Determination of verocytotoxin and *eae* gene loci by multiplex PCR in *Escherichia coli* O157: H7 isolated from human faeces in Northern Ireland: a four-year study of trends, 1997–2000

J. L. D. CROTHERS, J. E. MOORE*, B. C. MILLAR, M. WATABE and P. J. ROONEY

Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast BT9 7AD, UK

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ABSTRACT

This study aims to determine the distribution and frequency of verocytotoxin genes in human faecal clinical isolates of *Escherichia coli* O157 in Northern Ireland during the period 1997–2000, using a special four-target multiplex polymerase chain reaction (PCR) assay. One hundred and thirty two isolates of *E. coli* O157:H7 cultured during the four-year period (1997 [n=28]; 1998 [n=25]); 1999 (n=43); 2000 [n=36]), representing approximately 79% of total E. coli O157 laboratory isolations throughout N. Ireland, are examined for the presence of verocytotoxin gene loci (VT1, VT2 and *eae*) using a multiplex PCR assay. These isolates originate from the four Regional Area Health Boards that constitute the healthcare system in N. Ireland as follows: Eastern (53.8%; n=71), Northern (34.1%; n=45), Western (6.8%; n=9) and Southern (5.3%; n=7). Results showed that over 80% of these isolates possessed the VT2 and *eae* gene loci, with the remainder being predominantly VT1-, VT2- and *eae*-positive. None possessed the VT1 gene locus alone. Development and adoption of this simple four-target (three virulence and one control gene loci) multiplex PCR assay and subsequent recording of resulting verocytotoxin-typing data in a database, permitted local, rapid determination of carriage of known molecular virulence determinants of *E. coli* O157 isolates, which may aid in outbreak-related epidemiological investigations or other longitudinal studies.

KEY WORDS: Escherichia coli O157. Polymerase chain reaction. Verocytotoxin.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC), the most common serotype of which is *E. coli* O157: H7, is the causative agent of infant diarrhoea, haemorrhagic colitis, thrombotic thrombocytic purpura (TTP) and haemolytic uraemic syndrome (HUS).[1] The organism's virulence is mediated through several determinants, of which toxin production is clinically one of the most important. They produce one or several powerful cytotoxins, Shiga-like toxin (SLT; also known as verocytotoxins [VT]), either VT2 or one of several VT2 variants and sometimes also VT1.[2] In addition, they have an additional gene locus, known as *eae*, which encodes an attaching and effacing protein (intimin) that enables the *E. coli* to attach intimately to the cells of the microvilli of the large intestine.[2]

While most of the isolation and diagnostic methods used in routine clinical laboratories are based on phenotypic characterisation of *E. coli* O157, polymerase chain reaction (PCR) detection methods have also been used, and a number of gene loci encoding verocytotoxin production have been developed.[3-5] As most O157 strains isolated from humans possess both verocytotoxin 1 and 2 (VT1 and 2) or verocytotoxin 2 alone, those genes have been previously sequenced and specific oligonucleotide primers designed for PCR amplification.[3-5]

Gannon *et al*.[6] reported that PCR using VT1 and VT2 primers originally applied to detect Shiga toxin-producing *E. coli* (STEC) in ground beef were used together with the DNA extracted from modified trypticase soy broth (mTSB) cultures. They demonstrated that an initial sample inoculum of as few as one colony-forming unit (cfu) of STEC per gram could be detected.

In addition, virulence factors for VTEC, such as *eae* and *hly*A genes, have also been targeted by PCR.[7] The *eae* gene that encodes intimin protein is on the locus for enterocyte effacement (LEE) pathogenicity island and involves VTEC attaching and effacing activity.[7]

Multiplex PCR has been used widely, as has simplex PCR, for the detection of *E. coli* O157 strains in foods[6] and animal faeces.[8] There have been a number of primer combinations for the multiplex PCR reported. The sets of the primers, VT1, VT2 and *eae* have been used most widely for the detection of VTEC and O157 strains. Fratamico *et al*.[8] demonstrated that multiplex PCR sensitivity was 1 cfu/g in food or bovine faeces, and results could be obtained within 24 h. Their study also demonstrated that multiplex PCR has as high a sensitivity as simplex PCR for the detection of these gene loci.

To date, there have been no reports of the distribution of VT and *eae* gene loci within the Northern Ireland *E. coli* O157 population, originating from symptomatic human patients with enteritis. Thus, the aim of this study is to

Correspondence to: Dr John E. Moore
Email: jemoore@niphl.dnet.co.uk

Table 1. Oligonucleotide primer sequences, location and size of PCR amplicons associated with individual PCR assays employed i n the determination of verocytotoxin, *eae* and 16S rDNA.

Primer	Gene target	Oligonucleotide sequence (5'–3')	Location within gene locus	Predicted size of amplified product (bp)	Reference
VT1(f)	VT1	A CTG GAT GAT CTC AGT GG	899 – 918*	601	6
VT1(r)		CTG AAT CCC CCT CCA TTA TG	1500–1481		
VT2(f)	VT2	CCA TGA CAA CGG ACA GCA GTT	714 – 734§	780	6
VT2(r)		CCT GTC AAC TGA GCA CTT TG	1493 – 1474		
eae(f)	Attaching/effacing	CG TCA CAG TTG CAG GCC TGG T	25446 – 25467†	1109	9
eae(r)		CGA AGT CTT ATC AGC CGT AAA GT	26555 – 26533		
P11P(f)	16S rDNA	GAG GAA GGT GGG GAT GAC GT	1139 – 1158‡	216	10
P13P(r)		AGG CCC GGG AAC GTA TTC AC	1354 – 1335		

* with respect to *E. coli* (GenBank Accession no. AF461166]
† with respect to *E. coli* (GenBank Accession no. AF022236)
§ with respect to *E. coli* (GenBank Accession no. AF461174)
‡ with respect to *E. coli* (GenBank Accession no. AF5278270

Table 2. Summary of distribution and frequency of verocyotoxin (VT) and *eae* gene loci in 132 clinical isolates.

	VT2	VT1 and VT2	Negative
1997	21 (70%)	6 (20%)	3 (10%)
1998	22 (88%)	2 (8%)	1 (4%)
1999	38 (86.4%)	4 (9.1%)	2 (4.5%)
2000	29 (80.5%)	6 (16.7%)	1 (2.8%)

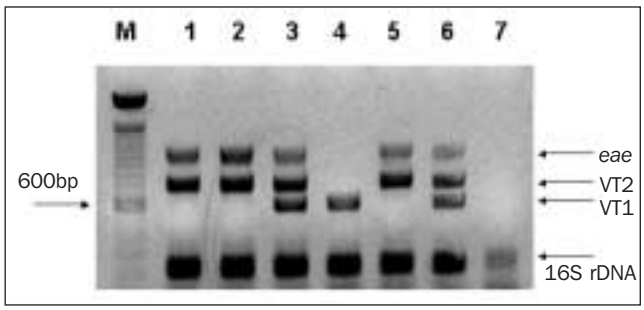

Fig. 1. Multiplex PCR amplification of verocytotoxin (VT) gene VT1 (601 bp), VT2 (780 bp; including variants) and the *eae* gene locus (1109 bp). The 16S rDNA amplicon (216 bp) represents a positive extraction control in all cultures extracted. M: 100 bp molecular weight marker; lane 1: *E. coli* O157 VT2$^+$, *eae*$^+$; lane 2: *E. coli* O157 VT2$^+$, *eae*$^+$; lane 3: *E. coli* O157 VT1$^+$, VT2$^+$, *eae*$^+$; lane 4: positive control (WT1, *E. coli* O157 VT1$^+$, *eae*$^-$); lane 5: positive control (WT2, *E. coli* O157 VT2$^+$, *eae*$^+$); lane 6: positive control (*E. coli* O157 NCTC 12079, VT1$^+$, VT2$^+$, *eae*$^+$); lane 7: negative control (molecular grade water).

determine the frequency of these gene loci in *E. coli* O157 isolates throughout N. Ireland for the period 1997–2000, using a special multiplex PCR assay for VT1, VT2 and *eae* gene loci, in addition to 16S rDNA PCR.

Materials and methods

E. coli O157 isolates (n=132) were recovered over the period 1997–2000 from faecal samples originating from human patients with enteritis submitted by general practitioners and environmental health officers in the community, as well as from in-patient hospital wards in N. Ireland. Isolates were received from all nine clinical microbiology hospital laboratories in N. Ireland and were isolated on either tellurite-cefixime sorbitol MacConkey agar (TC-SMAC; Oxoid CM0813 plus Oxoid SR0172, Oxoid, Basingstoke, UK) or sorbitol MacConkey agar (SMAC; Oxoid CM0813), depending on the individual laboratory's protocol.

Isolates were confirmed as *E. coli* using the API 20E identification scheme (bioMérieux, Les Halles, France) and were confirmed as serotype O157:H7 with specific antisera and a latex agglutination method (17PL071; Prolab Diagnostics, Canada). Three control strains of *E. coli* were used and included WT1 (E. coli O128; VT1-positive, *eae*-negative), WT2 (*E. coli* O157; VT2- and *eae*-positive) and *E. coli* O157 NCTC 12079 (VT1-, VT2- and *eae*-positive). All isolates were stored at –80°C at the Northern Ireland Public Health Laboratory, Belfast City Hospital.

Isolates were grown in pure culture on Columbia agar base (Oxoid CM 0331) supplemented with 5% (v/v) defibrinated horse blood at 37°C for 24 h. Using a 2 mm plastic loop, a colony was picked off and emulsified in 500 µL 1 x TAE (0.04 mol/L Tris acetate [pH 8.3], 0.001 mol/L EDTA) buffer in a 1.5 mL Eppendorf microfuge tube and was placed in a boiling waterbath for 10 min and used within 24 h for PCR amplification. All positive control isolates were extracted using the High Purity PCR Template DNA extraction kit (Roche Diagnostics, Lewes, UK) in accordance with the manufacturer's instructions, and were stored at 4°C.

A multiplex PCR assay was designed to co-amplify the VT1, VT2 and *eae* gene loci concurrently, as well as a partial region of the 16S rDNA gene locus, based on pre-existing primer sequence information. The nucleotide sequence of each primer (Applied Biosystems, Warrington, England) and corresponding locations within the VT1, VT2 and *eae* gene loci are shown in Table 1. The conserved primers (P11P and P13P; Table 1) were used to amplify a highly conserved

Table 3a. Detailed breakdown of phenotypic and genotypic isolate characteristics of human clinical isolates of *E. coli* 0157 for 1997.

Isolate reference*	Sex§	Age	Isolation date	API 20E profile	Verotoxin gene	*eae* gene	Phagetype
E97/1N	M	2½y	03/03/97	5144172	VT2	Pos	49
E97/2N	F	1y	06/03/97	5144172	VT2	Pos	49
E97/3N	M	4y	07/03/97	5144172	VT2	Pos	49
E97/4N	M	57½y	02/05/97	5144172	VT2	Pos	14
E97/5N	F	22y	09/05/97	5144172	VT2	Pos	14
E97/6E	F	54y	07/05/97	5144172	VT2	Pos	14
E97/7E	F	25½y	17/05/97	5144172	VT2	Pos	32/14
E97/8E	M	Adult	22/05/97	5144162	Negative	Pos	32
E97/9E	F	4m	23/05/97	5144152	VT2	Pos	14
E97/10N	F	36y	31/05/97	5144172	VT2	Pos	32
E97/11E	F	27y	06/06/97	5144172	VT2	Pos	2
E97/12E	F	34y	10/06/97	5144172	VT1&VT2	Pos	14
E97/13E	F	7½y	12/06/97	5144172	VT2	Pos	49
E97/14E	F	4y	05/08/97	5144172	VT2	Pos	8
E97/15E	F	30y	21/08/97	5144172	VT1&VT2	Pos	8
E97/16E	F	2y	26/08/97	5144172	VT2	Pos	49
E97/17S	F	66y	21/08/97	5144162	VT2	Pos	32
E97/18S	M	78y	19/08/97	5144172	VT2	Pos	2
E97/19E	F	49y	29/08/97	5144172	VT1&VT2	Pos	14
E97/20E	M	1y	04/09/97	5144172	VT2	Pos	RDNC*
E97/21E	F	50y	12/09/97	5144162	VT2	Pos	21
E97/22N	M	8y	12/09/97	5144172	VT2	Pos	14
E97/23N	F	38y	20/09/97	5144162	VT2	Pos	21
E97/24E	M	8m	06/10/97	5144572	Negative	Pos	RDNC
E97/25N	F	2y	08/10/97	5144172	VT1&VT2	Pos	14
E97/26N	F	10m	11/10/97	5144172	VT1&VT2	Pos	14
E97/27N	F	1½y	21/10/97	5144172	VT1&VT2	Pos	14
E97/28S	M	42y	28/10/97	5144142	VT2	Pos	32
E97/29S	M	91y	03/11/97	5144142	VT2	Pos	32
E97/30E	M	31y	08/12/97	5044512	Negative	Neg	RDNC

Isolate reference is suffixed with geographical originator code defining Area Health Board location:
(E) Eastern, (N) Northern, (S) Southern, (W) Western. UN1: bovine source. UN2: effluent source. ND: not determined.

partial region of the 16S rRNA gene. This amplification was included to verify the presence of target DNA following extraction from the pure culture.

Amplification of bacterial DNA was carried out in 25 µL volumes containing 5.0 mmol/L $MgCl_2$, 10 µmol/L Tris hydrochloride (pH 8.3), 50 mmol/L KCl, 1 µL template DNA, 0.2 mmol/L (each) dATP, dGTP, dCTP and dTTP (Pharmacia Biotech, Germany), 0.15 µmol each primer and 2.5 units *Thermus aquaticus (Taq)* DNA polymerase (Stoffel fragment; Applied Biosystems). Following empirical optimisation, PCR amplifications were carried out in a DNA thermal cycler (BioRad, California, USA) consisting of one cycle of 3 min at 96°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C, with a final extension of 5 min at 72°C.

Negative controls (1µL molecular grade water; Biowhittaker, Walkersville, Maryland, USA) and positive controls (1 µL extracted genomic DNA from the three control strains used) were included in each run.

Following amplification, 15 µL amounts were removed from each reaction mixture and examined by electrophoresis (80 V, 45 min) in gels composed of 1% (w/v) agarose (Gibco, UK) in TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]), stained with ethidium bromide (5 µg/100 mL). Gels were visualised under ultraviolet (UV) illumination using a gel image analysis system (UVP Products, England) and all images were archived as digital graphic files (*.bmp).

All isolates were phagetyped as previously described.[11]

Results

One hundred and thirty-two isolates of *E. coli* O157 from the four-year period 1997–2000 (1997 [n=28], 1998 [n=25], 1999 [n=43], 2000 [n=36]) were received and examined as described above. These represented 79% of total provincial *E. coli* O157 reported to the Communicable Disease Surveillance Centre (CDSC, N. Ireland),[12] with the remaining

Table 3b. Detailed breakdown of phenotypic and genotypic isolate characteristics of human clinical isolates of *E. coli* 0157 for 1998.

Isolate reference	Sex	Age	Isolation date	API 20E profile	Verotoxin gene	*eae* gene	Phagetype
E98/1W	M	2y	06/02/98	5144172	VT2	Pos	49
E98/2E	M	73y	22/02/98	5144142	Negative	Pos	34
E98/3E	F	79y	10/05/98	5144162	VT2	Pos	32
E98/4N	M	77y	23/05/98	5144142	VT2	Pos	32
E98/5N	F	84y	27/05/98	5144142	VT2	Pos	32
E98/6E	M	46y	10/06/98	5144172	VT1&VT2	Pos	8
E98/7W	F	1y	07/07/98	5144172	VT2	Pos	32
E98/8N	F	1y	22/07/98	5144162	VT2	Pos	32
E98/9E	M	12½y	29/07/98	5144142	VT2	Pos	14
E98/10E	F	9y	29/07/98	5144142	VT2	Pos	14
E98/11N	M	17y	03/08/98	5144162	VT2	Pos	32
E98/12S	F	63y	05/08/98	ND	VT2	Pos	14
E98/13E	M	6m	27/08/98	5144172	VT2	Pos	49
E9814E	F	4½y	30/08/98	5144172	VT2	Pos	49
E98/15E	M	2y	30/08/98	5144172	VT2	Pos	49
E98/16E	F	35y	31/08/98	5144172	VT2	Pos	49
E98/17N	F	3y	29/08/98	5144162	VT2	Pos	32
E98/18E	UN1	UN1	01/09/98	5144172	VT2	Pos	49
E98/19E	F	56y	05/09/98	5144172	VT1&VT2	Pos	14
E98/20E	F	1y	07/09/98	5144162	VT2	Pos	32
E98/21E	M	3y	06/11/98	5144162	VT2	Pos	32
E98/22N	F	1y	01/12/98	5144162	VT2	Pos	32
E98/23E	M	2½y	26/12/98	5144162	VT2	Pos	21/28
E98/24E	M	12y	31/12/98	5144162	VT2	Pos	21/28
E98/25W	F	11m	28/08/98	ND	VT2	Pos	32
E98/26W	F	1½y	22/10/98	ND	VT2	Pos	14

Isolate reference is suffixed with geographical originator code defining Area Health Board location:
(E) Eastern, (N) Northern, (S) Southern, (W) Western. UN1: bovine source. UN2: effluent source. ND: not determined.

35 (21%) *E. coli* O157 isolates not referred for verocytotoxin typing. These isolates originated from the four Regional Area Health Boards that constitute the healthcare system in Northern Ireland in the following proportions: Eastern 53.8% (n=71), Northern 34.1% (n=45), Western 6.8% (n=9) and Southern 5.3% (n=7).

The multiplex assay proved successful for the co-amplification of the VT1, VT2 and *eae* gene loci, as well as of the 16S rDNA partial fragment, from both purified cultures and controls in a single master mix (Fig. 1), and was able to determine the distribution and frequency of carriage of these virulence determinants (Table 2 [summary]; and Tables 3a–d [yearly breakdown]). In addition, Tables 3a–d also detail the API 20E profiles and phagetypes obtained for all the isolates examined.

During this period (1997–2000), no isolate demonstrated the sole presence of the VT1 gene locus. The association between the presence of virulence (VT1, VT2 and *eae*) gene loci and phagetype is shown in Table 4.

Six API 20E profiles were recorded for the *E. coli* O157 isolates examined and the frequency of these and their homology scores are detailed in Table 5. Although there was a biochemical variation in the individual assays that constitute the API 20E scheme, all isolates were identified as *E. coli* by this method. Of these, the majority (84.4%) of all isolates gave one of two profiles (5144162 or 51441720 and there was no association between API profile and phagetype.

Discussion

Verocytotoxigenic *E. coli* O157 emerged as an important gastrointestinal bacterial pathogen in the late 1990s, due to the morbidity and mortality associated with it, which has led to the description of the disease in most developed countries.[1] To date, there have been no reports in the literature of the disease in N. Ireland. Therefore, this study examined the carriage of associated virulence gene loci in *E. coli* O157 in the N. Ireland population, using a special multiplex PCR approach, and compared the results with phagetyping data.

Up to 1996, there were less than 10 laboratory reports of *E. coli* O157 in N. Ireland each year. However, with the local introduction of Advisory Committee on the Microbiological Safety of Food (ACMSF) recommendations that all stool samples should be tested for *E. coli* O157, laboratory reports began to increase. In recent years (1999–2000), the average annual figure has increased to 54 reports, equating to an attack rate of 3.21 cases/100,000 population. The greatest

Table 3c. Detailed breakdown of phenotypic and genotypic isolate characteristics of human clinical isolates of *E. coli* 0157 for 1999.

Isolate reference	Sex	Age†	Isolation date	API 20E profile	Verotoxin gene	*eae* gene	Phagetype
E99/1N	F	6m	11/01/99	5144162	VT2	Pos	32
E99/2N	F	2y	14/01/99	5144162	VT2	Pos	32
E99/3N	F	3y	14/01/99	5144162	VT2	Pos	32
E99/4N	F	35y	20/01/99	5144162	VT2	Pos	32
E99/5N	M	5y	22/01/99	5144162	VT2	Pos	32
E99/6E	F	9y	28/01/99	5144162	VT2	Pos	32
E99/7E	M	39y	28/01/99	5144162	VT2	Pos	32
E99/8E	M	14m	28/01/99	5144162	VT2	Pos	32
E99/9E	M	5y	28/01/99	5144162	VT2	Pos	32
E99/10E	F	35y	28/01/99	5144162	VT2	Pos	32
E99/11E	F	8y	28/01/99	5144162	VT2	Pos	32
E99/12E	F	2y	31/01/99	5144162	VT2	Pos	32
E99/13E	F	1y	31/01/99	5144162	VT2	Pos	32
E99/14E	M	34y	31/01/99	5144162	VT2	Pos	32
E99/15E	F	5y	31/01/99	5144162	VT2	Pos	32
E99/16E	M	10y	31/01/99	5144162	VT2	Pos	32
E99/17E	M	3y	05/02/99	5144162	VT2	Pos	32
E99/18N	M	3y	03/03/99	5044512	Negative	Pos	RDNC
E99/19N	M	9y	25/02/99	5144162	VT2	Pos	21/28
E99/20N	M	7m	10/05/99	5144142	VT2	Pos	32
E99/21E	F	14y	21/06/99	5144172	VT2	Pos	RDNC
E99/22N	F	35y	02/07/99	5144172	VT2	Pos	21/28
E99/23E	M	4y	05/07/99	5144142	VT2	Pos	21/28
E99/24E	M	3y	05/07/99	5144142	VT2	Pos	21/28
E99/25E	M	32y	07/07/99	5144142	VT2	Pos	21/28
E99/26E	F	22y	15/07/99	5144172	VT2	Pos	47
E99/27N	M	18y	16/07/99	5144172	VT2	Pos	32
E99/28E	F	37y	16/07/99	5144172	VT2	Pos	47
E99/29N	M	47y	20/07/99	5144172	VT2	Pos	32
E99/30N	F	53y	22/07/99	5144172	VT2	Pos	32
E99/31E	M	69y	11/08/99	5144172	VT1&VT2	Pos	32
E99/32S	M	43y	10/08/99	5144172	VT2	Pos	8
E99/33S	F	43y	13/08/99	5144172	VT2	Pos	RDNC
E99/34N	M	69y	13/08/99	5144172	VT1&VT2	Pos	32
E99/35N	M	2y	19/08/99	5144172	VT2	Pos	32
E99/36N	M	33y	02/09/99	5144172	VT2	Pos	2
E99/37N	F	65y	07/09/99	5144172	VT2	Pos	49
E99/38N	M	5y	15/09/99	5144172	VT1&VT2	Pos	14
E99/39E	M	44y	20/09/99	5144172	VT2	Pos	32
E99/40E	F	43y	27/09/99	5144142	VT2	Pos	32
E99/41E	M	43y	06/10/99	5144172	Negative	Pos	8
E99/42E	F	16y	20/11/99	5144172	VT2	Pos	2
E99/43W	F	2y	23/11/99	5144172	VT1&VT2	Pos	1
E99/44N	F	63y	21/01/99	ND	VT2	Pos	8

Isolate reference is suffixed with geographical originator code defining Area Health Board location:
(E) Eastern, (N) Northern, (S) Southern, (W) Western. UN1: bovine source. UN2: effluent source. ND: not determined.

prevalence is seen in children and babies aged between one and four years (16.6 cases/100,000 population[12]), with the majority of such cases being sporadic in nature. Furthermore, we examined all isolates reported in 2000 from across N. Ireland, but were unable to demonstrate the co-existence of any other faecal pathogen in O157-positive faeces.

Following the *E. coli* O157 outbreak in Wishaw in Scotland during November and December, 1996, the Chief Medical

Table 3d. Detailed breakdown of phenotypic and genotypic isolate characteristics of human clinical isolates of *E. coli* 0157 for 2000.

Isolate reference	Sex	Age	Isolation date	API 20E profile	Verotoxin gene	*eae* gene	Phagetype
E00/1N	F	1½y	17/01/00	5144162	VT2	Pos	21/28
E00/2N	F	35y	10/01/00	5144162	VT2	Pos	21/28
E00/3N	M	4½y	18/01/00	5144162	VT2	Pos	21/28
E00/4E	F	2½y	24/03/00	5144162	VT2	Pos	21/28
E00/5E	M	2½y	27/03/00	5144162	VT2	Pos	21/28
E00/6E	F	5m	31/03/00	5144162	VT2	Pos	21/28
E00/7E	F	37½y	03/04/00	5144162	VT2	Pos	21/28
E00/8E	M	3ly	03/04/00	5144162	VT2	Pos	21/28
E00/9N	M	37½y	01/04/00	5144162	VT2	Pos	21/28
E00/10N	F	35½y	01/04/00	5144162	VT2	Pos	21/28
E00/11N	M	2½y	01/04/00	5144162	VT2	Pos	21/28
E00/12E	UN2	UN2	15/04/00	5144162	VT2	Pos	21/28
E00/13E	F	2½y	17/04/00	5144162	VT2	Pos	32
E00/14W	M	1y	18/05/00	5144162	VT2	Pos	32
E00/I5E	F	1½y	23/05/00	5144162	VT2	Pos	32
E00/16E	F	31y	23/05/00	5144162	VT2	Pos	32
E00/17E	M	60y	16/06/00	5144172	VT2	Pos	14
E00/18E	F	Ly	19/06/00	5144162	VT2	Pos	4
E00/19N	M	32y	13/07/00	5144172	VTI & VT2	Pos	8
E00/20W	M	1y	17/07/00	5144172	VT2	Pos	32
E00/21E	F	54y	26/07/00	5144172	VT1 & VT2	Pos	33
E00/22E	M	2y	03/08/00	5144162	VT2	Pos	21/28
E00/23N	M	1y	08/08/00	5144142	VT2	Pos	32
E00/24E	F	27y	19/08/00	5144172	VT2	Pos	4
E00/25N	F	35y	16/08/00	5144162	VT2	Pos	32
E00/26E	F	25y	06/09/00	5144172	VT1 & VT2	Pos	32
E00/27E	M	6y	04/10/00	5144162	VT2	Pos	21/28
E00/28E	F	43y	09/10/00	5144162	VT2	Pos	21/28
E00/29E	F	9y	10/10/00	5144162	VT2	Pos	21/28
E00/30E	F	30y	10/10/00	5144162	VT2	Pos	21/28
E00/31E	M	29y	16/10/00	5144142	VT2	Pos	21/28
E00/32N	M	2½y	18/10/00	5144172	VT1 & VT2	Pos	14
E00/33N	M	42y	21/10/00	5144172	VT1 & VT2	Pos	14
E00/34E	F	27½y	23/10/00	5144162	VT2	Pos	32
E00/35E	F	2y	25/10/00	5144162	VT2	Pos	32
E00/36W	F	57y	13/11/00	5144172	VT1 & VT2	Pos	8
E00/37W	F	1y	07/12/00	5144172	Negative	Pos	RDNC

Isolate reference is suffixed with geographical originator code defining Area Health Board location:
(E) Eastern, (N) Northern, (S) Southern, (W) Western. UN1: bovine source. UN2: effluent source. ND: not determined.

Officer in Northern Ireland published a report entitled *Review of Communicable Disease Control in Northern Ireland*, which recognised the need for a rapid and local real-time laboratory response to outbreak control (5.6 – *Development of molecular epidemiology*). Furthermore, this report detailed that the development and introduction into routine practice of modern molecular diagnostics, including *E. coli* O157, would also be supported.[13]

In response to this, an in-house multiplex PCR assay was developed, based on previously published assays for the detection of VT1, VT2 and *eae* gene loci.[6, 9] In addition to these, a fourth molecular target gene (a partial region of the 16S rRNA gene locus) was also included to act as a positive control for the DNA extraction stage from the pure culture.

As 'time-to-detection' was an important criterion in the design of the assay, a simple DNA extraction protocol was introduced, based on boiling of the isolate being examined in pure culture. Hence, it was important to ensure that sufficient quantity and quality of DNA was extracted that was free from any intrinsic PCR inhibitor(s) and that this bacterial genomic DNA was made available for the downstream PCR amplification stage.

Table 4. Association between presence of virulence (VT1, VT2 and *eae*) gene loci and phagetype of *E. coli* O157.

Phagetype	VT2	VT1 & VT2	Negative
1	0	1	0
2	4	0	0
4	2	0	0
8	2	4	1
14	10	9	0
21	2	0	0
21/28	24	0	0
32	48	3	1
33	0	1	0
34	0	0	1
34/14	1	0	0
47	2	0	0
49	11	0	0
RDNC*	3	0	4

* RDNC: reacts with but does not conform to standard phage patterns.

Table 5. Variation and frequency in API 20E scores of *E. coli* O157 isolates examined.

API 20E profile	% score	Frequency (% total isolates)
5144162	95.7	55 (42.0)
5144172	89.6	53 (40.5)
5144142	96.3	14 (10.7)
5144152	97.7	1 (0.75)
5144572	99.5	1 (0.75)
5044512	85.5	2 (1.5)
5154172	99.5	3 (2.3)
5044172	98.8	2 (1.5)

A universal or 'broad-range' set of highly conserved oligonucleotide primers was employed,[10] which targeted a 216 bp region of the 16S rRNA *E. coli* gene. Thus, any subsequent multiplex PCR reaction that failed to yield a 216 bp amplicon was repeated and the results voided until a positive 16S rDNA signal was obtained.

Subsequent analysis of most *E. coli* O157 isolates referred to the Northern Ireland Public Health Laboratory from the primary hospital diagnostic laboratories across N. Ireland has largely demonstrated that over 80% of these isolates possess the VT2 and *eae* gene loci, with the remainder being predominantly VT1-, VT2- and *eae*-positive.

In conclusion, development and adoption of this simple four-target (three virulence and one control gene loci) multiplex assay and subsequent recording of verocytotoxin typing data in a database has permitted local, rapid determination of the carriage of known molecular virulence determinants of human *E. coli* O157 isolates. Comparison of local human verocytotoxin typing data similar to local food and animal data should be encouraged and may aid in outbreak-related epidemiological investigations or other longitudinal studies.

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