim and Thompson 1987). Overnight nutrient broth cultures (Oxoid CM1), steamed for 1 h were used as O antigens. Following repeated passage through semisolid medium, suspensions observed microscopically as fully motile were treated with 0·05% (v/v) formaldehyde and these served as H antigens. Strains which failed to achieve motility were considered nonmotile and designated H-.

All the strains were tested for their ability to produce verocytotoxin(s) (VT) by the Vero cell assay (Konowalchuk *et al.* 1977), using the filter (0·2 μm; Supor Acrodisc Syringe Filter; Gelman Sciences, Ann Arbor, MI, USA) sterilized supernatant of an 18 h culture in tryptone soya broth (Oxoid CM129). The Vero cell assay results were confirmed by the Enzyme Linked Immunosorbent Assay (ELISA) technique (Acheson *et al.* 1990) using sheep hydatid cyst fluid (obtained from Dr M. Lightowlers, School of Veterinary Science, University of Melbourne, Veterinary Clinical Centre, Werribee, Vic., Australia) to capture toxin. Monoclonal antibody 13C4, which is specific for VT1 (STI), obtained from the American Type Culture Collection (ATCC No. CRL 1794) and 11E10, which is specific for

VT2 (STII), obtained from Dr A. O'Brien, Armed Services University of the Health Sciences, Bethesda, MD, USA) were used to confirm the toxins serologically (Perera *et al.* 1988). In addition, the presence of genes for toxin production was confirmed by PCR analysis of positive strains, using the method of Paton and Paton (1998).

The strains were also tested to determine their sensitivities to a range of antimicrobials by the plate/replicator method (Bettelheim *et al.* 2003). The antimicrobials included ampicillin (32 μg l^{-1}), streptomycin (25 μg l^{-1}), tetracycline (20 μg l^{-1}), chloramphenicol (0·01 μg l^{-1}), sulphathiazole (0·55 μg l^{-1}), trimethoprim (0·05 μg l^{-1}), kanamycin (10 μg l^{-1}), nalidixic acid (50 μg l^{-1}), spectinomycin (50 μg l^{-1}), gentamicin (2·5 μg l^{-1}), and ciprofloxacin (2 μg l^{-1}). The antimicrobials were incorporated in lysed blood Iso-sensitest agar (Oxoid CM471). Plates and cultures were tested using a Clements antibiotic sensitivity replicator with 32 prongs.

To perform the antimicrobial test, the *E. coli* isolates and controls were grown up in double strength Nutrient Broth (Oxoid CM1) for 1·5 h at 37°C, in an orbital shaker at 100 rev min^{-1}. The organisms were then diluted 1 : 10 (v/v) in tryptone water (Oxoid CM87), and added to the wells of the antibiotic sensitivity replicator, which were filled with 0·5 ml of nutrient broth containing 0·05% agar. The *E. coli* isolates were then inoculated, by means of the replicator, onto each of the antimicrobial agar plates, and then incubated at 37°C overnight. Where little or no growth of the *E. coli* on the plate was observed, this result was recorded as demonstrating susceptibility to that specific antimicrobial. This method had originally been developed to determine the resistance patterns of *Salmonella* isolates, therefore, two standard strains, *S.* Heidelberg, *S.* Hadar with known resistances were used as positive controls and a fully sensitive strain of *S.* Typhimurium, was used as negative control (Bettelheim *et al.* 2003).

Statistical analysis

A cluster analysis of serotype data obtained from the second faecal collection was undertaken according to dietary treatment group and the number of *E. coli* isolates obtained of each serotype identified, using the Statistica data analysis software package (StatSoft® Version 6.1, series 0403; Statsoft Inc., Tulsa, OK, USA).

RESULTS

The serotypes of *E. coli*

A total of 474 *E. coli* isolates, from 60 bovine faecal specimens collected from 30 animals at two time points, were serotyped. Among these isolates, 52 different serotypes were identified, comprising of 27 O groups and 19 H types, as well as four nonmotile (H-) strains. In addition, 11 serotypes were identified with nontypable O antigens and two serotypes had O antigens which were autoagglutinable or rough (OR). A diverse variety of serotypes were found in each faecal specimen, and out of the eight colonies selected from primary isolation plates for each faecal specimen, 3·6 ± 0·22 (mean ± S.E.M.) serologically distinct types were identified. At both collection time points, serotypes most frequently isolated included: Ont:H8, O8:H2, O146:H21, O42:H21, O2:HR, Ont:H16, and O93:H19 (Tables 1 and 2).

Of all the serotypes identified, one-third were found to share the same O antigen, but carried different flagella antigens including, O2, O5, O42, O84, O93, O107, O114, O150, O154 and OR. The most frequently shared O antigen was the O2 type. In contrast, a large proportion of serotypes identified (73%), were characterized as having different O antigens but sharing flagellar antigens. For example, there were 12, 8, and 5, different O types associated with the H8, H21, and H2 flagellar antigens, respectively. Nonmotile isolates were only identified amongst four serotypes (O84, O130, O154 and four *E. coli* isolates with the same nontypable O antigen).

Effect of diet and collection period on *E. coli* serotypes

From the 240 *E. coli* strains isolated at time point 1, 31 serotypes were identified (Table 1). Twenty of these serotypes were also isolated at the second time point (Table 2), with the remaining 11 serotypes unique to the first time point. These unique serotypes included six different O antigens (O41, O42, O91, O107, O150 and O159) and two serotypes with nontypable O antigens. Only two H antigens (H49 and H8/40) were not also observed in *E. coli* isolates obtained at time point 2. The most predominant serotypes identified at time point 2, when all 30 cattle were fed the G diet, included: O8:H2 and Ont:H8 [both isolated from 11 cattle (37%) respectively], and O146:H21 [isolated from 10 cattle (33%)].

From the 231 *E. coli* strains isolated at time point 2, 41 serotypes were identified (Table 2). The serotypes most frequently isolated from cattle, were found to vary with the diet provided. From the 11 cattle fed the R diet, the serotype isolated from the greatest number of animals [five animals (45·5%)] was O2:HR, followed by O2:H8 and O10:H45, which were both isolated from three animals (27·3%). Similarly, from the 10 cattle fed the M diet, the most frequently isolated serotype was also O2:HR, found in three animals (33·3%). From the nine cattle fed the G diet, the most frequently isolated serotypes were O168:H8 and O42:H21, both isolated from five animals (55·6%), respectively.

Table 1 *Escherichia coli* serotypes isolated from faecal samples collected at the first sampling when all cattle were fed a high grain diet

Serotype	No. of isolates (no. of cattle)	Antibiotic resistances† (no. of isolates; no. of cattle)	Unusual fermentation characteristics‡ (no. of isolates; no. of cattle)	α-Haemolysin (no. of isolates; no. of cattle)	Enterohaemolysin (no. of isolates; no. of cattle)
O2:HR	9 (5)			9 (5)	
O8:H2	22 (11)			22 (11)	
O28:H8	5 (4)				
O41:H8*	1 (1)				
O42:H8*	3 (2)	T (1, 1)			
O42:H21	20 (8)	T (3, 1); Tm (2, 1)		2 (1)	
O84:H2	8 (4)			8 (4)	
O88:H25	5 (2)	T (1, 1)		2 (1)	
O91:H49*	2 (1)				
O93:H19	3 (2)	Su (2, 1); Su, Tm (1, 1)	NSF (2, 1)	1 (1)	
O100(rel):H21	25 (6)	T (5, 2)			
O107:H8*	1 (1)	T (1, 1)		1 (1)	
O107:H16*	10 (6)				
O107:HR*	6 (2)				
O146:H21	26 (10)	A, T (2, 1)	NSF (25, 8)	1 (1)	
O150:H8*	6 (1)	T (6, 1)		6 (1)	
O150:H21*	1 (1)			1 (1)	
O154:H-	1 (1)				
O154:H8	13 (4)	Su, Tm (1, 1);Tm (2, 1)		2 (1)	
O159:H8*	1 (1)				
O168:H8	3 (1)				
Ont:H-	2 (2)			1 (1)	
Ont:H7*	1 (1)				
Ont:H8	32 (11)	A, T (16, 5);T (1, 1)		1 (1)	
Ont:H8/40*	2 (2)	A, T (1, 1)			
Ont:H16	16 (7)		NLF (2, 2)		
Ont:H19	2 (1)				2 (1)
Ont:H21	6 (4)	T (1, 1)			
Ont:H38	1 (1)				
OR:H8	2 (1)	A, T (1, 1)			
OR:H21	5 (3)	T (2, 1)			

*Serotypes not identified in collection time point 2.

†Antibiotics testing include ampicillin (A), streptomycin, tetracycline (T), chloramphenicol, sulphathiazole (Su), trimethoprim (Tm), kanamycin, nalidixic acid, spectinomycin, gentamicin, and ciprofloxacin.

‡Unusual biochemical reactions including nonlactose fermenter (NLF) and nonsorbitol fermenter (NSF).

When the total number of serotypes identified per dietary treatment group, were compared, cattle fed the M diet were found to have greater faecal serotype diversity, than cattle fed R or G diets. Of the total number of serotypes identified at time point 2, 30 serotypes (73%) were isolated from cattle fed the M diet. Fewer serotypes were isolated from cattle fed G [17 serotypes (41·5%)] or R [21 serotypes (51·2%)] diets. Only six serotypes (O8:H2, O93:H19, O100r:H21, O154:H8, Ont:H8 and Ont:H16) were isolated from cattle across all three dietary groups. Serotypes isolated from cattle fed the R diet, were more likely to be also isolated from cattle fed the M diet (15 serotypes in common) than in cattle fed the G diet (seven serotypes in common).

To further determine the effect of diet on faecal serotype diversity, cluster analysis of the serotypes isolated from each dietary treatment group, incorporating the number of *E. coli* isolates obtained of each serotype, was undertaken (Fig. 2). Using this method of diversity analysis, serotypes isolated from cattle fed the R and M diets were more closely associated than serotypes isolated from cattle fed the G diet.

When the number of animals each serotype was isolates from was plotted according to the dietary treatment group of the animal (Fig. 1), it was evident that serotypes identified from cattle fed the M or R diets, were more often isolated from only one animal. In contrast, serotypes from cattle fed the G diet could often be isolated from as many as five animals.

Table 2 *Escherichia coli* serotypes isolated from faecal samples collected at the second sampling when cattle were fed one of three dietary treatments, including high grain (G), molasses + roughage (M) and roughage (R) diets

Serotype	Diet	No. of isolates (no. of cattle)	Antibiotic resistances‡ (no. of isolates; no. of cattle)	Unusual fermentation characteristics§ (no. of isolates; no. of cattle)	α-Haemolysin (no. of isolates; no. of cattle)	Enterohaemolysin (no. of isolates; no. of cattle)
O2:H8	M, R	8 (5)				
O2:H29*	R	7 (2)			2 (1)	
O2:H39	M, R	2 (2)			2 (2)	
O2:HR	M, R	28 (8)	A (1,1)		28 (8)	
O5:H6	M	3 (1)		NLF (3, 1)		
O5:H51	R	1 (1)				
O8:H2	G, M, R	11 (6)			11 (6)	
O10:H45	M, R	6 (4)			6 (4)	
O28:H8	G, M	12 (4)				
O42:H21	G, M	12 (7)	T (3, 2)			
O75:H2	M	1 (1)	A (1, 1)		1 (1)	
O84:H-*	R	2 (1)				2 (1)
O84:H2	M, R	2 (2)			2 (2)	
O88:H25	G, M	10 (3)			3 (2)	
O93:H2	M	1 (1)	Su (1,1)	NSF (1,1)		
O93:H19	G, M, R	13 (7)	Su (13, 7)	NSF (12, 7)		
O100(rel):H21	G, M, R	6 (4)				
O104:H8	G	2 (2)				
O114:H6	M, R	7 (5)		NLF (7, 5)		
O114:H7*	M	2 (1)		NLF (2,1)		2 (1)
O130:H-*	M	1 (1)			1 (1)	
O130/134:H38*†	M	1 (1)			1 (1)	
O146:H21	G, M	7 (2)		NSF (7, 2)		
O152:H16	G	2 (1)				
O154:H-	G	2 (1)				
O154:H8	G, M, R	4 (3)				
O160:H43	M, R	2 (2)	Su (1,1)			
O168:H8*	G, M	15 (6)	A, Su (1, 1)			
O174:H21*	M	1 (1)				
O175:H21*	M	3 (1)			3 (1)	
Ont:H-	M	2 (2)	A (1,1)		1 (1)	
Ont:H2	R	2 (1)			2 (1)	
Ont:H8	G, M, R	17 (7)	Su (1, 1) A, T (6, 5)			
Ont:H14	R	4 (1)		NLF (4, 1)		
Ont:H16*	G, M, R	8 (6)		NLF (3, 2)		
Ont:H18	M, R	6 (3)				
Ont:H19	M, R	6 (2)				5 (2)
Ont:H21	M	1 (1)				
Ont:H38	G	1 (1)				
OR:H8	G, R	10 (3)	A, T (2, 2)			
OR:H21	G	3 (1)	A, T (3, 1)			

*Isolate found to contain VTEC genes.

†O130/134 = O antigen exhibits both O130 and O134 specificities.

‡Antibiotics testing include ampicillin (A), streptomycin, tetracycline (T), chloramphenicol, sulphathiazole (Su), trimethoprim, kanamycin, nalidixic acid, spectinomycin, gentamicin, and ciprofloxacin.

§Unusual biochemical reactions including nonlactose fermenter (NLF) and nonsorbitol fermenter (NSF).

There also appeared to be a high degree of variability in the *E. coli* serotypes identified for individual animals, irrespective of whether they were retained on the same dietary treatment or whether the diet was changed. When the identity of the eight faecal *E. coli* isolates obtained from individual animals were compared over time, no more than

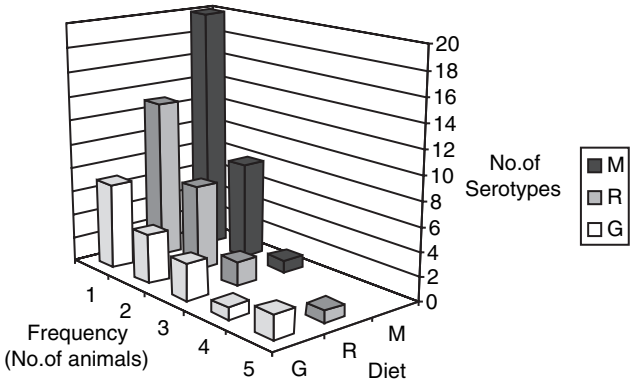

Fig. 1 Effect of diet on serotype diversity in faecal samples obtained at collection time point 2. The number of different serotypes which were observed in a certain number of animals (frequency) plotted according to dietary treatment groups of roughage (R), roughage + molasses (M) and grain (G)

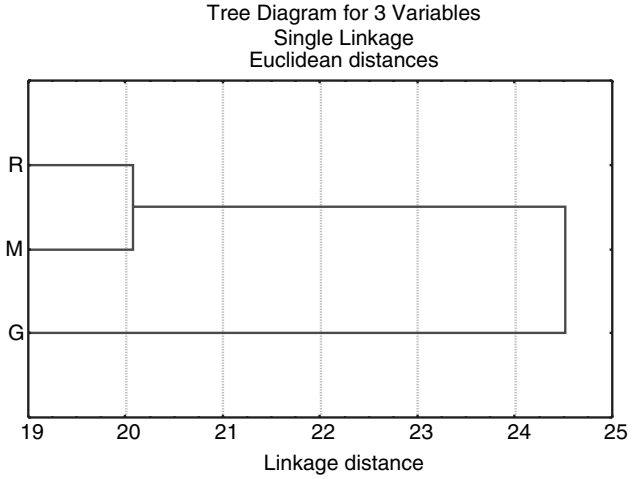

Fig. 2 Cluster analysis of all serotypes obtained from faecal collection 2, grouped according to dietary treatment on the basis of the number of *Escherichia coli* isolates obtained for each serotype identified. Roughage (R), roughage + molasses (M) and grain (G)

three identical faecal serotypes were isolated from any animals between the two collection time points.

Animals that were continuously fed the G diet, tended to have less serotype variability over time. Between the two time points, seven animals (77·8%) of the nine animals retained on the G diet, were found to repeatedly contain isolates of at least one serotype. Serotypes retained over time included: O8:H2, O28:H8, O42:H21, O93:H19, O126:H21, O154:H-, O154:H8, O168:H8, O100r:H21 and Ont:H8, however, only serotypes Ont:H8 and O42:H21 were isolated from more than one animal at any time.

Of the cattle that changed diet from G to either R or M diets, only four animals from each dietary group (36·4% of animals fed R and 40% of animals fed M) were found to have isolates of one serotype in common between the two collection periods. None of these animals were found to have more than one serotype in common. Serotypes retained despite a change to the R diet included: O100r:H21, Ont:H16, and Ont:H8 and serotypes retained despite a change to the M diet included: O146:H21, O8:H2 and Ont:H8. Isolates of serotype Ont:H8 were the most frequently identified, irrespective of diet.

Antibiotic sensitivity of *E. coli* serotypes

The antibiotic sensitivity of the 474 *E. coli* isolates was determined, and 83 isolates (17·5%) belonging to 20 serotypes, were identified as being resistant to at least one of the 11 antibiotics tested (Tables 1 and 2). Antibiotic resistant strains belonging to approximately one-third of these serotypes [six serotypes (30%)], were isolated from more than one animal within the herd. Many strains identified with the same serotype, were obtained with or without an antibiotic resistance phenotype, when isolated either from different animals [14 (93·8%) of the 15 serotypes identified in more than one animal] or from a single animal [seven (54%) of the 13 serotypes where multiple isolates of the same serotype were obtained from a single faecal sample]. In contrast, all isolates of the commonly occurring serotype O93:H19, were found to have an antibiotic resistance phenotype. The specific antibiotics to which these O93:H19 isolates were resistant however, was found to vary, with 15 isolates resistant to sulphathiazole and one isolate resistant to sulphathiazole and tetracycline.

When all cattle were fed the G diet (Table 1), isolates from 14 serotypes (45% of the total number of serotypes identified for time point 1), obtained from 12 cattle (40%), were found to be resistant to at least one of the antibiotics tested. Resistance to tetracycline was the most common, being found in isolates belonging to 13 serotypes, arising from 12 steers. Antibiotic resistant isolates of serotype Ont:H8 were found most frequently (16 isolates from five animals) but most isolates with an antibiotic resistant phenotype, were of serotypes found only in low numbers in individual steers.

At time point 2 (Table 2), 14 cattle (46·7%) were found to contain isolates from 11 different serotypes (26·8% of the total number of different serotypes identified for this time point), resistant to at least one of the antibiotics tested. Antibiotic resistant *E. coli* were isolated from all dietary treatment groups, with four cattle (36·4%) from the R diet group, five cattle (50%) from the M diet group, and five cattle (55·6%) from the G diet group, positive for antibiotic resistant strains. Isolates resistant to sulphathiozole (Su) were the most widespread amongst the herd, irrespective of the dietary treatment, with three animals from the R and M

fed groups, and four animals from the G fed group found to contain Su resistant isolates.

Unusual fermentation characteristics

The majority of *E. coli* isolates [405 isolates (85·4%)] collected for either time point were able to ferment both lactose and sorbitol and none of the isolates lacked the ability to ferment both lactose and sorbitol. Isolates from eight serotypes were unable to ferment lactose or sorbitol. Most isolates found to be nonlactose fermenters (NLF) or nonsorbitol fermenters (NSF), were of serotypes found only in low numbers, in individual steers.

The inability to ferment sorbitol (Table 1) was most widespread in isolates obtained when all cattle were fed the G diet [27 isolates (11·3%)] with this predominance attributed to the abundance of a single serotype (O146:H21) within the herd [eight animals (26·7%)]. The only NLF isolates, were obtained from two animals (6·7%) and were of serotype Ont:H16.

When cattle were fed three dietary treatments (Table 2), 19 isolates (8·2%) were unable to ferment lactose, and 20 isolates (8·7%) were unable to ferment sorbitol. Most NLF isolates were identified as serotype O114:H6, detected in five (16·7%) of animals and most NSF isolates were identified as serotype O93:H19, isolated from seven (23·3%) of animals.

α-Haemolysin and enterohaemolysin

The ability of the 474 *E. coli* isolates to produce α-haemolysin and/or enterohaemolysin was determined and no isolates were found that produced both (Tables 1 and 2). One-quarter of the total number of isolates, belonging to almost half (46·2%) of the different serotypes identified, were shown to produce α-haemolysin. In contrast, the ability to produce enterohaemolysin was relatively rare, and was detected in only 11 (2·3%) of the total number of isolates, belonging to just 3 (5·8%) serotypes.

Isolates producing α-haemolysin were obtained frequently and were detected in faecal samples from 19 (63·3%) and 17 (56·7%) animals at time points 1 and 2, respectively. Alternatively, faecal samples from only 1 (3·3%) and four (13·3%) animals sampled at time points 1 and 2, respectively, contained enterohaemolysin-producing *E. coli*. Interestingly, all enterohaemolysin-producing isolates of O114:H7 and O84:H- were identified as VTEC strains (Table 3).

Isolates of serotypes O2:HR and O8:H2 were the most predominant α-haemolysin-producing strains identified, irrespective of time point or diet. Among the 30 animals, O2:HR and O8:H2 isolates were obtained from a maximum of eight animals (26·7%) and 11 animals (36·7%), respectively, at any particular time point. In addition, all of the 37 O2:HR and 33 O8:H2 isolates identified produced α-haemolysin.

The number of animals containing α-haemolysin-producing isolates were higher in treatment groups fed R [eight (72·7%)] or M [six (60%)] diets than the G diet [three (33·3%)]. Although isolates producing enterohaemolysin were rare, nine isolates within three serotypes (O84:H-, O114:H7 and Ont:H19) were identified among cattle fed R or M diets, but none were identified in cattle fed the G diet.

The verocytotoxigenic *E. coli* (VTEC) isolated

Despite the fact that there was no selection for toxigenicity, 17 VTEC isolates belonging to nine different serotypes (1·7% of the total number of serotypes identified), were

Table 3 Serotypes found to contain VTEC genes and the corresponding number of animals from which the same serotypes without VTEC genes were isolated

Serotype	No. of animals yielding VTEC serotype (no.)	No. of animals yielding same non-VTEC serotype (no.)	Characteristics of VTEC serotypes	
			VT produced	Haemolysin
O2:H29	2*	1	2	–
O84:H-	1	0	1	EHly
O114:H7	1	0	2	EHly
O130:H-	1	0	1,2	α-H
O130/134:H38‡	1	0	1,2	α-H
O168:H8	1	5†	1	–
O174:H21	1	0	2	–
O175:H21	1	4†	1,2	α-H
Ont:H16	1	9†	2	–

*Non-VTEC strains of the same serotype isolated from a single faecal sample.
†Non-VTEC strains of the same serotype found in animals at two collection time points.
‡O130/134 = O antigen exhibits both O130 and O134 specificities.
EHly, enterohaemolysin; α-H, α-haemolysin.

isolated from nine (15%) of the 60 faecal specimens examined (Table 3). All of the VTEC serotypes were isolated from time point 2, and VTEC serotypes were only isolated from cattle fed either the R or M diets.

Isolates from four serotypes that were found to contain VTEC genes, were also isolated from faecal samples in a non-VTEC state. In one instance, both non-VTEC and VTEC strains of serotype O2:H29, were isolated from a single faecal sample. In addition, isolates from a number of other serotypes, though not verocytotoxigenic in this study, have been reported in the literature around the world as VTEC (Table 4), including: O2:H8, O2:H39, O8:H2, O28:H8, O84:H2, O88:H25, O91:H49, O146:H21, O150:H8, O154:H- and O168:H8.

The majority of the VTEC isolated were sensitive to the full range of antibiotics tested, with only one isolate of serotype O168:H8, being resistant to ampicillin and sulphathiazole. Only one VTEC isolate of serotype O114:H7, was unable to ferment lactose and all the VTEC isolated were able to ferment sorbitol. Three of the VTEC produced α-haemolysin and two of the VTEC produced enterohaemolysin.

DISCUSSION

A high degree of diversity in the *E. coli* population was observed from individual animals within the herd, with more than three different serotypes isolated from each faecal sample, irrespective of the diet or time point at which the faecal sample was collected. Although isolates of the same serotype were found in animals fed diets based on different forms of fermentable carbohydrate, and a relatively limited number of H antigens were identified, there was still a great deal of serotype variability between individual animals within the herd.

A dietary effect on serotype diversity was observed and verified by cluster analysis, albeit based on data from one faecal collection time point. Animals fed a grain diet high in starch tended to contain fewer serotypes, spread across more animals within the herd and serotypes identified were not as predominant in animals fed diets based on high concentrations of cellulose or sucrose (M and R diets respectively). The diet high in sucrose in the form of molasses, contained the greatest variety of different serotypes, with often little similarity between serotypes isolated from different animals maintained only on this diet.

A larger scale study involving a greater number of cattle per dietary treatment group and an increased number of faecal sample collections from which *E. coli* could be isolated would be desirable to further investigate these conclusions. The technical limitations of the current investigation, highlight the necessity of developing new molecular-based ecology tools to study *E. coli* diversity and community structure in environmental samples. Previous investigations into the diversity of *E. coli* populations have involved the isolation and characterization of individual *E. coli* strains

Table 4 Reports on the sources of the VTEC and potential VTEC O:H serotypes (based on http://www.sciencenet.com.au/vtectable.htm)

			Reported source(s)					
Serotype	VTEC in current study	Reported in the literature as VTEC	Healthy cattle	Other healthy animals	Animal disease	Meat	Healthy humans	Human disease
O2:H8		+	A	–	–	–	–	–
O2:H29	+	+	A, O	–	–	O	–	O
O2:H39		+	O	–	–	–	–	–
O8:H2		+	O	–	–	O	–	O
O28:H8		+	A	–	–	–	–	–
O84:H-	+	+	O	–	–	–	–	O
O84:H2		+	O	–	A	–	–	O
O88:H25		+	O	–	–	O	–	O
O91:H49		+	O	–	–	O	–	–
O93:H19		+	A	–	–	–	–	O
O114:H7	+		–	–	–	–	–	–
O130/134:H38	+		–	–	–	–	–	–
O146:H21		+	O	O	–	–	–	O
O150:H8		+	–	–	–	O	–	–
O154:H-		+	A	–	–	–	–	O
O168:H8		+	O,A	–	–	O	–	–
O174:H21	+	+	O	O	–	O	–	–

A, isolated in Australia.

O, isolated in places other than Australia.

using methods such as pulsed field gel electrophoresis (PFGE) (Akiba *et al.* 2000; Jarvis *et al.* 2000; McLellan *et al.* 2003; Grozdanov *et al.* 2004) rather than using molecular methods to examine the *E. coli* population directly from an environmental or faecal sample.

It is difficult to correlate the findings of the current study with previous reports investigating the effect of diet on bovine faecal *E. coli* serotype diversity because in the current study, *E. coli* isolates were serotyped irrespective of whether they contained VTEC virulence factors. In a previous Australian investigation involving the longitudinal monitoring (117d) of cattle as they progressed through a feedlot (Midgley *et al.* 1999), the diversity of VTEC isolated from faecal samples was found to decrease with time, and the VTEC population became dominated by a single serotype (O136:H16), not isolated in the current investigation. In another Australian study (Hornitzsky *et al.* 2002), VTEC were isolated from cattle on pasture (roughage-based diet) and feedlot (high grain) diets. In this study, it was suggested that within healthy cattle there is a dynamic, population of fluctuating VTEC serotypes. There was no direct indication that either pasture or feedlot diets favoured the proliferation of individual VTEC strains. It was suggested, however, that as in the current investigation, diet may be a contributing factor in determining the diversity of the VTEC population, with fewer VTEC serotypes isolated from the faeces of cattle on feedlot diets, compared with cattle on pasture-based diets. In addition to diet, it was suggested that *E. coli* serotype diversity may also be influenced by factors such as stress, hormonal levels and anatomical development of the gastrointestinal tract (age of the animal).

The high level of diversity in faecal *E. coli* serotypes isolated from individual steers in this study was similar to observations made in studies on the diversity of serotypes isolated from human faeces (Bettelheim *et al.* 1972). Serotypes identified in this trial also represent the population of predominant *E. coli* strains shed in faeces. Due to the intrinsic limitations of the methodology, the high level of diversity within the commensal bovine *E. coli* population observed may therefore be an underestimate, with less predominant strains such as VTEC, present as a subpopulation within individual cattle. An examination of the bovine faecal serotypes identified showed that they do not correspond to serotypes frequently isolated from humans (Bettelheim 1997). These results confirm previous findings that there may be a distinct, species-specific bovine *E. coli* microflora (Beutin *et al.* 1997; Hornitzsky *et al.* 2002; Djordjevic *et al.* 2004) and that human colonization by many of the predominant non-VTEC bovine serotypes identified in this study, may therefore be unlikely.

Interestingly, the VTEC serotype previously shown to be most commonly found in Australian cattle (Hornitzsky *et al.* 2002), and second most common in cattle from France and Japan (Pradel *et al.* 2000; Kobayashi *et al.* 2001), serotype O113:H21, was not isolated from any cattle within our study. This finding may reflect the variability in *E. coli* population that can occur between herds fed similar diets, such as high grain feedlot diets, even within the same country. It may also be a further indication that VTEC strains are not always found as the predominant strains within bovine faecal samples (Duffy 2003), and were therefore not frequently isolated in the current investigation, which did not specifically select for VTEC subpopulations (VTEC isolated from only 15% of faecal samples).

The VTEC isolated in the current investigation belonged to serotypes, which have rarely been identified as human pathogens and the most common human pathogenic VTEC, i.e. O157:H7, O111:H- and O26:H11 were not found. In fact two VTEC serotypes: O114:H7 and O130/O134:H38 have never been reported (http://www.sciencenet.com.au/vtectable.htm) from any source. Many of the non-VTEC serotypes which were isolated, have been reported as VTEC both in Australia as well as around the world, with the majority of these VTEC serotypes previously isolated from healthy cattle, meat and human patients with gastrointestinal disease (Table 4).

There appeared to be a high degree of clonal variation in the serotypes identified in this investigation. Many of the *E. coli* isolates with the same O:H combination, were also isolated as strains with different phenotypic characteristics, differing either in their susceptibility to the antibiotics examined, their ability to ferment lactose of sorbitol, to produce either α-haemolysin or enterohaemolysin, or even the presence of VTEC genes. This suggests that many isolates of the same serotype, were phenotypically different and therefore of heterogeneous genotype. Previous studies that differentiated non-VTEC faecal *E. coli* isolates on the basis of genetic diversity found within the *fliC* gene, encoding the H antigen (Aslam *et al.* 2003) and 16S rDNA and PFGE of genomic DNA (Jarvis *et al.* 2000) have also shown that the genetic diversity of commensal *E. coli* populations in cattle is very great, with genetic subtypes shared among animals, or sometimes unique to an animal within a herd.

Few previous investigations have been undertaken to characterize the phenotypic characteristics of non-VTEC bovine *E. coli* isolates. A previous study into the prevalence of antibiotic resistance amongst non-VTEC populations of *E. coli* from bovine (26 non-VTEC isolates of *E. coli*), ovine, porcine and human origins found that resistances to suphathiazole, ampicillin and tetracycline were observed most frequently, particularly in non-VTEC obtained from sick animals and man (Bettelheim *et al.* 2003). In the present study where many more non-VTEC isolates were examined, isolates resistant to at least one of the antibiotics tested were identified from almost half of the herd, at either faecal

sample collection time point, however only 17·5% of the 474 *E. coli* isolates examined were found to be resistant to at least one of the antibiotics tested. As found in other studies (Threlfall *et al.* 2000; Bettelheim *et al.* 2003), resistance to the antimicrobials tetracycline, ampicillin and sulphathiazole were most common. It was interesting that resistances to these antibiotics were observed, as to the best of our knowledge, the cattle examined had no history of antibiotic treatment. This suggests that antimicrobial resistances may have been introduced to the commensal *E. coli* population as a result of gene mobility, noted in previous studies (Aminov *et al.* 2002; Sherley *et al.* 2004) rather than by the direct selective pressure caused by exposure to antimicrobials.

Previous investigations have shown a close association between production of verocytotoxin and enterohaemolysin and that α-haemolysin-producing *E. coli* do not usually also produce verocytotoxins (Beutin *et al.* 1989; Bettelheim 1995). In the current investigation, although only a very low number of VTEC were isolated, three of the VTEC (O130:H-, O130/134:H38 and O175:H21) were found to also produce α-haemolysin, whereas isolates of only two of the VTEC serotypes identified, O84:H- and O114:H7, were found to also produce enterohaemolysin. *Escherichia coli* strains containing α-haemolysin were frequently isolated from animals within the herd, indicating that the ability to produce this toxin may be endemic within the commensal *E. coli* populations of bovine faeces. Production of the pathogenic virulence factor enterohaemolysin however, was rarely identified (present in only 2·3% of all *E. coli* isolates), with isolates of only one serotype (Ont:H19) found to also carry VTEC genes, indicating that enterohaemolysin was not commonly found within the predominant commensal *E. coli* population shed in faeces. It is interesting that the number of animals containing α-haemolysin-producing isolates were higher in treatment groups fed the R or M diets, than the G diet. This suggests that diet may have an effect on the subpopulations of *E. coli* containing this gene.

Both nonpathogenic and pathogenic *E. coli* strains may be able to colonize the human gut, differing only in the presence of functional genes allowing for increased fitness of the bacteria, enabling successful colonization of the host or encoding for specific virulence traits (Mason and Richardson 1981; Grozdanov *et al.* 2004). This suggests that the commensal *E. coli* population of cattle shed in faeces represent a reservoir of phenotypically heterogeneous *E. coli* serotypes, some of which may acquire verocytotoxigenicity, and thereby become potential human pathogens. Further investigations into the distribution of *E. coli*, both VTEC as well as non-VTEC, in healthy domestic food animals are warranted to clarify these hypotheses. This study confirms the importance of studying both commensal and pathogenic *E. coli* in individual animals in order to gain a better understanding of their ecological niches.

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