

Prevalence and characteristics of *Escherichia coli* serotype O157:H7 and other verotoxin-producing *E. coli* in healthy cattle

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SUMMARY

From February to July of 1994, 328 faecal samples from 32 herds were collected and verotoxin-producing *Escherichia coli* (VTEC) found on 84% of the farms. The proportion of animals infected varied from 0–63%. VTEC were recovered from 52 (20%) of 257 cows and from 16 (23%) of 71 calves. Although the VTEC belonged to 25 different serogroups, 7 (O8, O20, O22, O77, O113, O126 and O162) accounted for 46% of strains. Nearly 45% of the 83 bovine VTEC strains belonged to serogroups associated with haemorrhagic colitis and haemolytic uraemic syndrome in humans. However, only 2 (2%) of 83 VTEC strains isolated from cattle belonged to enterohaemorrhagic *E. coli* (EHEC) serotypes (O26:H11 and O157:H7), and only 8 (10%) were positive for the attaching and effacing *E. coli* (*eae*) gene sequence. Polymerase chain reaction (PCR) showed that 17 (20%) of VTEC strains carried VT1 genes, 43 (52%) possessed VT2 genes, and 23 (28%) carried both VT1 and VT2 genes. Characterization of VTEC isolates revealed a heterogeneous population in terms of serogroup and toxin type in the positive herds. This study confirms that healthy cattle are a reservoir of VTEC, but, the absence of *eae* genes in most bovine VTEC strains suggests that they may be less virulent for humans than *eae*-positive EHEC.

INTRODUCTION

Verotoxin-producing *Escherichia coli* (VTEC) are recognized as a cause of haemorrhagic colitis (HC) and the haemolytic-uraemic syndrome (HUS), in humans who contract infection following the consumption of contaminated foodstuffs like meat and unpasteurized milk [1–4]. Epidemiological investigations revealed that cattle frequently excrete VTEC in their faeces and thus may represent a source of infection [5–10]. However, the epidemiology of the organism remains unclear. Identification of the source of contamination of foods with VTEC is important to the understanding of the epidemiology of human

VTEC infection and devising strategies for its control. To date most surveys of bovine VTEC infection have expressed the prevalence of infection as the proportion of infected animals in the population. Equally important, from a public health standpoint, is the determination of farm-level prevalence of infection, an aspect of the epidemiology of VTEC which has so far received little attention [11–13].

VTEC from different sources and geographical areas belong to many different O serogroups. However, most of the documented outbreaks of HC and HUS were attributed to only a few serotypes (e.g. O26:H11, O111:H- and O157:H7 or H-) which have been designated enterohemorrhagic *E. coli* (EHEC)

[1–4, 14]. It is not known whether all variants of VTEC are equally pathogenic for humans. However, the relatively low incidence of non-EHEC VTEC disease in humans suggest that VT production alone may not be sufficient for VTEC to cause disease [15, 16]. A factor that may affect virulence of VTEC is the ability to cause attaching and effacing lesions in the intestinal mucosa [15–17].

In this study the prevalence of VTEC infection in cattle in the province of Lugo (Galicia, Northwest Spain) was investigated at the herd and individual animal levels.

MATERIALS AND METHODS

Specimens collection and *E. coli* strains

Thirty-two farms in the province of Lugo (Galicia, Northwest Spain) were visited once between February and July of 1994. Both beef and dairy cattle were sampled. A single faecal swab was obtained from selected cows and from all calves aged 4 months or less. The swabs were placed in transport medium and taken to laboratory for immediate processing. They were used to inoculate lactose-MacConkey agar and cefixime tellurite sorbitol MacConkey (CT-SMAC) medium [7]. From each sample five suspect *E. coli* colonies (lactose positive, lactose negative or sorbitol negative) were chosen, identified and examined for verotoxin (VT) production. Identification was based on biochemical tests, including hydrogen sulphide, citrate, urease and indole. All VTEC strains were confirmed as *E. coli* using the API-20E system (bioMérieux, France). Reference *E. coli* strains, used as positive and negative controls, included: 933 (O157:H7 VT1⁺, VT2⁺ and *eae*⁺), H19 (O26:H11, VT1⁺ and *eae*⁺) and K12-185 (non-toxicogenic). Strains were stored at room temperature in nutrient broth with 0.75% of agar.

Production and detection of verotoxins in Vero and HeLa cells

For production of verotoxins, bacteria were grown for 20 h in Tryptone Soya Broth with mitomicin C at 37 °C (shaken at 200 rpm) and then centrifuged (6000 g) for 30 min at 4 °C. The Vero and HeLa cell culture assays were performed using nearly confluent cell monolayers grown in plates with 24 wells. At the time of assay, the growth medium (RPMI with

polymixin sulphate) was changed (0.5 ml per well) and 75 µl undiluted culture supernatant added. Cells were incubated at 37 °C in a 5% CO₂ atmosphere and the morphological changes in cells observed after 24 and 48 h of incubation using a phase contrast inverted microscope [18].

Detection of VT1, VT2 and *eae* sequences by PCR

Bacteria were harvested from CFA agar, suspended in 200 µl of sterile water, incubated at 100 °C for 10 min to release the DNA, and centrifuged. The supernatant was used in the PCR reaction as described below. Base sequences, locations and predicted sizes of amplified products for the specific oligonucleotide primers used in this study are shown in Table 1 [19, 20]. Oligonucleotide primers were synthesized using a Gene Assembler Special (Pharmacia, LKB Biotechnology Inc.) according to the manufacturers instructions. Amplification of bacterial DNA was performed using 50 µl volumes containing 10 µl of the prepared sample supernatant; the oligonucleotide primers (90 ng for *eae* and VT2 primers; 150 ng for VT1 primers); 0.2 mM (each) dATP, dGTP, dCTP, and dTTP; 10 mM Tris HCl (pH 8.8); 1.5 mM MgCl₂; 50 mM KCl; and 1 U of DynaZyme DNA polymerase (Finnzymes OY, Finland). The reaction mixtures were overlaid with an equal volume of mineral oil. The conditions for the PCR were 94 °C for 2 min for initial denaturation of DNA within the sample followed by 30 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (primer annealing) and 72 °C for 1 min (DNA synthesis) performed with a thermal cycler (model Gene ATAQ Controller; Pharmacia, LKB Biotechnology Inc, Sweden). The amplified product was visualized by standard submarine gel electrophoresis using 10 µl of the final reaction mixture on a 2% agarose (agarose MP, Boehringer Mannheim, Germany) gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA). The samples were electrophoresed for 40 min at 140 V. Amplified DNA fragments of specific sizes were located by UV fluorescence after staining with ethidium bromide (0.5 µg/ml). Molecular size markers (*Hae* III digest of ϕ x174DNA) were included in each gel.

Serotyping

The isolates were serotyped using a modification [21] of the method of Guinée and colleagues [22]. O (O1–O171) and H (H1–H56) antisera were absorbed

Table 1. Primers used in PCR to amplify specific fragments from genes for VT1, VT2 and *eae*

Primer*	Oligonucleotide sequence (5'-3')	Location within gene†	Size of amplified product (bp)
VT1a	CAGTTAATGTGGTGGCGAAG	215-234	894
VT1b	CTGCTAATAGTTCTGCGCATC	1089-1109	
VT2a	CTTCGGTATCCTATTCCCGG	288-307	478
VT2b	GGATGCATCTCTGGTCATTG	747-766	
<i>eae</i> -1	ACGTTGCAGCATGGGTAAGTC	1054-1074	815
<i>eae</i> -2	GATCGCAACAGTTTCACCTG	1869-1849	

* References: VT1 and VT2 (20) and *eae* (19).

† In nucleotides.

Table 2. Prevalence of VTEC strains in healthy cattle

Age group	Number of animals	Number of animals with VTEC strains				
		Total	VT1 ⁺	VT2 ⁺	VT1 ⁺ VT2 ⁺	<i>eae</i> ⁺
Cows	257	52 (20%)	7 (3%)	33 (13%)	16 (6%)	2 (1%)
Calves	71	16 (23%)	7 (10%)	6 (8%)	4 (6%)	5 (7%)

with the corresponding cross-reacting antigens to remove the non-specific agglutinins. The antisera were obtained from the National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands). The O antigen was established in all VTEC detected, whereas the flagellar antigen was determined in VTEC belonging to serogroups O26, O111 and O157.

Statistical methods

Results were evaluated using the χ^2 test with Yates' correction for continuity.

RESULTS

Prevalence of VTEC in healthy cattle

A total of 1469 *E. coli* colonies from 328 animals from 32 different farms were investigated for verotoxin production in Vero and HeLa cells. VTEC were recovered from 52 (20%) of 257 cows and from 16 (23%) of 71 calves (Table 2) from 27 (84%) of 32 farms examined. The estimated proportion of animals infected on each farm ranged from 0-63% (Table 3).

Serogroups and EHEC serotypes

A total of 83 VTEC strains were identified in this study, 60 from cows and 23 from calves. VTEC strains

belonged to 25 different serogroups; however, 46% (38 of 83) were among 7 serogroups (O8, O20, O22, O77, O113, O126, O162) (Table 4). In 16 (24%) animals with VTEC, 2 (14 animals) or 3 (2 animals) serogroups were identified. In many farms a variety of VTEC strains with different O serogroups were detected (Table 3). Only two VTEC strains, isolated from calves, belonged to EHEC serotypes (O26:H11 and O157:H7).

Verotoxins (VT1 and VT2) synthesized by VTEC strains and attaching and effacing *E. coli* (*eae*) sequences

PCR demonstrated that 17 (20%) of VTEC strains carried VT1 genes, 43 (52%) possessed VT2 genes, and 23 (28%) carried both VT1 and VT2 genes (Table 4). VT1⁺ strains were more prevalent in VTEC obtained from calves than from cows (39% vs. 13%) ($P < 0.05$), whereas VT2⁺ strains were more commonly recovered from cows (58% vs. 35%). In contrast, similar proportions of VT1⁺VT2⁺ strains were observed in adult (28%) and young (26%) animals (Table 5). Only 8 (10%) of the 83 VTEC strains were positive for *eae* genes. Presence of *eae* gene was significantly more frequent among VTEC strains isolated from calves (26%) than from cows (3%) ($P < 0.01$).

Table 3. *Distribution of VTEC strains in the 32 farms investigated*

Farm	Number VTEC+/ Number of animals examined		Serogroups of VTEC
	Cows	Calves	
1	1/7	0/2	NT* (1)†
2	2/5	0/5	O8 (1), NT (2)
3	1/8	1/2	O77 (1), O171 (1)
4	2/10	—	O113 (1), NT (1)
5	0/4	2/5	O22 (1), O88 (1)
6	0/8	0/2	—
7	5/8	—	O8 (1), O22 (1), O77 (1), NT (2)
8	2/7	0/2	O4 (1), O113 (1)
9	3/7	0/1	O2 (1), O20 (2)
10	2/8	0/1	O116 (1), O162 (1), NT (1)
11	1/8	1/2	O138 (1), NT (2)
12	1/8	0/2	O113 (1)
13	2/6	0/4	O77 (1), O113 (1), NT (1)
14	0/4	0/1	—
15	2/10	0/1	O8 (1), O96 (1)
16	3/10	—	O113 (3), NT (1)
17	3/9	0/1	O138 (1), O140 (1), O168 (1), NT (2)
18	2/8	0/2	O22 (1), NT (1)
19	1/9	1/3	O15 (1), O20 (1), O149 (1), NT (1)
20	2/6	1/4	O7 (1), O77 (1), NT (1)
21	1/7	1/3	O22 (1), O26 (1)
22	2/9	1/2	O8 (1), O81 (1), NT (3)
23	1/6	3/6	O20 (1), O77 (1), O116 (1), O126 (3), NT (1)
24	3/8	0/2	O77 (2), O162 (1)
25	3/10	—	O22 (2), O136 (1)
26	1/6	1/1	O77 (1), O113 (1)
27	0/8	0/2	—
28	0/8	—	—
29	1/15	0/5	O136 (1)
30	0/5	0/5	—
31	1/5	4/5	O9 (1), O15 (1), O157 (1), NT (2)
32	4/20	—	O20 (1), O110 (1), O162 (1), NT (1)

* Not typeable strains.

† Number of animals yielding VTEC strains belonging to indicated serogroups.

DISCUSSION

The results of this study indicate that VTEC infection is widespread among healthy cattle in the province of Lugo. VTEC strains were present in similar proportions in adult and young animals (20% vs. 23%). In contrast, to some previous studies, in which VTEC were more frequently recovered from young dairy cattle [9, 12, 13]. In a study, comparable in many respects to this one, Wilson and co-workers [12] estimated the prevalence of VTEC infection in cows and calves on Ontario (Canada) dairy farms at 9% and 25% respectively. While, Wells and colleagues [9] in the USA found VTEC in 8% of adult cows and

19% of heifers and calves, and Montenegro and co-workers [11] in Germany identified VTEC in 17% of cows and 9% of bulls.

PCR demonstrated that 20% of bovine VTEC strains isolated in this study carried VT1 genes, 52% possessed VT2 genes, and 28% carried both VT1 and VT2 genes. Several other authors have reported a similar distribution in VTEC from healthy cattle [6, 9, 10, 11]. In contrast, Pohl [23], Wieler [24], Dorn and colleagues [25] observed that the majority of VTEC isolated from calves with diarrhoea in Belgium, Germany and USA produced VT1 only while in previous studies [5, 26, 27] we found that 72% were VT1+, 23% were VT2+ and 5% were VT1+VT2+ (data

Table 4. Serogroups, type of VT and *eae* gene in VTEC strains recovered from healthy cattle

Serogroup	Number of VTEC strains					
	Total		VT1 ⁺	VT2 ⁺	VT1 ⁺ VT2 ⁺	<i>eae</i> ⁺
O2	1	(1/0/1)*	0	1	0	0
O4	1	(1/0/1)	0	1	0	0
O7	1	(0/1/1)	1	0	0	0
O8	4	(3/1/4)	0	4	0	0
O9	1	(0/1/1)	0	1	0	0
O15	2	(2/0/2)	0	1	1	0
O20	5	(4/1/4)	0	1	4	0
O22	6	(5/1/5)	0	4	2	0
O26	1	(0/1/1)	1	0	0	1
O77	8	(6/2/7)	1	2	5	1
O81	1	(1/0/1)	0	0	1	0
O88	1	(0/1/1)	0	1	0	0
O96	1	(1/0/1)	0	0	1	0
O110	1	(1/0/1)	0	1	0	1
O113	8	(8/0/6)	1	7	0	0
O116	2	(1/1/2)	0	2	0	0
O126	4	(1/2/1)	1	0	3	0
O136	2	(2/0/2)	2	0	0	0
O138	2	(1/1/2)	2	0	0	1
O140	1	(1/0/1)	0	1	0	0
O149	1	(0/1/1)	1	0	0	0
O157	1	(0/1/1)	0	1	0	1
O162	3	(3/0/3)	0	2	1	0
O168	1	(1/0/1)	1	0	0	0
O171	1	(0/1/1)	0	1	0	0
NT†	23	(16/6/16)	6	12	5	3
Total	83	(52/16/27)	17 (20%)	43 (52%)	23 (28%)	8 (10%)

* Number of cows, calves and farms yielding VTEC strains belonging to indicated serogroups.

† Not typeable strains.

Table 5. Type of VT, *eae* gene and EHEC in bovine VTEC strains

Characteristics of VTEC strains	Number of VTEC strains from	
	Cows (n = 60)	Calves (n = 23)
VT1 ⁺	8 (13%)	9 (39%)
VT2 ⁺	35 (58%)	8 (35%)
VT1 ⁺ VT2 ⁺	17 (28%)	6 (26%)
<i>eae</i> ⁺	2 (3%)	6 (26%)
EHEC*	0 (0%)	2 (9%)

* Enterohaemorrhagic *E. coli* serotypes were recovered in two cases: one strain O26:H11 VT1⁺*eae*⁺ and one strain O157:H7 VT2⁺*eae*⁺.

not shown). Little is known about the putative pathogenic role of VTEC in cattle. Although VTEC strains have been isolated from a high proportion of

both healthy and diarrhoeic calves [5–13, 23–28], recent studies suggest that strains that produce VT1 only, and possess the *eae* gene, in addition may be involved in calf diarrhoea [24, 25, 29]. Interestingly we found the *eae* genes significantly more frequently in VTEC isolated from diarrhoeic calves (59%) (23 of 39) than in those from healthy cattle (10%) ($P < 0.001$). Dorn and co-workers [25] suggests that the higher prevalence of VT2-producing *E. coli* than VT1 *E. coli* infections among adult cattle might result in a greater passive transfer of antibodies to VT2 than VT1, resulting in lower incidence of clinical illness due to VT2-producing VTEC strains in calves. This interesting hypothesis requires further investigations.

The production of VT by *E. coli* strains of bovine origin has been detected in 79 O serogroups, although 20 (O4, O8, O22, O25, O32, O45, O82, O84, O103, O111, O113, O116, O121, O136, O146, O153, O157,

O171, O172 and OX3) are more prevalent among VTEC strains isolated from healthy cattle [1–13, 30]. Twenty-five VTEC serogroups were identified in this study and included four serogroups (O77, O96, O140, O162) not previously described. Fourteen (O2, O4, O7, O8, O9, O15, O10, O22, O26, O110, O113, O126, O157 and O168) of 25 serogroups have been associated with human disease (CH and HUS) [1–4, 30] and this is in agreement with previous reports [9, 10, 11]. Only 2 (2%) strains of the 83 VTEC belonged to EHEC serotypes. In contrast, EHEC serotypes were observed in 11 (28%) of 39 VTEC strains isolated from calves with diarrhoea [5, 26, 27]. The results of this study, and others [5, 8, 23, 27], indicate therefore, that EHEC strains are isolated sporadically from faeces of cattle but appear more prevalent in animals with diarrhoea than in healthy animals.

The number of VTEC serotypes associated with human disease is increasing, although EHEC O157:H7 continues to be the main cause of HC and HUS [31, 32]. We detected this enteropathogen in only 1 (0.3%) of the 228 animals investigated. This is consistent with other surveys of VTEC in cattle, which suggests that EHEC O157:H7 is uncommon in cattle [6, 7, 11, 12]. On the other hand, the prevalence of EHEC O157:H7 infection among cattle is higher on farms linked epidemiologically to cases of O157:H7 associated with illness in humans [7, 9]. The methods used for isolating EHEC O157:H7 greatly influence the isolation rates. Recently, Zhao and co-workers [13], using a sensitive ELISA procedure, detected EHEC O157:H7 in 18 (28%) of 64 herds investigated in 15 states of USA. This, and previous studies [9], revealed that EHEC O157:H7 is more frequently carried by calves and heifers than by adult cattle.

The other objective of this study was to characterize VTEC from healthy animals for their virulence factors and thereby for a possible relationship with types of *E. coli* known to be pathogenic to humans since is not known whether all variants of VTEC are equally pathogenic for humans. However, the relatively low incidence of non-O157 VTEC disease in humans suggests that verotoxin production alone may not be sufficient for VTEC to cause disease in humans [15]. Since intimate adherence to intestinal epithelial cells and effacing of microvilli are also virulence determinants of human pathogenic EHEC strains. The *eae* genes are essential for causing attaching and effacing lesions and are present in most human EHEC strains [15–17]. In contrast, only 8 (10%) of 83 bovine VTEC

investigated in this study were positive for *eae*. The low prevalence corresponds to recently published data [29, 33]. The *eae* genes were found frequently in VTEC from cattle and humans with diarrhoea, whereas *eae*-negative VTEC were more common in healthy cattle [15, 29, 33]. Our results indicate that *eae*-positive VTEC are more commonly found among calves than among adult cows, confirming that young animals are a more important reservoir of pathogenic VTEC strains.

Having confirmed that the *eae* gene is an essential virulence factor in EHEC, it is probably the majority of bovine VTEC strains isolated from healthy cattle in this study would not be pathogenic for humans. This would explain, in part, why VTEC are rarely isolated in Spain from the stools of diarrhoeic patients in spite of being so prevalent in cattle. Further studies of VTEC strains isolated from cattle are required to assess their role as human pathogens.

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