Sequence heterogeneity of the *eae* gene and detection of verotoxin-producing *Escherichia coli* using serotype-specific primers

M. LOUIE[1]. J. DE AZAVEDO[1]. R. CLARKE[2]. A. BORCZYK[3], H. LIOR[4],
M. RICHTER[5] AND J. BRUNTON[1]*

[1] *Departments of Microbiology and Medicine, The Toronto Hospital, University of Toronto and Samuel Lunenfeld Research Institute at Mount Sinai Hospital, Toronto, Ontario*
[2] *Agriculture Canada, Health of Animals Laboratory, Guelph, Ontario*
[3] *Central Public Health Laboratory, Toronto*
[4] *Laboratory Centre for Disease Control, Ottawa*
[5] *Provincial Laboratory of Public Health for Northern Alberta, Edmonton, Alberta, Canada*

SUMMARY

The distribution of the *Escherichia coli* attaching and effacing (*eae*) gene in strains of verotoxin-producing *E. coli* (VTEC) isolated from cattle and humans was studied. The majority of strains isolated from humans with bloody diarrhoea or HUS and cattle with severe diarrhoea were *eae* positive (82 and 83% respectively). In contrast. 59% of VTEC isolated from asymptomatic cattle were *eae* negative and of the remaining 41% that were *eae* positive, the majority were serotype O157.H7. The nucleotide sequence of the 3' end of the *eae* gene of enteropathogenic *E. coli* (EPEC) of serotype O55.H7 was found to be almost identical to that of serotype O157.H7. Specific primers are described which detect the *eae* sequences of VTEC serotypes O157.H7, O157.H−, and EPEC serotypes O55.H7 and O55.H−. The nucleotide sequence of the 3' end of the *eae* gene of serotype O111.H8 differed significantly from that of O157.H7. Primers were developed to specifically identify the *eae* sequences of VTEC serotypes O111.H− and O111.H8. We conclude that whereas the majority of VTEC associated with disease in cattle and humans possess the *eae* gene, the gene itself may not be necessary to produce haemorrhagic colitis and HUS. Sequence heterogeneity in the 3' end of *eae* alleles of VTEC permits specific identification of subsets of these organisms.

* Corresponding author: Dr James L. Brunton, The Toronto Hospital. Department of Microbiology. NU 13–124. 200 Elizabeth Street, Toronto. Ontario M5G 2C4.

INTRODUCTION

Verotoxin-producing *Escherichia coli* (VTEC) cause a wide spectrum of clinical disease in humans including non-bloody diarrhoea, haemorrhagic colitis (HC), the haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopoenic purpura [1–4]. Serotype O157.H7, the prototype of enterohaemorrhagic *E. coli* (EHEC) [5], is the most common cause of HC and HUS in North America and Western Europe. This serotype is also the most frequent cause of outbreaks in daycare centres and other institutions [6–9]. Other VTEC strains of diverse serotypes including those causing diarrhoea and HUS in humans are frequently isolated from animal faeces, meat and dairy products [10–17]. Cattle have been implicated as the principal reservoir for VTEC [16, 18, 19].

Recognition of the specific types of VTEC which are important causes of human disease, in animal and food samples presently depends on serotyping. Other diagnostic methods have been developed on the basis of the virulence properties of EHEC such as the DNA probe pCVD419 which detects the 60 megadalton plasmid of EHEC strains [20] and probes which detect the genes encoding Verotoxin 1 and Verotoxin 2 [21]. However, in animal specimens, toxin production does not distinguish VTEC which are important causes of human disease from those which are rarely human pathogens [11, 14]. Clearly other methods of identifying specific serogroups of VTEC would be useful.

Most VTEC strains which cause disease in humans and cattle colonize the intestine in a characteristic manner known as attaching and effacing (AE) [22–24]. This lesion is characterized by intimate adherence, effacement of microvilli and polymerization of actin in the underlying cytoplasm. Accumulation of polymerized actin can be demonstrated *in vitro* by the fluorescent actin staining (FAS) test [25]. The *eae* (*E. coli* attaching effacing) gene from an enteropathogenic *E. coli* (EPEC) strain has been cloned [26]. This gene which encodes a 94 kDa outer membrane protein called intimin, has been shown to be necessary but not sufficient for formation of the AE lesion [27, 28]. We and others have cloned and sequenced a similar *eae* gene from an EHEC strain of serotype O157.H7 [29, 30]. Although the EPEC and EHEC *eae* genes are highly conserved at the 5′ and central regions, they differ considerably at the 3′ end. The aim of this study was to determine the distribution of *eae* alleles in serotypes of VTEC and to see if the variation in the nucleotide sequence in the 3′ end could be used to develop allele-specific DNA probes and PCR primer sets.

METHODS

Bacterial strains

As summarized in Table 1, 146 well characterized VTEC strains of multiple serotypes and different sources were selected, to determine the serotype distribution of the *eae* gene. This group included 38 human strains consisting of 25 strains from cases of HUS and 13 strains from cases of bloody diarrhoea (Dr M. Karmali). A further 35 human VTEC strains were isolated from human faecal cultures (for which there was no clinical information). The remaining VTEC strains comprised 37 strains from asymptomatic cattle (isolated during surveys of

Table 1. *Verotoxin-producing* E. coli *strains used in PCR and colony hybridizations*

| | | Isolates | | | *eae* Gene* |
| | | Cattle | | | Based on |
VTEC	Human No.	Diseased No.	Not diseased No.	Total No.	C1–C2 probe No.
O?.H16. 19. 21	--	4	4	8	0
O5.H⁻	—	8	1	9	9
O26.H11	6	7	1	14	14
O103.H2	—	2	2	4	4
O111.H⁻	8	4	--	12	12
O111.H8	2	2	—	4	4
O111.H11	—	6	—	6	6
O113.H21	3	—	3	6	0
O115.H18	—	2	—	2	0
O117.H4	2	—	—	2	0
O118.H16. 30	1	1	—	2	2
O121.H19	2	—	—	2	2
O145.H⁻	1	—	3	4	2†
O153.H21. 25	—	—	2	2	0
O156.H⁻	--	—	4	4	0
O157.H7	41	—	9	50	50
O157.H⁻	5	—	—	5	5
VTEC‡	2	—	8	10	0

* Presence of *eae* gene based on hybridization studies using the highly conserved central fragment C1–C2.

† *Eae* gene detected in two cattle O145 strains.

‡ Other VTEC (single isolates) include: cattle strains of serotypes O1.H20. O2.H29. O6.H34. O22.H16. O40.H8. O43.H2. O126.H8. O136.H16 and human strains of serotypes O18.H7. O91.H21.

beef cattle, dairy cows and veal calves) and 36 strains from calves with severe diarrhoea (isolated from faecal samples of sick animals referred from provincial laboratories across Canada where routine diagnostic procedures failed to isolate other known enteric pathogens).

Sixty-three verotoxin (VT) negative strains that represented the classical human EPEC serotypes as defined by the World Health Organization (WHO) [31] were used to test the specificity of O111.H8 and O157.H7 *eae* allele specific probes and primers. In addition, seven VT-negative serogroup O157 human strains were tested. Other bacterial strains used as negative controls included 10 non-pathogenic human faecal *E. coli*, 10 *E. coli* strains isolated from urine, 2 enterotoxigenic *E. coli*, 2 enteroinvasive *E. coli*, 2 *Shigella* sp., 1 *Yersinia enterocolitica* and 1 *Helicobacter pylori*. Bacterial strains were stored in citrated glycerol at −70 °C and were subcultured on unsupplemented Luria agar plates [32] prior to use.

Primers and probes

Table 2 shows the primers and probes used in polymerase chain reaction (PCR) and hybridization experiments. Synthetic oligonucleotide primers C1, C2, A2, S1, S2. P1EH and P2EH were based on the EHEC *eae* gene sequence [29]. Primers

Table 2. *Primers and probes. All primers were based on the EHEC* eae *gene. serotype O157.H7 except P40. P20 and B1 which were based on the* eae *gene of serotype O111.H8*

	Sequence (5′–3′)	Sense (+) Antisense (−)	Location within *eae* gene (BP)	Size of amplified product (KB)
Primers				
C1	TCGTCACAGTTGCAGGCCTGGT	+	803	1·1
C2	CGAAGTCTTATCCGCCGTAAAGT	−	1912	
P1EH	AAGCGACTGAGGTCACT	+	2442	0·45
P2EH	ACGCTGCTCACTAGATGT	−	2917	
S1	GCAGTAGCAAATGGTAAGGAT	+	2213	0·80
S2	TCTACACAAACCGCAT	−	3010	
P40	ACGTTACTGGTGACTTA	+	2508	0·40
P20	TATTTTATCAGCTTCAGT	−	2917	
Probes				
A2	GACCTCTTGACATTGT	−	2520	
B1	GCTGCAATATGGTCAGT	+	2539	

P40, P20, and B1 were based on the O111.H8 *eae* gene sequence determined in this study. Oligonucleotides A2 and B1 were used as internal probes for Southern hybridizations. Primers were purchased from the DNA Synthesis Laboratory. Samuel Lunenfeld Research Institute, Toronto. Ontario.

Polymerase chain reaction

Several bacterial colonies were selected, resuspended in 50 μl 1 × PCR buffer (Perkin Elmer Cetus, Norwalk, CT) and boiled for 10 min prior to use as template DNA in PCR assays. Amplification reactions of 50 μl were set up using 1 mM deoxynucleotide triphosphates, 1 × PCR buffer. 1 unit *Taq* polymerase (GeneAmp. Perkin Elmer Cetus, Norwalk, CT), 20 pmol of each primer and 1 μl template DNA. Amplification was performed in a Thermal Cycler, Model No. 480 (Perkin Elmer Cetus) for 40 cycles as follows: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. A negative control which contained all components of the reaction mixture, with the exception of template DNA, was included in each set of samples tested. After amplification, 10 μl of the reaction mixture was analysed by agarose gel electrophoresis.

Hybridizations

The 1·1 kb *eae* probe C1–C2 was made by PCR amplification using strain CL8 (serotype O157.H7) as template and primers C1 and C2. The 0·45 kb probe. P1EH–P2EH, specific for O157 strains was similarly amplified using primers P1EH and P2EH. The 0·40 kb probe, P40–P20, specific for O111 strains was amplified using strain RC541 (serotype O111.H8) as template and primers P40 and P20. DNA fragments used as probes were labelled with [α^{32}P] dATP using the Random Primed DNA Labelling Kit (Boehringer Mannheim). Oligonucleotide probes were end-labelled with [γ^{32}P] ATP using T4 polynucleotide kinase [32]. Colony blot and Southern hybridizations were performed overnight at 42 °C in

$6 \times$ SSC ($1 \times$ SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), $5 \times$ Denhardt's Reagent, 0.5% sodium dodecyl sulfate (SDS), 50% formamide and $100\ \mu g$ of denatured herring sperm DNA [32]. Colony blots hybridized with probes labelled by random priming were washed as follows: 5 min at room temperature in $2 \times$ SSC and 0.5% SDS, 15 min at RT in $2 \times$ SSC and 0.1% SDS, 30 min $\times 2$ at $68\ ^{\circ}$C in $0.1 \times$ SSC and 0.5% SDS, and at RT in $0.1 \times$ SSC. The Southern blot hybridized with the end-labelled A2 oligonucleotide was washed twice in $6 \times$ SSC and 0.2% SDS at RT for 1 min, twice at $30\ ^{\circ}$C for 2 min, and once in $2 \times$ SSC and 0.2% SDS at $34\ ^{\circ}$C for 3 min. The blot hybridized with the B1 oligonucleotide was similarly washed but the final wash was at $37\ ^{\circ}$C for 3 min.

Sequencing

DNA for sequencing the $3'$ regions of the *eae* genes of strain AB9483.91 (serotype O55.H7), strain CL8 (serotype O157.H7) and strain RC541 (serotype O111.H8) was made by PCR amplification using bacterial colonies as template, as described above, and primers S1 and S2 (Table 2). Specific PCR products (0.8 kb) were excised from 1% agarose gels and the DNA was extracted by freezing the gel fragments at $-70\ ^{\circ}$C spinning them through $0.22\ \mu m$ cellulose acetate filters (CoStar Spin-X, Cambridge, MA) followed by phenol extraction and ethanol precipitation [32]. Direct PCR sequencing was carried out using the dsDNA Cycle Sequencing System (BRL Life Technologies, Gaithersburg, MD). The 0.8 kb purified PCR fragments were also subcloned into the vector pAMP™1 (BRL Life Technologies, Gaithersburg, MD) and sequencing was performed using single stranded DNA as previously described [29]. Sequence analysis was performed using the Wisconsin Genetics Computer Group Sequence Analysis Software Package Version 7.1 [33]. The $3'$ *eae* gene sequences of serotypes O55.H7 and O111.H8 have been submitted to the Genbank database.

Tests for verotoxin

All bacterial strains (except negative control isolates) were tested for verotoxin production by cytotoxicity assays on Vero cells as described previously [11]. The presence of VT genes was confirmed using VT specific oligonucleotide primers in PCR assays [34].

RESULTS

Serotype distribution of eae gene

The conserved central region of the O157.H7 *eae* gene was used to determine the serotype distribution of *eae* alleles in the 146 VTEC strains isolated from humans and cattle. For colony blot hybridization, a 1.1 kb *eae* probe C1–C2 was produced by labelling a fragment amplified by PCR from the conserved central region, using primers C1 and C2 and DNA from serotype O157.H7 strain CL8 as template. This amplicon contained the 1.1 kb *Stu*1 and *Sal*1 fragment and has 98% sequence similarity with the *eae* probe described by Jerse and colleagues [26]. The results are presented in Table 1. *Eae* probe positive strains included serotypes O5.H⁻, O26.H11, O103.H2, O111.H⁻, H8, H11, O118.H16, H30, O121.H19, O145.H⁻, and O157.H7, H⁻. The presence or absence of the *eae* gene in strains of a

given serotype was consistent, with the exception of those of serotype O145.H⁻ where 1/1 human and 1/3 cattle strains were *eae* negative. Other bacterial isolates (28 strains) used as controls were all *eae* probe negative.

Human strains

Clinical histories were available for 38 strains which were isolated from patients with bloody diarrhoea or HUS. Of these, *eae* positive strains were of serotype O26.H11 (4), O111.H⁻ (5), O111.H8 (1), O118.H30 (1), O121.H19 (2). O157.H⁻ (2), O157.H7 (16), whereas *eae* negative strains were of serotypes O18.H7 (1), O91.H21 (1), O113.H21 (3), O117.H4 (1), and O145.H⁻ (1). The number of isolates of each serotype is indicated in parentheses. No clinical history was available for the remaining 35 strains of human origin.

Cattle strains

A total of 36 VTEC strains were isolated from cattle which were being investigated for severe diarrhoea or HC. No other enteric pathogens were isolated from these animals. Of these, 30 isolates were *eae* positive; they belonged to serotypes O5.H⁻ (8), O26.H11 (7), O103.H2 (2), O111.H⁻ (4), O111.H8 (2). O111.H11 (6) and O118.H16 (1). Six, including 4 O-untypable strains and 2 O115.H18 strains, were *eae* negative.

Thirty-seven VTEC strains were isolated from asymptomatic cattle during the course of surveys of cattle faecal samples. *Eae* positive strains included serotypes O5.H⁻ (1), O26.H11 (1), O103.H2 (2), and O145.H⁻ (2) and O157.H7 (9). *Eae* negative strains isolated from asymptomatic cattle included serotypes O?.H21 (3), O113.H21 (3), O156.H⁻ (4) and single isolates of serotypes O?.H19. O1.H20, O2.H29, O6.H34, O22.H16, O40.H8, O43.H2, O126.H8, O136.H16. O145.H⁻, O153.H21, O153.H25.

Nucleotide sequence of the 3′ end of eae *alleles*

To design primers which would detect specific VTEC serotypes, attempts were made to amplify and determine the nucleotide sequence of the 3′ regions of the *eae* alleles of selected VTEC strains of serotypes O5.H⁻, O26.H11, O111.H8. O111.H11, O118.H30 and an EPEC strain of serotype O55.H7. Using primers S1 and S2, specific 0·8 kb PCR amplification products were obtained only from a bovine isolate serotype O111.H8, and an EPEC strain of serotype O55.H7. The products were sequenced (data not shown) and the sequences were compared to those of the EPEC and EHEC *eae* genes which have been reported. The nucleotide sequences have been deposited in Genbank under the accession numbers L08095 (O55.H7) and L06255 (O111.H8). The deduced amino acid sequences are presented in Figure 1. Although there was significant nucleotide sequence (82%) similarity in the 3′ end of the *eae* alleles of serotypes O111.H8 and O157.H7. multiple amino acid substitutions were predicted in the putative protein products. The deduced amino acid sequence of the carboxyl end of the *eae* protein of the O55.H7 strain was virtually identical to that of the O157.H7 strain. differing only at position 770, 772, 773 and 904 (Fig. 1). By comparison, the deduced amino acid sequence of the O157.H7 strain reported by Yu and Kaper [30] only differed from the O55.H7 sequence at position 904.

```
O157.H7  VKVMKNGQPVNNQSVTFSTNFGMFNGKSQTQATTGND-GRATITLTSSSAGKATVSATVS    740
O111.H8  .....E....QGH..V.T.............NA.TGSD......................    741
O55.H7   ................................................-.............  743
O127.H6  .....GDK..S..E...T.TL.---KL.NSTEK.DTN-.Y.KV....TTP..SL...R..    737


O157.H7  DGAE-VKATEVTFFDELKIDNKVD-IIGNN-VK-RSMLPNIWLQYGQFKLKASGGDGTYS    796
O111.H8  G.N-D...P......G..:......-.L.K.-.T--GD..............V..N....    796
O55.H7   ....-..................-......-.--.GE.......................   798
O127.H6  .V.VD...P..E..TT.T..DGNIE.V.TG-..--GK..TV......VN......N.K.T    794


O157.H7  WYSENTSIATVDAS-GKVTLNGKGSVVIKATSGDKQTVSYTIKAPSYMI-----K-VDKQ    849
O111.H8  .H....N.....E.-.....K...TA..NV...............N...-----R-.GNK    849
O55.H7   ..............-..................................-----.-....  851
O127.H6  .R.A.PA..S....S.Q...KE..TTT.SVI.S.N..AT...AT.NSL.VPNMS.R.TYN    854


O157.H7  -AYYADAMSICKNL---LPSTQTVLSDIYDSWGAANKYSHYSSMNSITAWIKQTSSEQRS    905
O111.H8  -.S..N...F.G..---...S.....NV.N.R.P..G.D..R..Q....R.T..EADKI.    905
O55.H7   -..............---........................................S.    907
O127.H6  D.VNT-----...FGGK...S.NE.ENVFKA.......EY:K.SQT.IS.VQ..AQDAK.    909


O157.H7  GVSSTYNLITQNPLPGVNVNTPNVYAVCVE*                             936
O111.H8  ...T..D......HKD.Q..A.........*                             936
O55.H7   ..........................                                  931
O127.H6  ..A...D.VK....NNIKASES.A..T..K*                             940
```

Fig. 1. Alignment of the deduced amino acid sequences at the 3′ end of: EHEC *eae* O157.H7 sequence [29]. VTEC *eae* O111.H8 sequence. VTEC *eae* O55.H7 sequence, EPEC *eae* O127.H6 sequence [26]. The O111.H8, O55.H7 and O127.H6 amino acid sequences are compared with O157.H7 (strain CL8). Only positions which differ from the O157.H7 sequence are shown; identical position are displayed as dots (...) and spaces are represented by dashes (———). Amino acids of the O111.H8 and O55.H7 sequences were assigned numbers based on the alignment with the O157.H7 sequence.

Allele specific eae *probes*

Based on differences between the O157.H7 and O111.H8 *eae* gene sequences, primers P1EH, P2EH and primers P40, P20 were selected. Using an EHEC O157.H7 strain (CL8) as template, primers P1EH–P2EH were designed to amplify by PCR a 0·45 kb probe specific for O157 strains. This probe hybridized only with VT-positive strains of serotypes O157.H⁻ and O157.H7 but not with VT-negative and *eae* negative O157 strains of the following H serotypes: H⁻ (1), H16 (3). H19 (1), H25 (1) and H42 (1) (data not shown). The probe hybridized with EPEC strains of serotypes O55.H⁻ and O55.H7, but not with other *eae* positive O55 strains. The probe also did not hybridize with any other EPEC strains which hybridized to the C1–C2 probe from the conserved central region (data not shown, see below).

A 0·40 kb probe P40–P20 was generated by PCR amplification using the O111.H8 strain RC541 as template. This probe hybridized only with VT-positive O111.H⁻ and O111.H8 strains but not with VT-positive O111.H11 nor with the VT-negative strains of serotypes O111.H⁻ and O111.H2 which hybridized with

Table 3. *PCR amplification products derived from various VTEC and EPEC
O-serogroups using specific 3′ end primer sets*

		Specific PCR amplification products with primers	
		---	---
Serogroup	Verotoxin	P1EH–P2EH*	P40–P20*
O5	+	0/9	0/9
O26	+	0/14	0/14
O55	−	11/16†	0/16
O103	+	0/4	0/4
O111‡	+	0/22	16/22
	−	0/6	0/6
O118	+	0/2	0/2
O121	+	0/2	0/2
O145	+	0/4	0/4
O157	+	28/28	0/28

* Strains positive for the *eae* gene were further tested using primer sets P1EH–P2EH and P40–P20.

† EPEC O55.H⁻ and H7 strains but no H6, H34 and H44 strains yielded a specific amplification product.

‡ All VT-positive O111.H⁻ and H8 but no VT-positive O111.H11 strains yielded a specific amplification product. EPEC strains of serotypes O111.H⁻ and O111.H2 did not yield a specific amplification product.

the C1–C2 probe (data not shown). The P40–P20 probe also did not hybridize with EPEC strains which were *eae* positive as determined with the C1–C2 probe (data not shown, see below).

Allele specific PCR

The same primer sets were used in PCR assays to test strains from *eae* positive VTEC serogroups (Table 3). Primers P1EH and P2EH produced amplicons only from VT-positive O157.H⁻, O157.H7 and VT-negative O55.H⁻ and O55.H7 strains. All other *eae* positive strains gave negative results when tested with these allele specific primers. Similarly, primers P40 and P20 produced amplicons only from VT-positive O111.H⁻ and O111.H8 strains. All other *eae* positive strains gave a negative result with these primers. The sizes of specific amplification products obtained in the PCR studies are shown in Table 2. Lack of a specific product of the expected molecular size was considered as negative. The specificity of amplification products was confirmed by probing them with labelled oligonucleotide primers A2 and B1 (Fig. 2). Oligonucleotide A2 derived from a site on the 3′ end of an O157.H7 *eae* gene hybridized only with the 0·45 kb amplification product of VT-positive O157.H7, O157.H⁻ strains, and O55.H7 (Fig. 2, lanes 1, 5 and 6 respectively) and O55.H⁻ strains. The 0·40 kb amplification product of O111.H8 (lane 8) and O111.H⁻ (lanes 10–11) strains hybridized with the internal probe B1 derived from a site on the 3′ end of an O111.H8 *eae* gene.

Eae *distribution in VT-negative isolates of serogroups O55 and O111*

Because of our interest in the O157/O55 and the O111 alleles we also studied VT-negative isolates of serogroups O55 (24 strains) and O111 (12 strains) in more detail. Verotoxin negative isolates of the following O55 serotypes contained *eae*
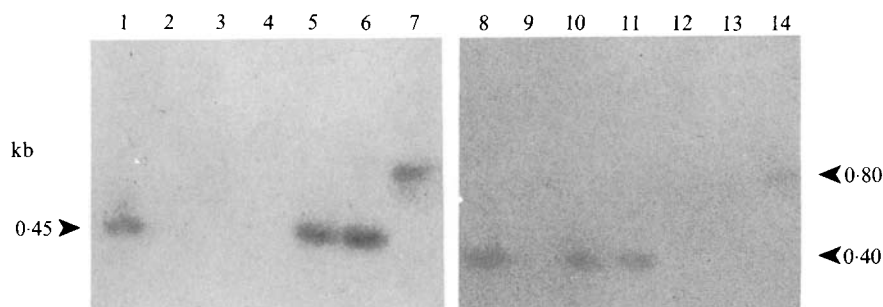
Fig. 2. Southern blot analysis of amplification products using specific internal probes A2 based on O157 sequence (lanes 1–7) and B1 based on O111 sequence (lanes 8–14). Probe A2 hybridized with a 0·45 kb product amplified from serotypes O157.H7 (lane 1), O157.H⁻ (lane 5), O55.H7 (lane 6) using primers P1EH–P2EH and with a 0·8 kb product from serotype O157.H7 using primers S1–S2 (lane 7). A2 did not hybridize with amplification products of serotype O111.H8 using primers P40–P20 (lane 2) and serotype O40.H8 using primers P1EH–P2EH (lane 3) or a 1·1 kb product of serotype O157.H7 using primers C1–C2 (lane 4). Probe B1 hybridized with a 0·40 kb product amplified from serotypes O111.H8 (lane 8) and O111.H⁻ (lanes 10–11) using primers P40–P20. and a 0·8 kb product from serotype O111.H8 using primers S1–S2 (lane 14). B1 did not hybridize with amplification products of serotypes O111.H11 (lane 9), O5.H⁻ (lane 12) and O26.H11 (lane 13) using primers P40–P20.

alleles: O55.H⁻ (4/7), O55.H6 (1/1), O55.H7 (7/7), O55.H34 (1/1) and O55.H44 (3/3). Of these only O55.H⁻ (4/7) and O55.H7 (7/7) hybridized with the specific probe P1EH–P2EH from the 3′ end of the O157.H7 allele (Table 3). Serotypes O55.H⁻ (3/7), O55.H1 (1/1), O55.H2 (1/1), O55.H4 (1/1), O55.H11 (1/1) and O55.H21 (1/1) did not hybridize with the conserved C1–C2 probe. For VT-negative isolates of serogroup O111, serotypes O111.H⁻ (4/4) and O111.H2 (2/2) hybridized with the C1–C2 probe while serotypes O111.H12 (0/4) and O111.H21 (0/2) did not. None of the VT-negative O111 strains hybridized with the O111.H8 allele specific probe P40–P20 (Table 3). Representative strains (27 isolates) of the classical EPEC serotypes as defined by WHO were also tested. All EPEC strains of serotypes O26.H⁻ (2), O26.H11 (2), O86.H⁻ (2), O114.H2 (2), O119.H6 (2), O127.H⁻ (2), O127.H6 (1), O128ab.H2 (1), O142.H6 (2), O158.H23 (1) and 1/2 O86.H34 strains hybridized with the C1–C2 probe, whereas strains of serotypes O86.H2 (2), O125.H21 (1), O126.H27 (2), O127.H9 (1) and O127.H21 (2) did not. None of the *eae* positive strains hybridized with the allele specific probes P1EH–P2EH or P40–P20.

DISCUSSION

Many, but not all VTEC strains possess the *eae* gene. Our results extend the findings of Willshaw and colleagues and add VTEC of serotypes O111.H8, O111.H11, O118.H16, O118.H30 and O121.H19 to the group known to contain *eae* homologues [35]. Except for non-motile strains, all isolates of a single O.H serotype were concordant with regard to the presence or absence of the *eae* gene in both studies. This is an example of the previously recognized association of specific virulence genes with individual O.H serotypes [36, 37]. Non-motile strains of serogroups O145 and O111ac which differ with respect to *eae* status, may be derivatives of different H serotypes.

All studies of VTEC colonization in animal models such as gnotobiotic piglets. calves and rabbits have shown adherence by the classical AE lesion [22–24. 38–40]. Barrett and colleagues found that non-O157 VTEC isolates from humans were more likely to be *eae* positive than were animal isolates, and suggested that the *eae* gene is an important virulence marker for human disease [41]. However. they did not study strains isolated from cattle which were being investigated for severe diarrhoea and HC. In the present study 30 cattle with severe diarrhoea or HC were infected with *eae* positive strains of serotypes O5.H⁻, O26.H11. O103.H2. O111.H⁻, O111.H8, O111.H11 and O118.H16 whereas only six symptomatic cattle were infected with *eae* negative strains. Faecal cultures from asymptomatic cattle yielded 15 *eae* positive strains (nine of which were serotype O157.H7 and six were of other serotypes) and 22 *eae* negative strains of various serotypes. The isolation of O157.H7 strains from asymptomatic cattle has been noted before [18, 19, 42]. This indicates that disease in cattle is more likely to be associated with *eae* positive organisms of specific serotypes and that the *eae* gene in these strains may be a potential virulence marker.

It appears that while the *eae* gene is carried by a group of VTEC which are particularly virulent in humans and cattle, its presence *per se* is not predictive of virulence of a particular strain in a specific animal host. Whereas the majority (82 %) of isolates from humans with HUS or bloody diarrhoea were *eae* positive. important exceptions included VTEC of serotypes O18.H7. O91.H21. O113.H21, O117.H4 and O145.H⁻. With the exception of serotype O91.H21. these serotypes were not represented among the VTEC strains reported by Willshaw and colleagues [35]. It is noteworthy that *E. coli* associated with oedema disease of swine do not produce A/E lesions [43]. and do not contain the *eae* gene [44]. Thus the *eae* gene is clearly not necessary for production of the VTEC-associated systemic complications of HUS in humans, and oedema disease of swine. Additional studies using animal models will be required to further characterize adherence patterns of *eae* negative VTEC in the gastrointestinal tract.

The nucleotide sequence of the O55.H7 allele was 99·8 % similar to that of the O157.H7 allele at the 3′ end (Fig. 1) raising the possibility that these strains share some common evolutionary origin. Whittam and colleagues [45], using multilocus enzyme electrophoresis have demonstrated that EPEC of serotypes O55.H7 are very closely related to VTEC of serotypes O157.H7. The nucleotide sequence variation at the 3′ end of the *eae* alleles allowed us to construct probes and PCR primer sets which specifically detected VTEC of serotypes O157.H7 and O157.H⁻ strains. These reagents also yielded positive results with EPEC of serotypes O55.H7 and O55.H⁻ but were negative when tested against all other EPEC strains including other O55 serotypes. It is possible that they might also detect VTEC of serotype O55.H7, but such strains have not been isolated in North America to date (H. Lior, unpublished data). The O157 *eae* primers 19–20 reported by Gannon and colleagues detected O157, O55 and O145 strains [44]. However, primer 20 is based on a sequence, 153 bp downstream of the termination codon of the *eae* allele of serotype O157.H7.

A second probe and set of PCR primers specifically detected VTEC of serotypes O111.H8 and O111.H⁻ but not VTEC of serotype O111.H11 nor *eae* positive

EPEC strains of serogroup O111. The O111ab lipopolysaccharide (LPS) of EPEC is antigenically different from the O111ac LPS characteristic of VTEC, suggesting that there are significant differences between EPEC and VTEC of serogroup O111 [35, 46].

Our studies were extended to VT-negative isolates of serogroups O55 and O111. Whereas O55.H⁻, O55.H6 and O55.H7 are considered to be classical EPEC serotypes, some authors feel that virtually all H-types of serogroup O55 are associated with diarrhoea [37, 47]. Hybridization studies demonstrated *eae* alleles in O55 isolates of serotypes H⁻, H6 and H7 (as expected) as well as H34 and H44. It seems possible that serotypes O55.H34 and O55.H44 should be classified as EPEC on this basis. However, further studies would be required to demonstrate intestinal colonization and AE lesion formation in animal models and epidemiologic association with diarrhoea in humans. Other EPEC O55 serotypes, and serotypes O111.H12 and O111.H21 strains were *eae* negative. Strains of serotype O111.H21 and a single isolate of O55.H4 have previously been shown to exhibit enteroaggregative *E. coli* (EAggEC) adherence characteristics and to hybridize with the EAggEC probe [48, 49].

In conclusion, the study of *eae* gene distribution will be a useful method for subclassification of EPEC and VTEC. The mechanism of adherence of *eae* negative VTEC and EPEC needs to be studied. There is clearly significant variation in the 3′ end of *eae* alleles which might allow the development of serotype-specific diagnostic tools. Use of allele-specific probes and PCR primers may be of help in identifying small numbers of VTEC of specific serotypes in cultures of animal and human faeces and food samples.

REFERENCES

1. Griffin PM, Ostroff, SM, Tauxe RV, et al. Illnesses associated with *Escherichia coli* O157: H7 infections: A broad clinical spectrum. Ann Intern Med 1988; **109**: 705–12.
2. Karmali MA. Infection by verocytotoxin-producing *Escherichia coli*. Clin Microbiol Rev 1989; **2**: 15–38.
3. Pai CH, Ahmed N, Lior H, Johnson WM, Sims HV, Woods DE. Epidemiology of sporadic diarrhoea due to verocytotoxin-producing *Escherichia coli*: A two-year prospective study. J Infect Dis 1988; **157**: 1054–7.
4. Riley LW, Remis RS, Helgerson SD, et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 1983; **308**: 681–5.
5. Levine MM. *Escherichia coli* that cause diarrhoea: Enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent. J Infect Dis 1987; **155**: 377–89.

6. Belongia EA. MacDonald KL. Blaser MJ. Soler JT. Ammend DA. Osterholm MT. Outbreak of *Escherichia coli* O157: H7 in a day care center: patterns of transmission and control methods. Program Abstr. 1989; 29th Intersci Conf Antimicro Agent Chemother abstr 10.

7. Carter AO. Borczyk AA. Carlson JAK. et al. A severe outbreak of *Escherichia coli* O157: H7 associated hemorrhagic colitis in a nursing home. N Engl J Med 1987: **317**: 1496–500.

8. Ryan CA. Tauxe RV, Hosek GW, et al. *Escherichia coli* O157: H7 diarrhea in a nursing home: clinical, epidemiological, and pathological findings. J Infect Dis 1986: **154**: 631–8.

9. Spika JS, Parsons JE. Nordenberg D. Gunn RA. Hemolytic uremic syndrome and diarrhea associated with *Escherichia coli* O157: H7 in a day-care center. J Pediatr 1986: **109**: 287–91.

10. Belongia EA. MacDonald KL. Parham GL. et al. An outbreak of *Escherichia coli* O157: H7 colitis associated with consumption of precooked meat patties. J Infect Dis 1991: **164**: 338–43.

11. Clarke RC, McEwen SA, Gannon VP, Lior H. Gyles CL. Isolation of verocytotoxin-producing *Escherichia coli* from milk filters in south-western Ontario. Epidemiol Infect 1989; **102**: 253–60.

12. Duncan L, Mai V, Carter A. Carlson JAK. Borczyk AA. Karmali MA. Outbreak of gastrointestinal disease–Ontario. Can Dis Wkly Rep 1987: **13-2**: 5-8.

13. Montenegro MA. Bulte M, Trumpf T, et al. Detection and characterization of fecal verotoxin-producing *Escherichia coli* from healthy cattle. J Clin Microbiol 1990: **28**: 1417–21.

14. Read SC, Gyles CL, Clarke RC. Lior H, McEwen S. Prevalence of verocytotoxigenic *Escherichia coli* in ground beef, pork, and chicken in southwestern Ontario. Epidemiol Infect 1990: **105**: 11–20.

15. Sherwood D. Snodgrass DR. O'Brien AD. Shiga-like toxin production from *Escherichia coli* associated with calf diarrhoea. Vet Rec 1985: **116**: 217–18.

16. Wells JG, Shipman LD, Greene KD, et al. Isolation of *Escherichia coli* serotype O157: H7 and other Shiga-like-toxin-producing *E. coli* from dairy cattle. J Clin Microbiol 1991: **29**: 985–9.

17. Wilson JB, McEwen SA. Clarke RC. et al. Distribution and characteristics of verocytotoxigenic *Escherichia coli* isolated from Ontario dairy cattle. Epidemiol Infect 1992: **108**: 423–39.

18. Borczyk AA, Karmali MA. Lior H, Duncan LMC. Bovine reservoir for verotoxin-producing *Escherichia coli* O157: H7. Lancet: 1987: **i**: 98.

19. Orskov F. Orskov I, Villar J. Cattle as a reservoir of verotoxin-producing *Escherichia coli* O157: H7. Lancet 1987: **ii**: 276.

20. Levine MM, Xu JG, Kaper JB, et al. A DNA probe to identify enterohemorrhagic *Escherichia coli* O157: H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. J Infect Dis 1987: **156**: 175–82.

21. Willshaw GA, Smith HR. Scotland SM. Field AM, Rowe B. Heterogeneity of *Escherichia coli* phages encoding Vero cytotoxins: comparison of cloned sequences determining VT1 and VT2 and development of specific gene probes. J Gen Microbiol 1987: **133**: 1309–17.

22. Moxley RA, Francis DH. Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect Immun 1986: **53**: 339–46.

23. Tzipori S, Wachsmuth IK, Smithers J. Jackson C. Studies in gnotobiotic piglets on non-O157: H7 *Escherichia coli* serotypes isolated from patients with hemorrhagic colitis. Gastroenterol 1988: **94**: 590–7.

24. Tzipori S, Gibson R. Montanaro J. Nature and distribution of mucosal lesions associated with enteropathogenic and enterohemorrhagic *Escherichia coli* in piglets and the role of plasmid-mediated factors. Infect Immun 1989: **57**: 1142–50.

25. Knutton S, Baldwin T. Williams PH, McNeish AS. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect Immun 1989: **57**: 1290–8.

26. Jerse AE, Yu J, Tall BD, Kaper JB. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc Natl Acad Sci USA 1990: **87**: 7839–43.

27. Donnenberg MS. Kaper JB. Enteropathogenic *Escherichia coli*. Infect Immun 1992: **60**: 3953–61.

28. Jerse AE, Kaper JB. The *eae* gene of enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid. Infect Immun 1991; **59**: 4302–9.

29. Beebakhee G, Louie M, De Azavedo J, Brunton J. Cloning and nucleotide sequence of the *eae* gene homologue from enterohemorrhagic *Escherichia coli* serotype O157: H7. FEMS Microbiol Lett 1992; **91**: 63–8.

30. Yu K, Kaper JB. Cloning and characterization of the *eae* gene of enterohemorrhagic *Escherichia coli* O157: H7. Mol Microbiol 1992; **6**: 411–17.

31. World Health Organization. Manual of investigation of acute enteric infections. Publication no. CDD/83.3 Rev. 1983; **1**: 27.

32. Sambrook J, Fritsch EF, Maniatis T. In: Molecular cloning. A laboratory manual. Nolan C, ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.

33. Devereux J, Haeberli P, Smithies O. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 1984; **12**: 387–95.

34. Read SC, Clarke RC, Martin A, et al. Polymerase chain reaction for detection of verocytotoxigenic *Escherichia coli* isolated from animal and food sources. Mol Cell Probes 1992; **6**: 153–61.

35. Willshaw GA, Scotland SM, Smith HR, Rowe B. Properties of Vero cytotoxin-producing *Escherichia coli* of human origin of O serogroups other than O157: H7. J Infect Dis 1991; **166**: 797–802.

36. Merson MH, Orskov F, Orskov I, Sack R, Huq I, Koster F. Relationship between enterotoxin production and serotype in enterotoxigenic *Escherichia coli*. Infect Immun 1979; **23**: 325–9.

37. Robins-Browne RM. Traditional enteropathogenic *Escherichia coli* of infantile diarrhea. Rev Infect Dis 1987; **9**: 28–53.

38. Janke BH, Francis DH, Collins JE, et al. Attaching and effacing *Escherichia coli* infection as a cause of diarrhea in young calves. J Am Vet Med Assoc 1990; **196**: 897–901.

39. Schoonderwoerd M, Clarke RC, van Dreumel TA, Rawluk SA. Colitis in calves: natural and experimental infection with a verotoxin-producing strain of *Escherichia coli* O111: NM. Can J Vet Res 1988; **52**: 484–7.

40. Sherman P, Soni R, Karmali M. Attaching and effacing adherence of verocytotoxin-producing *Escherichia coli* to rabbit intestinal epithelium in vivo. Infect Immun 1988; **56**: 756–61.

41. Barrett TJ, Kaper JB, Jerse AE, Wachsmuth IK. Virulence factors in Shiga-like toxin-producing *Escherichia coli* isolated from humans and cattle. J Infect Dis 1992; **165**: 979–80.

42. Griffin PM, Tauxe RV. The epidemiology of infections caused by *Escherichia coli* O157: H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol Rev 1991; **13**: 60–98.

43. Bertschinger HU, Pohlenz J. Bacterial colonization and morphology of the intestine in porcine *Escherichia coli* enterotoxemia (edema disease). Vet Pathol 1983; **20**: 99–100.

44. Gannon VPJ, Rashed M, King RK, Golsteyn Thomas EJ. Detection and characterization of the *eae* gene of Shiga-like toxin producing *Escherichia coli* using polymerase chain reaction. J Clin Microbiol 1993; **31**: 1268–74.

45. Whittam TS, Wolfe ML, Wachsmuth IK, Orskov F, Orskov I, Wilson RA. Clonal relationships among *Escherichia coli* strains that cause hemorrhagic colitis and infantile diarrhea. Infect Immun 1993; **61**: 1619–29.

46. Scotland SM, Willshaw GA, Smith HR, Gross RJ, Rowe B. Adhesion to cells in culture and plasmid profiles of enteropathogenic *Escherichia coli* isolated from outbreaks and sporadic cases of infant diarrhoea. J Infect 1989; **19**: 237–49.

47. Stenderup J, Orskov F. The clonal nature of enteropathogenic *Escherichia coli* strains. J Infect Dis 1983; **148**: 1019–24.

48. Knutton S, Shaw RK, Bhan MK, et al. Ability of enteroaggregative *Escherichia coli* strains to adhere in vitro to human intestinal mucosa. Infect Immun 1992; **60**: 2086–91.

49. Scotland SM, Smith HR, Said B, Willshaw GA, Cheasty T, Rowe B. Identification of enteropathogenic *Escherichia coli* isolated in Britain as enteroaggregative or as members of a subclass of attaching-and-effacing *E. coli* not hybridising with the EPEC adherence-factor probe. J Med Microbiol 1991; **35**: 278–83.