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Subtyping of virulence genes in verocytotoxin-producing *Escherichia coli* (VTEC) other than serogroup O157 associated with disease in the United Kingdom

C. Jenkins,[1] G. A. Willshaw,[1] J. Evans,[2] T. Cheasty,[1] H. Chart,[1] D. J. Shaw,[3] G. Dougan,[4] G. Frankel[4] and H. R. Smith[1]

Correspondence
C. Jenkins
claire.jenkins@hpa.org.uk

[1]Laboratory of Enteric Pathogens, Central Public Health Laboratory, Specialist and Reference Microbiology Division, Health Protection Agency, 61 Colindale Avenue, London, NW9 5HT, UK

[2]Scottish Agricultural College, Veterinary Science Division, Drummondhill, Stratherrick Rd, Inverness, IV2 4JZ, UK

[3]Centre for Tropical and Veterinary Medicine, University of Edinburgh, Roslin, Midlothian, EH25 9RG, UK

[4]Centre for Molecular Microbiology and Immunology, Flowers Building, Imperial College, London, SW7 2AZ, UK

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Verocytotoxin-producing *Escherichia coli* (VTEC) causes a wide spectrum of disease in humans, from mild diarrhoea to haemolytic uraemic syndrome (HUS). The verocytotoxin (*vtx*) and intimin (*eae*) genes of VTEC strains, other than those of serogroup O157, were subtyped to identify common properties that may be associated with increased pathogenicity. Strains were isolated from patients with HUS, those with diarrhoea or from asymptomatic individuals. Strains of VTEC that carried vtx_2 gene subtypes vtx_2 and vtx_{2c} were most commonly associated with HUS, whereas strains from patients with less severe disease and from the healthy control group were more likely to have vtx_{1c} or vtx_{2d} genes. The *eae* gene was detected more frequently in strains isolated from HUS patients than in those associated with cases of diarrhoea; β-intimin was the most common intimin subtype in strains isolated from both groups of patients. None of the strains from the healthy control group carried the *eae* gene.

INTRODUCTION

Verocytotoxin-producing *Escherichia coli* (VTEC), also known as Shiga toxin-producing *E. coli* (STEC), causes a wide spectrum of disease in humans, ranging from mild diarrhoea to severe diseases such as haemorrhagic colitis and haemolytic uraemic syndrome (HUS) (Karmali, 1989). Strains of VTEC that belong to serotype O157 : H7 and O157 non-motile (H−) strains are most commonly associated with human disease in the UK (Willshaw *et al.*, 2001). However, this reflects the fact that tests to detect non-O157 VTEC are not routinely available in most laboratories (Subcommittee of the PHLS Advisory Committee on Gastrointestinal Infections, 2000). Infections with non-O157 VTEC, such as those that belong to serogroups O26, O103, O111 and O145, are detected more frequently in countries where tests for VTEC include O157 and non-O157 serogroups (Paton *et al.*, 1996; Caprioli *et al.*, 1997; Schmidt *et al.*, 1999).

The capacity of VTEC to cause disease in humans is associated with its ability to express verocytotoxin (VT) (Melton-Celsa & O'Brien, 1998) and to form attaching and effacing lesions in the intestine (Frankel *et al.*, 1998). Certain strains of VTEC produce an enterohaemolysin that may also contribute to VTEC pathogenicity (Schmidt *et al.*, 1995). There are two major types of VT (VT1 and VT2) and VT2 can be divided into at least five subtypes [VT2, VT2c, VT2d, VT2e and VT2f (Scheutz *et al.*, 2001)]. In this paper, the VT2d subtype we refer to is that described by Piérard *et al.* (1998), not the 'activatable' VT2d toxin described by Melton-Celsa & O'Brien (1998). There are a number of different methods for differentiation of these subtypes (Bastian *et al.*, 1998) and the nomenclature used to describe them is currently under review (Scheutz *et al.*, 2001). VT1 genes (vtx_1) appear to be more homogeneous than vtx_2,

Abbreviations: HUS, haemolytic uraemic syndrome; LEP, Laboratory of Enteric Pathogens, London, UK; STEC, Shiga toxin-producing *Escherichia coli*; VT, verocytotoxin; VTEC, verocytotoxin-producing *E. coli*.

although variants have been described (Paton *et al.*, 1995; Koch *et al.*, 2001; Zhang *et al.*, 2002a). The variant vtx_1 gene, $vtx_{1_{OX3}}$ or vtx_{1c}, was initially described in VTEC strains that were isolated from sheep (Paton *et al.*, 1995) and was termed $vtx_{1_{OX3}}$, but has also been detected in human strains (Koch *et al.*, 2001; Zhang *et al.*, 2002a). Zhang *et al.* (2002a) have designated this variant VT1c, in keeping with the nomenclature applied to the VT2 variants.

The locus of enterocyte effacement (LEE) is located on the chromosome and contains genes that encode proteins required for the attaching/effacing phenotype. These include the *eae* gene, which encodes intimin, an outer-membrane protein that mediates close contact between bacteria and the target cell (Frankel *et al.*, 1998). There are five well-established intimin types: α, β, δ, γ and ε (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000); other types have also been described, including ζ (Tarr & Whittam, 2002; Zhang *et al.*, 2002b), θ (Tarr & Whittam, 2002), η, κ and ι (Zhang *et al.*, 2002b).

The aims of this study were to subtype the vtx_1, vtx_2 and *eae* genes carried by strains of non-O157 VTEC isolated from patients with diarrhoeal disease and from individuals in a healthy control group and to try to identify common properties in strains associated with HUS patients, compared to those isolated from patients with diarrhoea who did not develop HUS and from asymptomatic individuals.

METHODS

Bacterial strains. Seventy-seven strains of VTEC [28 from patients with HUS (group 1), 34 from patients with diarrhoea who did not develop HUS (group 2) and 15 from a healthy control group (group 3)] were obtained from the culture collection of the Laboratory of Enteric Pathogens (LEP), London, UK. Strains from groups 1 and 2 were isolated between 1983 and 2000 from faecal samples sent for tests for VTEC, either at the LEP or at hospital laboratories in the UK that sent samples to the LEP for confirmation and further tests (Scotland *et al.*, 1988; Kleanthous *et al.*, 1990; Willshaw *et al.*, 1992, 2001; Thomas *et al.*, 1994). Strains selected for this study were isolated from people who were resident in the UK and had not travelled abroad recently, as the aim was to characterize VTEC associated with human disease in the UK. Therefore, all strains of non-O157 VTEC in the LEP collection were analysed, with the exception of isolates sent from abroad. Strains from the healthy control group were isolated during a study on Infectious Intestinal Disease in England (Evans *et al.*, 2002). Forty-two of the strains were isolated from children (i.e. individuals younger than 16 years) and 24 strains were isolated from adults (i.e. individuals of 16 years and above). Data on the ages of the 11 remaining subjects were unavailable.

Control strains used in PCR and PCR-RFLP tests for subtyping of the vtx_1 and vtx_2 genes were: VT1, E3787 (O26 : H11) (Smith & Linggood, 1971); VT1c, E105991 (O26 : H11) (this study); VT2, EDL933 (O157 : H7) (O'Brien *et al.*, 1984); VT2c, E32511 (O157 : H−) (Schmitt *et al.*, 1991); VT2d, E25702 (O118 : H12) (Willshaw *et al.*, 1992); VT2e, E88949 (O9ab : H−) (Thomas *et al.*, 1994); and VT2f, E75191 (O128 : H−) (Gannon *et al.*, 1990). Control strains used in the tests for subtyping of intimin genes included: α-intimin, E2347 (O127 : H6) (Cravioto *et al.*, 1979); β-intimin, E3787 (O26 : H11) (Smith & Linggood, 1971); γ-intimin, E32511 (O157 : H−) (O'Brien *et al.*, 1984); θ-intimin, E52849 (O111 : H−) (Willshaw *et al.*, 1992); δ-intimin, ICC95 (O86 : H34) (Adu-Bobie *et al.*, 1998); ε-intimin, E5276 (O103 : H2) (Dorn *et al.*, 1989); and ζ-intimin, E147779 (E7477 : H25, a provisional new serotype) (Jenkins *et al.*, 2002).

Serotyping. Each isolate was serotyped by using the LEP serotyping scheme, which depends on identification of the heat-stable LPS somatic or 'O' antigens and the flagella 'H' antigen (Gross & Rowe, 1985). Strains that could not be serogrouped by using the serotyping scheme described above, i.e. those designated 'O?' and 'O rough', were typed by using PCR-RFLP as described by Coimbra *et al.* (2000). Rough strains do not express the 'O' antigen and therefore cannot be typed by using a phenotypic serotyping scheme.

Typing of *vtx* and intimin genes and detection of VTEC enterohaemolysin (*ehxA*). VT typing was carried out by using digoxigenin-labelled DNA probes for vtx_1 and vtx_2 (Thomas *et al.*, 1991). The vtx_1 and vtx_2 genes were subtyped by PCR with the following primer pairs: VT1c, Stx1c-1/Stx1c-2 (Zhang *et al.*, 2002); VT2 (subtype), P2/PVT2 (Thomas *et al.*, 1994); VT2c, P2/PVT2vh (Thomas *et al.*, 1994); VT2d, VT2-cm/VT2-f (Piérard *et al.*, 1998); VT2e, P2/PVT2e (Thomas *et al.*, 1994); and VT2f, 128-1/128-2 (Schmidt *et al.*, 2000). *vtx* genes that could not be typed by using these primer pairs were analysed by using the PCR-RFLP method described by Lin *et al.* (1993).

The *eae* gene and intimin subtypes α, β, γ and ε were detected by using PCR (Oswald *et al.*, 2000). Intimin genes that could not be subtyped by PCR were identified by using the PCR-RFLP method described by Oswald *et al.* (2000). VTEC enterohaemolysin (*ehxA*) genes were detected by using PCR (Schmidt *et al.*, 1995).

Statistical analysis. The distributions of each of the *vtx* variant genes, the *eae* gene and the *ehxA* genes between patients with HUS and individuals who did not develop HUS, i.e. patients with diarrhoea and asymptomatic subjects, were determined in order to identify genes associated with HUS. Fisher's exact tests were performed to test the significance of the association between detection of particular genes and disease symptoms. A χ^2 test of association was performed to assess the relationship between age-group and disease symptoms. In all cases, a P value of $< 0{\cdot}05$ was taken to indicate significance.

RESULTS AND DISCUSSION

Age of patients and asymptomatic individuals

Twenty-two (84 %) of the 26 VTEC strains isolated from patients with HUS (group 1) where the age of the patient was known were from children and four (15 %) were isolated from adults, whereas in group 2 (patients with diarrhoea who did not develop HUS), 18 (69 %) of 26 VTEC isolates were from children and eight (32 %) were from adults. In group 3, there were two children (13 %) and 12 adults (80 %). These data showed a significant association between age-group and development of HUS [$\chi^2 = 18{\cdot}076$, degrees of freedom (df) $= 2$, $P < 0{\cdot}001$].

Serotyping

In all three groups of strains, 33 different recognized serotypes were identified and 14 strains were designated 'O?' or 'O rough' (Tables 1–3). VTEC of serotypes O145 and O26 were isolated most frequently from groups 1 and 2, respectively. Outside the UK, USA and Canada, the most frequently reported non-O157 VTEC serotypes have been O26 : H11/H−, O103 : H2, O111 : H−and O145 : H− (Paton

Table 1. Strains of non-O157 VTEC isolated from HUS patients

No. strains isolated in parentheses. Abbreviations: A, adult; C, child; NK, not known; NT, not tested (test not appropriate). References: Scotland *et al.* (1988), Kleanthous *et al.* (1990), Willshaw *et al.* (1992, 2001), Thomas *et al.* (1994).

Serotype (28)	*rfb* pattern	Adult/child	*vtx* type	vtx_1 subtype	vtx_2 subtype	*eae* gene (subtype)	*ehxA* gene
O5 : H− (1)	NT	C	1	1	NT	+ (β)	+
O9ab : H− (1)*	NT	A	2	NT	2e	−	−
O26 : H11 (2)	NT	C	1	1	NT	+ (β)	+
O26 : H11 (1)	NT	A	1	1	NT	+ (β)	+
O55 : H7 (1)	NT	C	2	NT	2c	+ (γ)	−
O55 : H10 (1)	NT	C	2	NT	2	−	−
O101 : H− (1)*	NT	A	2	NT	2e	−	−
O104 : H2 (1)	NT	C	2	NT	2c	−	−
O105ac : H18 (1)	NT	C	1+2	1	2	−	+
O111ac : H− (1)	NT	C	1+2	1	2	+ (θ)	+
O115 : H10 (1)	NT	C	1	1	NT	−	−
O128ab : H2 (1)	NT	C	1+2	1c	2d	−	+
O128ab : H7 (1)	NT	C	2	NT	2	+ (γ)	−
O128ab : H25 (1)	NT	C	2	NT	2	+ (β)	+
O134 : H25 (1)	NT	A	2	NT	2	−	+
O145 : H25 (3)	NT	C	2	NT	2	+ (β)	+
O145 : H25 (1)	NT	NK	2	NT	2	+ (β)	+
O163 : H19 (2)	NT	C	2	NT	2c	−	+
O165 : H25 (1)	NT	C	2	NT	2+2c	+ (ε)	+
O168 : H− (1)	NT	C	2	NT	2+2c	−	−
O173 : H21 (1)	NT	C	2	NT	2+2c	−	+
E55992/88 : H−† (1)	NT	C	1+2	1	2d	−	+
O? : H21 (1)	P9	NK	2	NT	2c	−	−
O? : H40 (1)	P11	C	2	NT	2c	+ (θ)	−

*Same patient.

†Provisional new serotype.

et al., 1996; Caprioli *et al.*, 1997; Schmidt *et al.*, 1999). VTEC of serogroup O26 has emerged as a particularly significant cause of human disease (Caprioli *et al.*, 1997; Schmidt *et al.*, 1999) and has been associated with outbreaks in the Republic of Ireland (McMaster *et al.*, 2001). VTEC O26 has been isolated frequently from cattle and has the same characteristics as human strains of the same serotype (Wieler *et al.*, 1996; Zhang *et al.*, 2000) although, to date, there has been no direct evidence that bovine VTEC O26 causes human infection. VTEC of serogroup O128ab was detected in all three groups, although O128ab : H2 was the only serotype common to all three (Tables 1–3). VTEC O128ab : H2 that harbours the vtx_{1c} and vtx_{2d} genes has been isolated from sheep (Koch *et al.*, 2001; Ramachandran *et al.*, 2001).

Fourteen strains in this study could not be serogrouped by using the current serotyping scheme. Further analysis of the *rfb* gene showed that each strain had a different PCR-RFLP pattern, designated P1–P14 (Tables 1–3), indicating that they probably belonged to different serogroups and that the 'O?' strains do not represent a single newly emerging serogroup. PCR-RFLP *rfb* patterns have not been determined for all 173 established serogroups in the LEP serotyping scheme, so the 'O rough' strains could be variants of established O-types.

Typing of *vtx* genes

vtx subtyping results are shown in Tables 1–3. Of strains isolated from patients with HUS, 82 % carried vtx_2, of which 46 % had subtype vtx_2 and 32 % had vtx_{2c} (three strains had both vtx_2 and vtx_{2c} subtypes) (Table 1). vtx_{1c} genes were present in 32 % of strains isolated from patients with less severe diarrhoeal disease and 44 % of strains had vtx_{2d} genes (Table 2). Of the 15 strains of VTEC isolated from healthy individuals in the control group, 67 % carried the gene that encodes the VT1c variant and the vtx_{2d} gene was detected in 80 % of strains (Table 3). Statistical analysis of the results in our study also showed that vtx_2 and vtx_{2c} were associated with HUS ($P < 0{\cdot}0001$ and $P < 0{\cdot}003$, respectively) and that the

Table 2. Strains of non-O157 VTEC isolated from patients with diarrhoea who did not develop HUS

No. strains isolated in parentheses. Abbreviations: A, adult; C, child; NK, not known; NT, not tested (test not appropriate). References: Willshaw *et al.* (1992, 2001), Evans *et al.* (2002).

Serotype (34)	*rfb* pattern	Adult/child	*vtx* type	vtx_1 subtype	vtx_2 subtype	*eae* gene (subtype)	*ehxA* gene
O4 : H10 (1)	NT	NK	1+2	1	2d	–	+
O5 : H– (1)	NT	A	1	1	NT	+ (β)	+
O26 : H11 (4)	NT	C	1	1	NT	+ (β)	+
O26 : H11 (1)	NT	NK	1	1	NT	+ (β)	+
O26 : H11 (1)	NT	C	1	1c	NT	+ (β)	+
O52 : H25 (1)	NT	C	2	NT	2c	–	+
O76 : H7 (1)	NT	NK	2	NT	2	+ (θ)	+
O91 : H– (1)	NT	NK	1+2	1	2d	–	–
O91 : H10 (1)	NT	C	2	NT	2c	–	–
O105ac : H18 (1)	NT	A	1+2	1	2	–	+
O118 : H12 (2)	NT	NK	2	NT	2d	–	–
O118 : H12 (1)	NT	A	2	NT	2d	–	–
O128ab : H– (1)	NT	C	2	NT	2f	+ (β)	–
O128ab : H– (2)	NT	C	1+2	1c	2d	–	+
O128ab : H2 (3)	NT	C	1+2	1c	2d	–	+
O128ab : H2 (1)	NT	A	1+2	1c	2d	–	+
O128ab : H2 (2)	NT	NK	1+2	1c	2d	–	+
O128ab : H8 (1)	NT	C	1	1	NT	–	–
O? : H– (1)	P3	C	1	1	NT	+ (β)	+
O? : H2 (1)	P4	C	1	1	NT	+ (ε)	+
O? : H10 (1)	P5	C	2	NT	2	–	–
O? : H10 (1)	P6	A	1+2	1c	2d	–	+
O? : H19 (1)	P8	A	1	1c	NT	–	–
O rough : H– (1)	P12	A	2	NT	2c	–	–
O rough : H– (1)	P13	C	1+2	1	2d	–	+
O rough : H45 (1)	P14	A	2	NT	New type	–	–

vtx_{1c} and vtx_{2d} genes were associated significantly with individuals who did not develop HUS ($P < 0{\cdot}001$ for both vtx_{1c} and vtx_{2d}).

Other studies have shown that VT2 subtypes VT2 and VT2c were associated with HUS, but that strains that harbour the vtx_{2d} gene were rarely associated with this disease (Bonnet *et al.*, 1998; Piérard *et al.*, 1998; Boerlin *et al.*, 1999; Friedrich *et al.*, 2002). In this study, two strains with vtx_{2d} genes and two that carried vtx_{2e} genes were isolated from patients with HUS. However, it is not clear whether these isolates were the cause of the patients' HUS disease, or whether they represent 'side-findings' without any aetiological association with the underlying HUS and the true cause of the patients' disease was a strain of VTEC more commonly associated with HUS, such as VTEC O157 : H7 or O26 : H11. Detection of antibodies to the LPS of VTEC O157 or O26 in the patients' sera may help to clarify the cause of disease but, unfortunately, sera from these patients were not available.

vtx_2 genes from the O rough : H45 strain were untypable by using the primer pairs in this study. A fragment (868 bp) of the vtx_2 gene in this strain was amplified by using the primers described by Lin *et al.* (1993); further analysis showed that this vtx_2 gene had a novel PCR-RFLP pattern (data not shown).

One strain of serotype O26 : H11, isolated from a patient with diarrhoea, had the vtx_{1c} and β-intimin genes (Table 2). Human VTEC that contains vtx_{1c} is usually *eae*-negative and does not belong to the major non-O157 serotypes (Koch *et al.*, 2001; Zhang *et al.*, 2002). In order to clarify this unusual result, the serotype of this strain was retested by the *E. coli* reference laboratory at the LEP by using the serotyping scheme described by Gross & Rowe (1985) and was confirmed as *E. coli* O26 : H11. In addition, the *vtx* gene was analysed by using PCR-RFLP as described by Lin *et al.* (1993), using the restriction enzymes *Hin*dII and *Hha*I. The patterns were consistent with those of the vtx_{1c} control strain.

Intimin typing and VTEC enterohaemolysin

The intimin gene, important in the pathogenesis of human VTEC infection (Frankel *et al.*, 1998), was detected more frequently in strains isolated from patients with HUS than from those with less severe disease (50 and 32 %, respectively)

Table 3. Strains of non-O157 VTEC strains isolated from a healthy control group during the Infectious Intestinal Disease study (Evans *et al.*, 2002)

No. strains isolated in parentheses. Abbreviations: A, adult; C, child; NK, not known; NT, not tested (test not appropriate).

Serotype (15)	*rfb* pattern	Adult/child	*vtx* type	vtx_1 subtype	vtx_2 subtype	*eae* gene (subtype)	*ehxA* gene
O82 : H2 (1)	NT	C	1+2	1c	2d	−	+
O91 : H− (1)	NT	A	1	1	NT	−	+
O115 : H10 (1)	NT	C	1	1	NT	−	−
O118 : H12 (1)	NT	A	2	NT	2d	−	−
O128ab : H2 (3)	NT	A	1+2	1c	2d	−	+
O146 : H21 (1)	NT	A	1+2	1c	2d	−	+
O146 : H21 (1)	NT	NK	1+2	1c	2d	−	+
O162 : H6 (1)	NT	A	1+2	1c	2d	−	−
O162 : H8 (1)	NT	A	1+2	1c	2d	−	−
O? : H− (1)	P1	A	2	NT	2d	−	+
O? : H− (1)	P2	A	1+2	1	2d	−	−
O? : H18 (1)	P7	A	1+2	1c	2d	−	+
O? : H21 (1)	P10	A	1	1c	NT	−	+

and was not detected in any of the strains from the healthy control group (Tables 1–3). Statistical analysis showed that the intimin gene was significantly associated with cases of HUS ($P = 0{\cdot}04$). Other studies have also shown an association between HUS and the presence of the *eae* gene in strains of VTEC (Boerlin *et al.*, 1999; Eklund *et al.*, 2002). In this study, β-intimin was the intimin type detected most frequently in both groups 1 and 2. No single intimin subtype has been associated with HUS (Schmidt *et al.*, 1999; Oswald *et al.*, 2000), although there is evidence that intimin subtype mediates both tissue tropism and host specificity (Phillips *et al.*, 2000; Reece *et al.*, 2001). Other pathogenicity factors associated with attachment are being investigated, as 59 % of strains in this study did not carry the intimin gene. Paton *et al.* (2001) suggested that an outer-membrane protein, designated STEC autoagglutinating adhesin (Saa), may have a role in attachment in strains of VTEC that do not have the intimin gene.

Genes that encode VTEC enterohaemolysin were detected in 64 % of strains from HUS patients, in 68 % of those from patients with less severe disease and in 66 % of strains from the healthy control group (Tables 1–3). There was no association between the presence of *ehxA* and VTEC isolated from patients with HUS ($P = 0{\cdot}87$).

Subtyping of genes associated with the pathogenicity of VTEC infection, such as the *vtx* and *eae* genes, can provide information on the association of each strain with severe disease and on the bacterium–host relationship, e.g. certain *vtx* genes and intimin subtypes may be host-specific. In this study, strains of VTEC that carried the *eae* gene and the VT2 gene subtypes vtx_2 and vtx_{2c} were most likely to be associated with HUS. There was no association between the presence of *ehxA* and symptoms of HUS and the role of VTEC enterohaemolysin in the pathogenesis of disease remains unclear. None of the intimin types was found to be significantly associated with HUS. However, data on intimin subtyping may be important to clarify whether intimin type can influence tissue tropism and determine host specificity.

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