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Characterization of verocytotoxin-producing *Escherichia coli* O157 isolates from patients with haemolytic uraemic syndrome in Western Europe

A. E. HEUVELINK[1], N. C. A. J. VAN DE KAR[2], J. F. G. M. MEIS[1], L. A. H. MONNENS[2] AND W. J. G. MELCHERS[1]

Departments of [1]Medical Microbiology and [2]Paediatrics, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands

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SUMMARY

Fifty verocytotoxin (VT)-producing *Escherichia coli* (VTEC) strains of serogroup O157 were characterized by phage typing, polymerase chain reaction (PCR) for VT genes and the *E. coli* attaching and effacing (*eae*) gene, and random amplified polymorphic DNA–PCR (RAPD–PCR) fingerprinting. The collection represented isolates obtained from patients with diarrhoea-associated haemolytic-uraemic syndrome (D+ HUS) and their family contacts, isolated in the Netherlands, Belgium and Germany between 1989 and 1993. Based on isolates from separate families ($n = 27$) seven different phage types were identified, types 2 (44%) and 4 (33%) were predominant. Eighty-five percent of the strains contained only VT2 gene sequences and 15% both VT1 and VT2. All strains of the dominant phage types 2 and 4 carried the VT2 gene. Strains that belonged to the minor phage types 8, 14, 32 carried both VT1 and VT2 genes, with the exception of two isolates identified as phage types 49 and 54 which contained only VT2 genes. All O157 VTEC strains possessed the chromosomally-located *eae* gene, which indicates its usefulness as virulence marker. RAPD–PCR fingerprinting identified four distinct banding patterns, with one profile found among 79% of the strains. Based on the combined results of all typing methods used in this study, the collection of 50 O157 VTEC strains could be divided into nine distinct groups. Strains isolated from different persons within one family could not be distinguished by any of these methods. The data suggest that O157 VTEC strains are members of one clone that has become widely distributed.

INTRODUCTION

Verocytotoxin (VT)-producing *Escherichia coli* (VTEC) strains are implicated in the pathogenesis of the diarrhoea-associated form (D+) of haemolytic-uraemic syndrome (HUS), which is the most common cause of acute renal failure in childhood [1]. Although VTEC strains that belong to serotypes O157.H7 and O157 non-motile (O157.H$^-$) are most frequently isolated from stools of patients with HUS, at least 50 other human VTEC serotypes are known [2]. The exact mechanisms of the attachment of VTEC to the human intestinal mucosa are not

well understood. However, based on *in vitro* and animal studies a model based on intimate adhesion with localized effacement of the microvilli and disruption of the cellular cytoskeleton at the site of attachment has been proposed. The intimate attachment may be mediated by the chromosomally encoded *E. coli* attaching and effacing (*eae*) gene product, an outer membrane protein [3]. Additional bacterial factors may contribute to the attachment and effacement phenotype of VTEC strains [4]. Accumulated evidence suggests that HUS may result from the systemic action of VT on vascular endothelial cells [1]. VTEC strains produce two immunologically distinct VTs (VT1 and VT2), either singly or in combination. These are encoded by bacteriophages. Since these VTs are closely related to the Shiga toxin, they are often referred to as Shiga-like toxin (SLT-I and SLT-II) [5]. In the past few years, variants of VT2 (VT2c or SLT-IIc) have been identified [6].

E. coli O157.H7 has been isolated from cattle and foods of animal origin (contaminated meat, unpasteurized milk) [2]. Waterborne and person-to-person transmission are also believed to be modes of spread of this pathogen [7–9]. Several phenotypic and genotypic schemes have been developed to assist in epidemiological investigations. These include typing of the strains by VT profile [6, 10–12], plasmid profile [10, 11, 13], restriction enzyme digestion and electrophoresis of plasmid [14] and whole cell DNA [15], multilocus enzyme electrophoresis [16, 17], phage type [18–20] and antibiogram [9, 11]. In this study, O157 VTEC strains and O111 VTEC strains were characterized which had been isolated from D+ HUS-patients as well as from family contacts in three Western European countries. Besides phage type, the VTEC isolates were examined for toxin profile and the presence of the *eae* gene by a multiplex polymerase chain reaction (PCR). To determine the genetic relationship of the strains PCR-based fingerprinting was used.

METHODS

Clinical VTEC isolates

Fifty-two human faecal VTEC isolates were studied, including 30 isolates from 19 children with D+ HUS, nine isolates from family contacts ($n = 9$) of five of these HUS-patients, 11 isolates from family contacts ($n = 11$) of seven patients with D+ HUS from which no VTEC had been isolated, one isolate from an adult with D+ HUS, and one isolate from a child with acute, bloody gastroenteritis. The isolates obtained from patients from the Netherlands, Belgium and Germany were collected between 1989 and 1993. Forty-six strains had been characterized as serotype O157.H7, four as serotype O157.H$^-$ and two as serotype O111.H$^-$. As controls, 6 VT-negative *E. coli* O157 isolates from raw retail meats bought in the Netherlands and 8 both VT-positive and VT-negative *E. coli* strains belonging to serogroups O157 ($n = 3$), O111 ($n = 3$) and O26 ($n = 2$) obtained from Dr M. A. Karmali (Hospital for Sick Children, Toronto, Canada) and from Dr W. Jansen (National Institute of Public Health and Environmental Protection, Bilthoven. the Netherlands) were included. In contrast to the VT-positive *E. coli* O157 isolates, the VT-negative *E. coli* O157 strains were able to ferment sorbitol and possessed the enzyme β-glucuronidase. The bacterial cells were stored at -70 °C in glycerol-containing (10%) medium.

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Table 1. *Oligonucleotide primers used for the detection of verocytotoxin (VT) and* Escherichia coli *attaching and effacing* (eae) *gene sequences*

Primer	Nucleotide sequence	Location*	Size of PCR product (bp)
VT1-1	5′-GGCAGATGGAAGAGTCCGTGGGATTACGC-3′	1011–1039	179
VT1-2	5′-CACAATCAGGCGTCGCCAGCGCACTTGCT-3′	1161–1189	
VT2-1	5′-CCACATCGGTGTCTGTTATTAACCACACC-3′	408–436	372
VT2-2	5′-GCAGAACTGCTCTGGATGCATCTCTGGTC-3′	751–779	
eae-1	5′-TGCGGCACAACAGGCGGCGA-3′	2078–2097	629
eae-2	5′-CGGTCGCCGCACCAGGATTC-3′	2687–2706	

* The position numbers are those in the published nucleotide sequences of VT1 [22], VT2 [23] and *eae* [24].

Phage typing

Phage typing of the 50 O157 VTEC isolates was performed by Dr B. Rowe (Laboratory of Enteric Pathogens, Central Public Health Laboratory, London, United Kingdom).

DNA preparation

Bacteria were grown in Tryptic Soy Broth at 37 °C, overnight. Genomic DNA was isolated as follows: 1 ml of an overnight culture was centrifuged in a microcentrifuge, and the cells were resuspended in 250 μl STET buffer (233 mM sucrose, 50 mM-TRIS-HCl (pH 8·0), 20 mM-EDTA, 5% Triton X-100). Lysozyme was added to a final concentration of 1·7 mg/ml. The suspension was incubated at room temperature for 5 min, then heated at 100 °C for 1 min and put on ice for another 2 min. In succession, sodium dodecyl sulphate and proteïnase K were added to a final concentration of 0·3% and 0·5 mg/ml, respectively. Then, the solution was incubated at 55 °C for 2 h. Following extraction with phenol, 0·03 mg/ml RNAse A was added and the mixture was incubated at 37 °C for 20 min. The solution was extracted successively with phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol (24:1). The DNA was precipitated overnight and resuspended in 100 μl of distilled water [21]. An aliquot was electrophoresed in an agarose gel (1%) containing 0·1 μg/ml ethidium bromide to estimate the DNA yield and verify DNA integrity.

Polymerase chain reaction

The VTEC strains were examined for the presence of VT and *eae* gene sequences by performing a hot start multiplex PCR. Table 1 shows the sequences of the oligonucleotides. The oligonucleotide primer pairs targeted conserved sequences found in the genes encoding VT1, VT2 and types or variants of the VT2 family, and the *eae* gene product. PCR reaction mixtures (50 μl) contained 75 mM-TRIS-HCl (pH 9·0), 2·5 mM-$MgCl_2$, 20 mM-$(NH_4)_2SO_4$, 0·01% Tween-20, 0·2 mM-dNTPs each, 0·2 U of Taq DNA polymerase (Thermoperfectplus DNA polymerase, Integro, Zaandam, the Netherlands) and 20 pmol of the primers VT1-1, VT1-2, VT2-1, VT2-2, *eae*-1, *eae*-2 each. The amount of template DNA used was approximately 5 ng per reaction. Negative (no template DNA) and positive (5 ng

Table 2. *Primers used for polymerase chain reaction fingerprinting of verocytotoxin-producing* Escherichia coli

Primer	Nucleotide sequence	Percent G+C	(Reference)
1247	5′-AAGAGCCCGT-3′	60	[25]
1254	5′-CCGCAGCCAA-3′	70	[25]
1281	5′-AACGCGCAAC-3′	60	[25]
1283	5′-GCGATCCCCA-3′	70	[25]
1290	5′-GTGGATGCGA-3′	60	[25]
RP1-4	5′-TAGGATCAGA-3′	40	[26]
RP2	5′-AAGGATCAGA-3′	40	[26]
Soy	5′-AGGTCACTGA-3′	50	[27]
HLWL85	5′-ACAACTGCTC-3′	50	[28]
HLWL74	5′-ACGTATCTGC-3′	40	[28]
D8635	5′-GAGCGGCCAAAGGGAGCAGAC-3′	67	[25]
ERIC1R	5′-ATGTAAGCTCCTGGGGATTCAC-3′	50	[29]
ERIC2	5′-AAGTAAGTGACTGGGGTGAGCG-3′	55	[29]

of purified genomic DNA from VTEC O157.H7 reference strain EDL931) controls were included in each experiment. Amplification was performed in a DNA thermal cycler (Perkin–Elmer Cetus). The thermal profile involved an initial denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 1 min, primer annealing at 59 °C for 3 min, and primer extension at 72 °C for 3 min. The final step was a 10 min incubation at 72 °C. After amplification, 15 μl aliquots were analysed by electrophoresis in 1·5% agarose gels containing ethidium bromide (0·1 μg/ml), visualized by UV transillumination and photographed. Molecular mass markers (100 bp ladder; Pharmacia, Roosendaal, the Netherlands) were electrophoresed simultaneously.

PCR fingerprinting

PCR fingerprinting of bacterial DNA (50 ng) was performed in a 50 μl reaction volume containing 75 mM-TRIS-HCl (pH 9·0), 2·5 mM-$MgCl_2$, 20 mM-$(NH_4)_2SO_4$, 0·01% Tween-20, 0·2 mM-dNTPs each, 50 pmol of primer (see Table 2 for the primer sequences) and 0·2 U of Taq DNA polymerase (Thermoperfectplus DNA polymerase, Integro, Zaandam, the Netherlands). A negative control, consisting of the same reaction mixture but with no template DNA added, was included in each reaction. A Perkin–Elmer thermal cycler was used for amplification running either of three programmes. The cycling programme when using 10-nucleotide primers was four cycles of 94 °C for 5 min, 36 °C for 5 min, 72 °C for 5 min, 30 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min, and then 72 °C for 10 min [25]. The cycling programme when using primer D8635 was four cycles of 94 °C for 5 min, 40 °C for 5 min, 72 °C for 5 min (low stringency amplification), 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min (high stringency amplification), and final incubation at 72 °C for 10 min [25]. The primers ERIC1R and ERIC2 required a programme consisting of 35 cycles of 94 °C for 1 min, 25 °C for 1 min, 74 °C for 2 min, preceded by a 5-min incubation at 94 °C and followed by a final extension step at 74 °C for 10 min [30]. Amplified DNA (5 μl) was separated by gel electrophoresis in 1·5% agarose gels and visualized by ethidium bromide staining (0·1 μg/ml). A molecular size marker (100 bp ladder; Pharmacia, Roosendaal, the

Table 3. *Characterization of the human faecal VTEC strains by serotype, verocytotoxin (VT) genotype,* Escherichia coli *attaching and effacing* (eae) *gene, random amplified polymorphic DNA-polymerase chain reaction (RAPD–PCR) and phage type*

Family no.	Strain no.*	Serotype	Source†	Results for‡ VT1	VT2	*eae*	RAPD profile§	Phage type‖	Overall type¶
I	1	O157.H7	HUS-patient	−	+	+	A	4	1
II	2a	O157.H7	HUS-patient	−	+	+	B	4	2
	2b	O157.H7	HUS-patient	−	+	+	B	4	2
III	3a	O111.H⁻	HUS-patient	−	+	−	C	ND	
	3b	O111.H⁻	HUS-patient	−	+	−	C	ND	
IV	4	O157.H7	Family contact	−	+	+	B	2	3
V	5	O157.H7	Family contact	+	+	+	B	32	4
	6	O157.H7	Family contact	+	+	+	B	32	4
VI	7	O157.H7	HUS-patient	−	+	+	A	4	1
	8	O157.H7	Family contact	−	+	+	A	4	1
	9	O157.H7	Family contact	−	+	+	A	4	1
VII	10	O157.H7	Family contact	−	+	+	B	2	3
VIII	11a	O157.H7	HUS-patient	−	+	+	B	2	3
	11b	O157.H7	HUS-patient	−	+	+	B	2	3
	11c	O157.H7	HUS-patient	−	+	+	B	2	3
	12	O157.H7	Family contact	−	+	+	B	2	3
	13	O157.H7	Family contact	−	+	+	B	2	3
	14	O157.H7	Family contact	−	+	+	B	2	3
IX	15	O157.H7	Family contact	−	+	+	B	4	2
	16	O157.H7	Family contact	−	+	+	B	4	2
X	17	O157.H7	Family contact	+	+	+	B	14	5
XI	18	O157.H⁻	Family contact	−	+	+	B	4	2
	19	O157.H⁻	Family contact	−	+	+	B	4	2
XII	20	O157.H7	Family contact	−	+	+	B	4	2
	21	O157.H7	Family contact	−	+	+	B	4	2
XIII	22	O157.H7	HUS-patient	−	+	+	B	2	3
XIV	23	O157.H7	HUS-patient	−	+	+	B	2	3
XV	24	O157.H7	HUS-patient	−	+	+	B	2	3

Netherlands) was used for reference. Gels were photographed and interpretation of banding patterns was performed by visual inspection.

RESULTS

Phage typing

Among the 50 VTEC isolates of serogroup O157, seven phage types were identified (Table 3). Sequentially isolated O157 VTEC strains from one patient (Table 3, no. 2a–2b, 11a–11c, 27a–27g, 36a–36b) appeared to belong to the same phage type. The same was true for strains which had been isolated from different persons within one family. Based on *E. coli* O157 isolates from separate families phage type 2 was the most common (44% of the strains), followed by types 4 (33%), 8 (7%), 14 (4%), 32 (4%), 49 (4%), and 54 (4%).

Amplification of toxin and eae gene sequences

Figure 1 shows the DNA products generated by PCR amplification using the primers VT1-1, VT1-2, VT2-1, VT2-2, *eae*-1 and *eae*-2 in a multiplex PCR assay

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Table 3 (*cont.*)

Family no.	Strain no.*	Serotype	Source†	Results for‡ VT1	VT2	*eae*	RAPD profile§	Phage type‖	Overall type¶
XVI	25	O157.H⁻	HUS-patient (adult)	+	+	+	B′	8	6
XVII	26	O157.H7	HUS-patient	−	+	+	B	2	3
XVIII	27a	O157.H7	HUS-patient	−	+	+	B″	49	7
	27b	O157.H7	HUS-patient	−	+	+	B″	49	7
	27c	O157.H7	HUS-patient	−	+	+	B″	49	7
	27d	O157.H7	HUS-patient	−	+	+	B″	49	7
	27e	O157.H7	HUS-patient	−	+	+	B″	49	7
	27f	O157.H7	HUS-patient	−	+	+	B″	49	7
	27g	O157.H7	HUS-patient	−	+	+	B″	49	7
	28	O157.H7	Family contact	−	+	+	B″	49	7
XIX	29	O157.H7	HUS-patient	−	+	+	B	4	2
XX	30	O157.H7	HUS-patient	−	+	+	B	2	3
	31	O157.H7	Family contact	−	+	+	B	2	3
	32	O157.H7	Family contact	−	+	+	B	2	3
XXI	33	O157.H7	HUS-patient	−	+	+	B	2	3
	34	O157.H7	Family contact	−	+	+	B	2	3
XXII	35	O157.H7	HUS-patient (BEL)	−	+	+	B″	2	8
XXIII	36a	O157.H7	HUS-patient (BEL)	−	+	+	B	2	3
	36b	O157.H7	HUS-patient (BEL)	−	+	+	B	2	3
XXIV	37	O157.H7	HUS-patient (BEL)	−	+	+	B	2	3
XXV	38	O157.H7	HUS-patient (BEL)	−	+	+	B	4	2
XXVI	39	O157.H7	HUS-patient (GER)	−	+	+	B	4	2
XXVII	40	O157.H7	HUS-patient (GER)	−	+	+	B	54	9
XXVIII	41	O157.H⁻	Gastroenteritis-patient	+	+	+	B′	8	6

* a–g represent sequentially isolated strains from the same person.
† (BEL), patient from Belgium; (GER), patient from Germany.
‡ +, positive; −, negative.
§ B′–B‴ represent similar, but slightly different DNA patterns.
‖ ND, not done.
¶ Based on VT genotype, RAPD–PCR profile and phage type.

on several strains. Amplification products were generated from positive control strains, including a 179 bp fragment for VT1-positive strains (Table 4, no. 8, 10, 11, 13, 14), a 372 bp fragment for VT2-positive strains (Table 4, no. 7, 8, 10) and a 629 bp fragment for *eae*-positive strains (Table 4, no. 7, 8, 10–14). The *E. coli* O111.H⁻ control strain no. 10 previously identified by neutralization assays as a VT2-producer, also appeared to contain VT1 gene sequences. No amplification occurred when PCR was performed with negative controls (Table 4, no. 1–6, 9). Of the 50 human O157 VTEC isolates characterized in this study, 45 were positive for the VT2 gene only and five were positive for both VT1 and VT2 genes (Table 3). Sequentially isolated O157 VTEC strains from the same patient (Table 3, no. 2a–2b, 11a–11c, 27a–27g, 36a–36b), and VTEC strains which had been isolated from different persons within one family showed the same toxin profile. Based on isolates from separate families, 21 of the 23 VT2-positive *E. coli* O157 isolates were characterized by the predominant phage types 2 and 4. The two remaining VT2-positive O157 VTEC strains (Table 3, no. 27a–28, 40) and the four O157 VTEC positive for both VT1 and VT2 were characterized by the less frequently encountered phage types 8, 14, 32, 49 and 54. All 50 O157 VTEC strains contained

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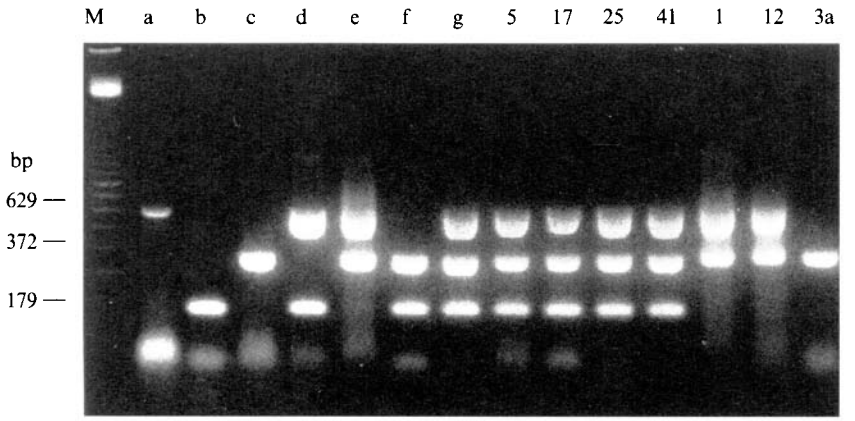

Fig. 1. Determination of VT and *eae* genes by PCR. The specific *E. coli* strains are indicated on top. a, enteropathogenic *E. coli* (O111.B4; *eae*); b, JB28 (VT1); c, CL15A (O113.H21; VT2c); d, CL13A (O145.H$^-$; VT1, *eae*); e, E32511 (O157.H$^-$; VT2c, *eae*); f. isolate from raw meat (O22.K$^-$; VT1, VT2), g, EDL931 (O157.H7; VT1+, VT2+, *eae*+). See Table 3 for details of strains no. 5–3a. VT1-positive amplification resulted in a 179 bp fragment, VT2 in a 372 bp fragment and *eae* in a 629 bp fragment. *M* = size marker (100 bp ladder).

Table 4. *Characterization of the* E. coli *control strains by serotype, verocytotoxin* (*VT*) *production, VT genotype,* E. coli *attaching and effacing* (eae) *gene and random amplified polymorphic DNA-polymerase chain reaction* (*RAPD–PCR*)

Strain no. (Source or reference no.)	Serotype	Production of VT	PCR results* for VT1	VT2	*eae*	RAPD profile
1 (chicken heart)	O157‡	–	–	–	–	F
2 (chicken meat)	O157‡	–	–	–	–	G
3 (chicken wings)	O157‡	–	–	–	–	G
4 (sausage)	O157‡	–	–	–	–	H
5 (raw chicken fillet)	O157‡	–	–	–	–	H
6 (raw turkey)	O157‡	–	–	–	–	I
7 (E32511)	O157.H$^-$	VT2c	–	+	+	B‴
8 (EDL931)	O157.H7	VT1+VT2	+	+	+	B‴
9 (–)	O157‡	–	–	–	–	J
10 (H30C5)	O111.H$^-$	VT2	+	+	+	K
11 (CL37A1)	O111.H8	VT1	+	–	+	K
12 [EPEC]†	O111.B4	–	–	–	+	L
13 (CL19)	O26.H11	VT1	+	–	+	M
14 (H19)	O26.H11	VT1	+	–	+	N

* +, positive; –, negative.
† EPEC, enteropathogenic *E. coli*.
‡ Sorbitol- and β-glucuronidase positive.

the *eae* gene sequences. The two O111.H$^-$ strains (Table 3, no. 3a, 3b) were only positive for the VT2 gene. Negative PCR results were obtained for VT1 and *eae* gene sequences.

PCR fingerprinting

PCR fingerprinting was initially performed with the 13 different primers (Table 2) on a random selection of 10 *E. coli* O157 strains in a pilot experiment. The

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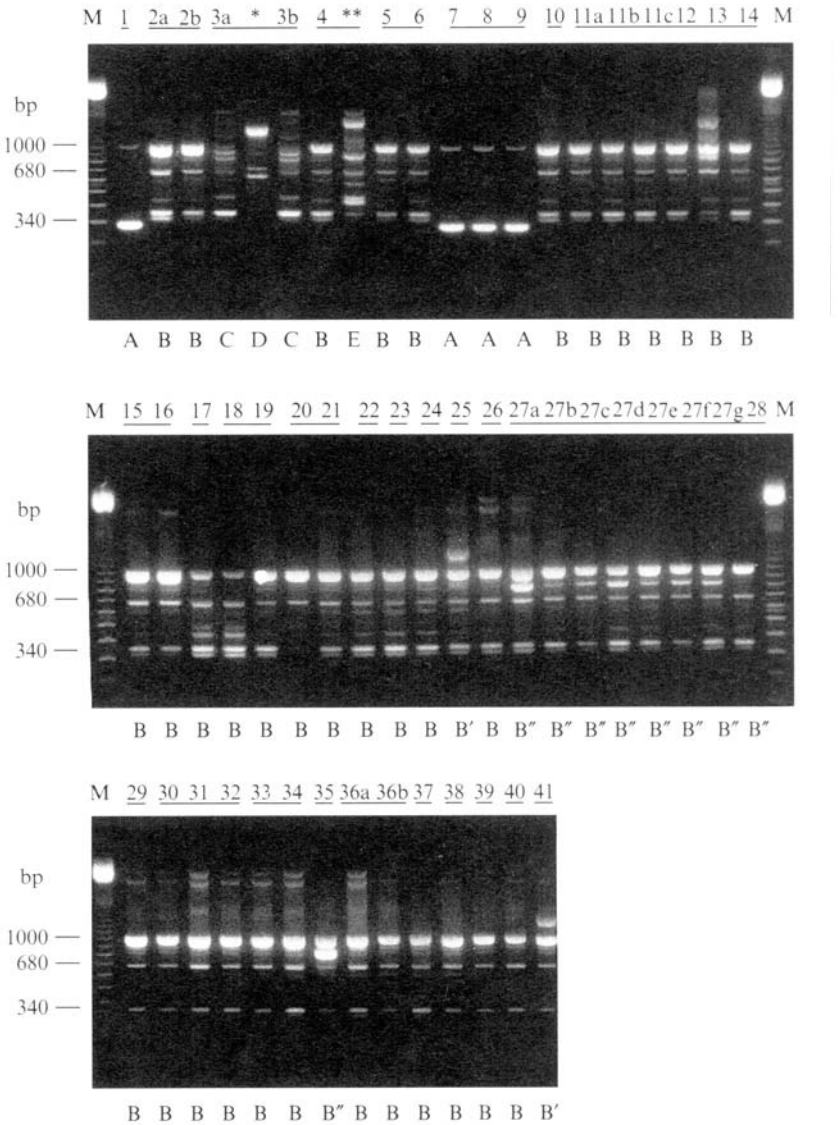

Fig. 2. Random amplified polymorphic DNA-polymerase chain reaction of human faecal verocytotoxin-producing *E. coli*. Numbers indicate the isolates and letters are RAPD–PCR profiles, as described in Table 3. Strains isolated from the same family are underlined. *M* = size marker (100 bp ladder). *, *Serratia marcescens*; ** *E. coli* O2.K$^-$.

primers were used either as a single or a pairwise combination. Primer 1247 gave the highest resolution and was therefore used for all strains listed in Tables 3 and 4. Each DNA preparation was tested in duplicate to ascertain the reproducibility of the PCR. Figures 2 and 3 show the random amplified polymorphic DNA–PCR (RAPD–PCR) profiles obtained for the 52 VTEC isolates and the 14 controls. respectively. Banding patterns were coded by letters A–N, also reported in Tables 3 and 4. Genotypic codes B–B''' represented similar but slightly different DNA banding patterns. Profile B shared four bands with the banding patterns referred

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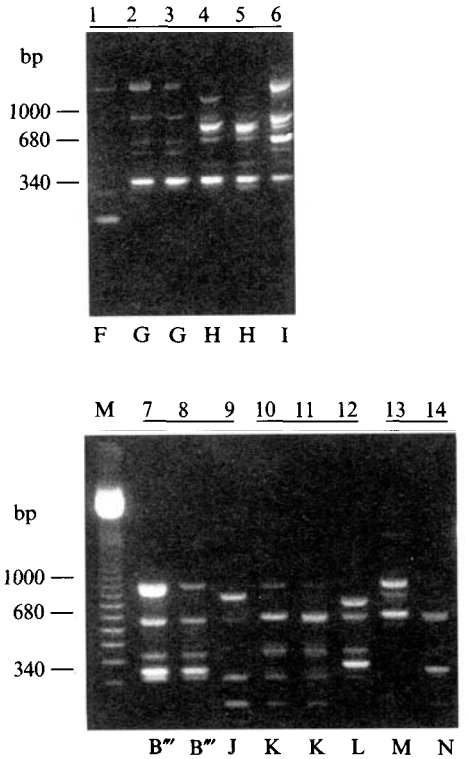

Fig. 3. Random amplified polymorphic DNA-polymerase chain reaction of the *E. coli* control strains. Numbers indicate the isolates and letters are RAPD–PCR profiles, as described in Table 4. Isolates of the same serotype are underlined. *M* = size marker (100 bp ladder).

to as B′, B″ and B‴: one major band at 1000 bp, a minor band at 680 bp and two minor bands near 340 bp. Profile B′ was characterized by a major band at 1200 bp. Whereas profiles B and B′ showed two minor bands in between 800 and 900 bp, profiles B″ and B‴ did not. Profile B″ was further characterized by one major band at 830 bp, and profile B‴ by the absence of a minor band near 600 bp. Figure 3 demonstrates that strains of different serotypes generated clearly distinct fingerprints. Furthermore, within a single serogroup different genotypes were encountered. Among the nine *E. coli* O157 isolates presented in Table 4, six distinct fingerprints were identified: the two VTEC strains no. 7 and no. 8 shared the same banding pattern, whereas for the seven VT-negative O157 strains five different patterns were obtained. The VT-negative isolate no. 12 stood apart from the VTEC isolates no. 10 and no. 11 (Table 4), though all three isolates belonged to serogroup O111. Although VTEC isolates no. 13 and 14 (Table 4) both belonged to serogroup O26, each produced distinct RAPD–PCR profiles. DNA fingerprints of the O157 VTEC strains isolated from the HUS-patients, from their relatives and from the patient with bloody enteritis showed highly monomorphic patterns (Fig. 2). Based on the fingerprint patterns, the collection of 50 isolates could be divided into four different groups: A, B, B′ and B″. Sequentially isolated O157 VTEC strains from one patient (Table 3, no. 2a–2b, 11a–11c, 27a–27g, 36a–36b), as well as strains

which had been isolated from different persons within one family showed the same banding patterns. Based on isolates from separate families, fingerprint profile B was observed for 21 of the 27 O157 VTEC isolates (79%), followed by profiles B′, B″ and A each for two (7%) of the strains. The B, B′ and B″ profiles were very similar to the B‴ profile produced by the O157 VTEC reference strains (Table 4, no. 7, 8). Some strains which were different by phage typing and VT genotyping could not be discriminated by RAPD–PCR fingerprinting, for example strains no. 4 and no. 17 (Table 3). However, some strains discriminated by RAPD–PCR fingerprinting could not be discriminated by phage typing nor by VT genotyping, for example strains no. 1 and no. 29 (Table 3). Combining the results of all typing methods used, the collection of 50 O157 VTEC strains could be divided into nine distinct groups (see the right part of Table 3). It has to be remembered that these 50 strains were isolated from 27 families. The two O111 VTEC strains (Table 3, no. 3a, 3b) showed a unique banding pattern, encoded by the letter C.

DISCUSSION

The predominant phage types identified among the O157 VTEC isolates characterized in this study correspond with the observation by Frost and co-workers [19]. Among a total of 1092 O157 VTEC strains isolated in the UK between 1989 and 1991, they identified types 2 (36·1%), 49 (29·6%), 1 (10·3%) and 4 (8·9%) the most frequently. Frost and colleagues [19] also observed that strains of phage types 2 and 49 carried only VT2 genes. It is remarkable that, unlike phage types 2 and 4, O157 VTEC phage type 49 has not been reported from North America [20].

The O157 VTEC isolates were also tested for their cytotoxicity by using the vero-cell assay (unpublished observations). Culture filtrates of all strains were neutralized by antibodies to VT2 only. PCR revealed that five of the O157 VTEC isolates contained VT1 gene sequences in addition to VT2 gene sequences. A possible explanation for the failure to detect VT1 in neutralization assays while PCR yielded positive results for the VT1 gene, might be that the VT1 gene does not become expressed. On the other hand, it has been observed that when both toxins are produced by the same strain, VT1 predominates in cell lysates and VT2 was the more active toxin in supernates [5]. Therefore, it might be possible that VT1 titres in culture supernates were too low to be detected in neutralization assays. The higher prevalence of infection among HUS-patients with VTEC producing only VT2 than infection with VTEC producing either only VT1 or a combination of VT1 and VT2, is consistent with other studies carried out in Western Europe and North-America [2, 31]. However, Rowe and co-workers [32] recently found that 22 of the 26 O157.H7 VTEC isolates from Canadian HUS-patients produced both VT1 and VT2 rather than VT2 only.

All O157 VTEC strains contained *eae*-specific DNA sequences. The presence of the *eae* gene has been closely associated with O157 VTEC that caused HUS and is assumed to be a virulence marker [33–36]. Our results strongly support this suggestion. Since spontaneous loss of VT genes during cultivation of human *E. coli* strains has been documented [37], the *eae* gene is possibly an even more reliable marker than the VT genes. The *eae* gene is responsible for intimate attachment to

and effacement of the intestinal epithelial cell microvilli, leading to damage of the epithelium [3]. This would allow the passage of bacterial products which are normally excluded from the circulation. It is not yet clear why the family members of the HUS-patients did not develop HUS, although they had been colonized with VTEC strains positive for the *eae* gene. The two *E. coli* O111 strains appeared to be negative for the *eae* gene. It is known that the *eae* gene is not always present in VTEC of serogroups other than O157 [38]. The possibility exists that the HUS-patient, from who the *E. coli* O111 strains had been isolated, was simultaneously infected with a second VTEC positive for the *eae* gene. Co-infection with two different serotypes of VTEC has been reported before [31, 39, 40].

Since *E. coli* O157.H7 and *E. coli* O157.H$^-$ are increasingly implicated in human diseases, a single and reliable method to compare these strains is needed. A good epidemiological typing scheme could assist in elucidating the route of transmission of these pathogens. Recently, PCR-based fingerprinting has been successfully used to study relatedness among epidemic isolates of various species as reviewed by Van Belkum [41]. This typing method uses a single or a pairwise combination of primers to amplify target genomic DNA by PCR, resulting in specific arrays of DNA products. Recently, Wang and co-workers applied RAPD–PCR to a limited number of O157 VTEC strains using five different arbitrary primers [42]. In contrast to Wang and colleagues we did not find the highest resolution with primer 1290, but with primer 1247. Although the isolates were obtained from different cases of HUS during a prospective study and no contact between the infected persons except for family contacts were present, genetic homogeneity in the O157 VTEC isolates was observed by RAPD–PCR fingerprinting. The collection of 50 O157 VTEC rendered only four different fingerprint patterns. Fingerprint profile B was obtained for 35 of the isolates. It was considered very similar to the patterns produced by the O157 VTEC reference strains E32511 and EDL931, which had been isolated in Britain and the USA respectively. The minor variations between the B, B′, B″ and B‴ patterns might be due to different sites of integration of phages into the bacterial chromosome. As group, fingerprint profiles B–B‴ were obtained for 27 (93%) of the 29 O157 VTEC strains tested (based on independent isolates; including the two reference strains E32511 and EDL931). It has to be emphasized that the primer with the highest resolution was used. Some O157 VTEC isolates produced a clearly different banding pattern, referred to as A. These findings suggest that the isolates were distinct from the strains producing profiles B–B‴, because it is highly unlikely that such different patterns have arisen by phage integration at a different site. The homogeneous DNA fingerprints observed in the present study for VTEC of serotypes O157.H7 and O157.H$^-$ correspond with previously reported studies on analysis of chromosomal DNA of these pathogens using multilocus enzyme electrophoresis and pulsed-field gel electrophoresis [15–17, 43]. A recent clonal expansion from a common progenitor strain has been suggested [17]. However, some authors have reported that epidemiologically independent O157 VTEC strains could be differentiated using pulsed-field gel electrophoresis or restriction fragment length polymorphism (RFLP) [44–46].

Based on the combined results of phage typing, VT genotyping and RAPD–PCR fingerprinting, the collection of 50 O157 VTEC strains (isolated from 27 families)

could be divided into nine distinct groups. Considering the phage typing and RAPD–PCR results of the present study and published data on typing of O157 VTEC, it seems likely that VTEC of serotypes O157.H7 and O157.H$^-$ are members of a single clone that has become widely distributed. Although the genomic DNA appears to be highly conserved, minor variations may occur by the integration of phages into the bacterial chromosome.

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