MOLECULAR DIAGNOSIS AND EPIDEMIOLOGY

Molecular characterisation of verocytotoxin-producing *Escherichia coli* of serogroup O111 from different countries

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A collection of epidemiologically unrelated verocytotoxin (VT)-producing *Escherichia coli* (VTEC) strains of serogroup O111 isolated from human patients and cattle with diarrhoeal disease in five different countries were characterised by determination of their VT genotypes, the presence of other virulence factors such as the intimin-coding *eae* gene and the enterohaemorrhagic *E. coli* (EHEC) plasmid, and their antibiotic susceptibility patterns. The genetic relatedness among isolates was evaluated by genomic DNA fingerprinting techniques such as restriction fragment length polymorphism analysis of ribosomal RNA genes (ribotyping) and pulsed-field gel electrophoresis. The results indicated that the VTEC O111 examined belong to two distinct clonal lineages. The first group was constituted mainly of non-motile, *eae*-positive, EHEC plasmid-positive isolates from both man and cattle. The second lineage was represented by an O111:H2 epidemic strain, isolated during an outbreak of haemolytic uraemic syndrome in France and exhibiting an unusual combination of virulence factors: VT production and aggregative adhesion to HEP-2 cells associated with an enteroaggregative *E. coli* (EAEC) plasmid.

Introduction

Verocytotoxin-producing *Escherichia coli* (VTEC) are an important cause of human illness such as diarrhoea, bloody diarrhoea and haemolytic uraemic syndrome (HUS) [1]. VTEC are common in the intestinal tract of cattle and other ruminants. Human infections are often a consequence of the ingestion of contaminated meat, dairy products or vegetables that have been inadequately cooked or processed [1, 2]. Although VTEC strains belonging to serogroup O157 are by far the most prevalent in human disease and have been associated with the most important food-borne outbreaks world-wide, strains belonging to other serogroups – usually referred to as non-O157 VTEC – have been isolated frequently from individuals with severe disease [1, 2]. Among non-O157 VTEC, those belonging to serogroup O111 have been included by Levine [3] among the enterohaemorrhagic *E. coli* (EHEC) group.

VTEC O111 strains have often been isolated from patients with diarrhoea and HUS [1, 2, 4]. They appear to have been reported most frequently from Australia since 1988 [5], when a family outbreak was reported from Perth, and again in 1990 when a single case of HUS was reported from Sydney [6]. Since then, institutional and community outbreaks have been reported in Japan [7], Italy [8], France [9], Spain [10] and a family outbreak in the USA [11]. An especially severe outbreak that involved predominantly EHEC O111 was reported from Australia [12], although it also included strains of EHEC O157, and might have been reported as an O157 outbreak if detailed molecular biological analyses had not been performed [13]. Moreover, a large community-wide outbreak of diarrhoea associated with an enteropathogenic *E. coli* (EPEC) O111 was reported in 1953–1954 at Fort Belvoir, VA, USA, before either the description of HUS or the discovery of VTEC [14]. The disease was particularly severe in children and nine infants with a clinical and pathological picture consistent with HUS died, thus suggesting that the EPEC O111 epidemic strain involved was actually a VT-producer.

Usually VTEC O111 express the flagellar antigen H8 or are non-motile (NM) [15], harbour plasmids related to the plasmid of EHEC O157 or EHEC plasmids [3, 4, 15, 16], and possess intimin-coding *eae* genes [1, 3, 4, 10, 12, 15], which are part of a chromosomal gene cluster termed LEE (for locus for enterocyte effacement), that is necessary for the production of attaching and effacing (AE) lesions in the intestinal mucosa [1]. However, VTEC O111:H2 strains presenting enteroaggregative adhesion to HEp-2 cells instead of the typical AE mechanism have been described recently [17].

The aim of this study was to assess the distribution of accessory virulence factors and antibiotic markers in a collection of VTEC O111 isolates from different sources in different countries and to study their genetic relatedness by means of genomic DNA fingerprinting techniques.

Materials and methods

Bacterial strains

The human *E. coli* O111 strains investigated in this study were isolated in different countries between 1985 and 1995 from epidemiologically unrelated cases of diarrhoea and HUS. Some of the strains have been described in previous studies [4, 18]. Isolates from outbreaks of HUS which occurred in Italy [8] and Australia [12] are included in this study. Strains RD1 and RD2 from France were representative of eight isolates from an outbreak of HUS [9, 17]. Bovine strains were isolated in Italy from calves with diarrhoea. The EPEC standard strain for the O111 antigen StokeW was included for comparison. VT production was tested by the Vero cell cytotoxicity assay as described previously [17]. Reference enteroaggregative *E. coli* (EAEC) strain 17-2 (O3:H2) and EHEC strain EDL933 (O157:H7) were used as controls.

Antimicrobial susceptibility testing

The disk diffusion method [19] was used to test the isolates for susceptibility to the following antibiotics: ampicillin (A), cephalothin, chloramphenicol (C), ciprofloxacin, kanamycin (K), gentamicin (G), streptomycin (S), sulphisoxazole (Su), tetracycline (T) and trimethoprim-sulphamethoxazole (Sxt). *Escherichia coli* ATCC 25922 was used as control.

Analysis of virulence factors

The presence of virulence factors was assessed by PCR amplification of the corresponding genes. Primer pairs KS7/KS8 and GK3/GK4 were used for VT1 and VT2, respectively [18]. PCR products obtained with primers GK3/GK4 were subjected to restriction endonuclease analysis with *FokI* and *HaeIII* to distinguish between VT2 and VT2c genes [18]. The intimin-coding *eae*

gene was detected with primer pair SK1/SK2 [20]. The *eae* gene was further characterised with primers LP2 and LP3 [21], in combination with SK1 as forward primer. LP2 and LP3 are homologous to the DNA sequences of the *eae* genes which encode for intimins of type α (prototype strain: EPEC O127:H6 E2348/69) and γ (prototype strain: EHEC O157:H7 EDL933), respectively [22]. The presence of EHEC plasmids was assessed by hybridisation with the EHEC probe, derived from plasmid pCVD419 [16]. DNA sequences of the EAEC plasmid associated with the aggregative pattern of adhesion [1] were detected with primer pairs Agg1/Agg2, which amplify a 630-bp region of the plasmid [23].

DNA fingerprinting

Restriction fragment length polymorphism (RFLP) analysis of ribosomal RNA (rRNA) genes (ribotyping) was performed according to Stull *et al.* [24]. Genomic DNA was digested with *HindIII* and subjected to electrophoresis in agarose 0.8% gel. The restriction fragments were then transferred to nylon membranes by capillary transfer and filters were hybridised with an rRNA digoxigenin-labelled DNA probe (Boehringer Mannheim, Germany). Labelling and hybridisation were performed under stringent conditions in accordance with the Boehringer manual. DNA hybrids were revealed by a DIG luminescent detection kit under the conditions indicated by the supplier (Boehringer Mannheim).

Pulsed-field gel electrophoresis (PFGE) of genomic DNA was performed according to Bender *et al.* [25]. Electrophoresis conditions were as follows: 2.2 s initial switch time, 48.5 s final switch time, 6 V/cm, TBE 0.5 $\times$ at 14°C.

Results

Analysis of virulence factors

The characteristics of the VTEC O111 strains included in this study are shown in Table 1. With the exception of the isolates from the HUS outbreak which occurred in France [9], all strains were homogeneous in many features, regardless of their source, country and year of isolation. All the isolates harboured *vt1* genes, together with the *vt2* determinant in eight strains; no isolate had *vt2c* sequences. All the strains possessed an *eae* intimin gene amplified by the PCR primers SK1/LP3, thus indicating homology to the sequence of type γ *eae* gene of EHEC O157:H7 [22]. Finally, all except three strains hybridised with the EHEC plasmid probe.

As previously described by Morabito *et al.* [17], the isolates from the French outbreak had neither the *eae* gene nor the EHEC plasmid markers, but gave positive results in a PCR specific for EAEC plasmids and showed aggregative adhesion to HEp-2 cells. In

Table 1. Sources and characteristics of VTEC O111 isolates

Strain no.	Country of isolation	Year	Source	H type	VT genotype	Adhesion gene	EHEC plasmid	Ribotype (<i>Hind</i> III)	R-type [†]
E-D31	Italy	1989	HUS	NM	<i>vt1/2</i>	<i>eae</i> (γ)	+	A	S,Su,T
E-D71	Italy	1990	Diarrhoea	NM	<i>vt1</i>	<i>eae</i> (γ)	—	A	S,Su,T,A,Sxt
E-D72	Italy	1990	Diarrhoea	NM	<i>vt1</i>	<i>eae</i> (γ)	—	A	S,Su,T,A
E-D73	Italy	1990	Diarrhoea	NM	<i>vt1</i>	<i>eae</i> (γ)	—	A	S,Su,T,A,Sxt
E-D82	Italy	1991	HUS	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,Su,T
E-D142	Italy	1992	HUS*	NM	<i>vt1/2</i>	<i>eae</i> (γ)	+	A	S,Su,T,G,Sxt
2/85	Germany	1985	HUS	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,T
4924/85	Germany	1985	HUS	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,T
3217/86	Germany	1986	HUS	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,T
3222/87	Germany	1987	HUS	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,Su,T
7042/87	Germany	1987	HUS	NM	<i>vt1/2</i>	<i>eae</i> (γ)	+	A	S,Su,T,G,Sxt
4856/88	Germany	1988	HUS	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,T
5736/88	Germany	1988	HUS	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,T
1899/90	Germany	1990	HUS	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,Su,T
928/91	Germany	1991	HUS	NM	<i>vt1/2</i>	<i>eae</i> (γ)	+	A	S,Su,T,G,Sxt
472/92	Germany	1992	HUS	NM	<i>vt1/2</i>	<i>eae</i> (γ)	+	A	S,Su,T
78/95	Germany	1995	HUS	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,Su,T
E-D227	Austria	1995	HUS	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,Su,T
RD1	France	1992	HUS*	H2	<i>vt2</i>	EAEC	—	B	A,K
RD2	France	1992	HUS*	H2	<i>vt2</i>	EAEC	—	B	A,K
FEC89056	Australia	1989	HUS	H8	<i>vt1/2</i>	<i>eae</i> (γ)	+	A	Susceptible
94127588	Australia	1994	HUS	NM	<i>vt1/2</i>	<i>eae</i> (γ)	+	A	Susceptible
95004975A	Australia	1995	HUS*	NM	<i>vt1/2</i>	<i>eae</i> (γ)	+	A	Susceptible
E-D47	Italy	1992	Cattle diarrhoea	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	S,Su,T,C,G
E-D285	Italy	1998	Cattle diarrhoea	NM	<i>vt1</i>	<i>eae</i> (γ)	+	A	Su

*Isolate from an outbreak. [†]Abbreviations as in *Materials and methods*.

addition, they presented the *vt2* genotype, never encountered among the *eae*-positive strains.

Antimicrobial susceptibility

Resistance to two or more drugs was found in all strains except the Australian isolates, which were susceptible to all the antimicrobial agents tested, and a bovine strain, which was resistant to sulphisoxazole alone (Table 1). The isolates from the French outbreak were resistant to ampicillin and kanamycin, while the other 19 strains were resistant to streptomycin and tetracycline and also showed resistance to sulphisoxazole (13 strains), trimethoprim-sulphamethoxazole (five), gentamicin (four) ampicillin (three) and chloramphenicol (one).

DNA fingerprinting analysis

Hybridisation of *Hind*III-digested genomic DNA with the rRNA probe showed that the O111 VTEC fell in two RFLP patterns (Fig. 1). Ribotype A was common to all the *eae*-positive isolates, including those from cattle. Ribotype B was common to the EAEC strains from the French outbreak (Table 1). Both the ribotypes were different to that of the EPEC O111 strain StokeW included for comparison (Fig. 1).

The genetic relatedness of the VTEC O111 isolates was also investigated by PFGE analysis of the genomic DNA cleaved with *Xba*I. No common patterns were observed among the epidemiologically unrelated, *eae*-positive strains, while a nearly identical profile was

shared by the EAEC strains from the outbreak in France (Fig. 2).

Discussion

E. coli O111 strains have been recognised as a cause of serious enteric disease for >40 years and serogroup O111 is considered to be one of the most important of the EPEC serogroups [26]. However, there is now evidence that *E. coli* O111 strains are heterogeneous in virulence traits and mechanisms of pathogenesis. Some isolates are still classified as EPEC, as they possess the chromosomal LEE locus and the EPEC adherence factor plasmids [1]. Others are categorised as entero-aggregative *E. coli* by virtue of their characteristic pattern of adherence to cell cultures and their ability to hybridise with the corresponding EAEC DNA probe [4, 27–29]. Finally, VT-producing strains are frequently isolated from cases of HUS and have been classified as EHEC [1–3]. Recently, a clonal analysis based on multilocus enzyme electrophoresis (MEE) confirmed the existence of extensive genetic variation among *E. coli* O111 isolates and identified four major bacterial clones, each characterised by the possession of different virulence factors [15].

In the present study, epidemiologically unrelated VTEC O111 strains isolated from different sources in different countries were examined to determine their patterns of virulence factors and antibiotic resistance markers and to define their genetic relatedness by genomic DNA fingerprinting techniques. The results of the study

Fig. 1. Ribotype patterns of *E. coli* O111 obtained by *Hind*III digestion. Lane 1, EPEC O111:NM strain StokeW; 2, 3, VTEC O111:NM *eae*+ strains from Italy; 4, VTEC O111:NM *eae*+ strain from Austria; 5, 6, enteroaggregative VTEC O111:H2 from France; 7, 8, VTEC O111 *eae*+ strains from Germany; 9, 10, VTEC O111 *eae*+ strains from Australia; 11, VTEC O111 *eae*+ strain from cattle.

Fig. 2. PFGE patterns of VTEC O111 obtained by digestion with *Xba*I. Lane 1, molecular size standards (λ ladder, BioRad Laboratories); 2, 3, 6, 7, 8, examples of VTEC O111:NM *eae*+ strains; 4, 5, enteroaggregative VTEC O111:H2 from the outbreak in France.

indicate that strains isolated in different countries and over an extensive period of time from both man and cattle belong to a unique clone, with the exception of an epidemic strain isolated during an outbreak of HUS in France [9, 17].

The main clone is constituted by the strains which belong to ribotype A and presents a stable combination

of virulence properties, as all the isolates possessed an *eae* intimin gene of type γ [22], and all except three were positive for EHEC plasmid markers. This combination is considered to be the hallmark of EHEC strains [1, 3] and corresponds to the virulence pattern shared by the isolates that belong to the EHEC clone ET8, which are distinguished by MEE analysis among *E. coli* O111 strains isolated in the USA and in South

America [15]. This suggests that the ribotype A clone described here corresponds to the EHEC clone ET8 found among the American strains.

The strains that belong to this EHEC clone were homogeneous in other features. All, except one possessing the H8 antigen, were non-motile and produced VT1 alone, or in combination with classic VT2, and all except four were resistant to streptomycin and tetracycline. These common features were also shared by the bovine strains, thus confirming that, as for VTEC O157 [1, 2], cattle may represent an important reservoir of VTEC O111 [12]. PFGE analysis showed a certain degree of variability within the EHEC clone, thus confirming its potential usefulness as a typing tool in epidemiological investigations.

The second clonal lineage found in this study corresponds to ribotype B and refers to epidemic O111:H2 strains from France that showed an unusual combination of virulence factors, i.e., VT production and aggregative adhesion to HEp-2 cells associated with the presence of an EAEC plasmid [17]. It is unclear whether this clone acquired the VT gene or the EAEC plasmid first. The lack of the chromosomal *eae* gene and the genomic DNA fingerprinting results seem to exclude its derivation from an EHEC O111 in which an EAEC plasmid has substituted the EHEC plasmid. Isolates of serogroup O111 are not very common among EAEC [28–31], but have been reported in some studies [4, 27–29]. One such strain, which already carried an EAEC plasmid, might have acquired a VT2 determinant in its chromosome. Very little is known about the possible role of this second clone in human disease, because the outbreak in France appears to be the only episode in which it has been reported so far. Despite the lack of the *eae* gene and of the EHEC plasmid, these VT-producing O111 EAEC strains seem to be as pathogenic for man as the classic EHEC strains. Therefore, *eae*-negative VTEC strains should also be examined for EAEC properties to evaluate their pathogenicity.

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