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Virulence factors and phenotypical traits of verotoxigenic strains of *Escherichia coli* isolated from human patients in Germany

Lothar Beutin[1], **Stojanka Aleksic**[2], **Sonja Zimmermann**[1], **Kerstin Gleier**[1]

[1] Escherichia coli National Reference Laboratory, Department of Microbiology, Robert Koch-Institut, Nordufer 20, D-13353 Berlin, Germany
[2] National Reference Laboratory for Enteritis, Hygienisches Institut, Marckmannstrasse 129a, D-20539 Hamburg, Germany

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Abstract. Fecal isolates of *Escherichia coli* which were collected from human patients in different parts of Germany between 1985 and 1992 were examined for production of verotoxins (VT). Among 2165 isolates 54 (2.5%) verotoxigenic *E. coli* (VTEC) were found. The 54 VTEC belonged to 13 different serotypes, 46 (85.2%) of these were enterohemorrhagic *E. coli* (EHEC) types as O157:H7, O157:H–, O145:H–, O111:[H8] and O26:[H11]. Of the 54 VTEC 50 (92.6%) hybridized with one or both of the DNA probes specific for VT1 and VT2. The 4 VTEC strains which were negative for VT1 and VT2 differed from all other VTEC by many phenotypical trains such as serotype, production of α-hemolysin and absence of EHEC-plasmid and "attaching and effacing" (*eae*)-specific DNA sequences. In contrast, VTEC which were positive for VT1, VT2 or both were frequently positive for *eae* sequences (92.0%), EHEC-plasmids (90.0%) and for production of enterohemolysin (88.0%). With enterohemolysin as an epidemiological marker more VTEC strains (81.5%) could be identified than with others such as the absence of β-glucuronidase activity (61.1%) or non-fermentation of sorbitol (48.1%). Case reports were available for 42 of the 54 VTEC strains. The clinical presentation of 42 cases with VTEC ranged from uncomplicated diarrhea to severe diseases as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). However, bloody diarrhea, HC and HUS were more associated with the O157 group than with other VTEC groups.

Introduction

Verotoxin (Shiga-like toxin)-producing strains of *Escherichia coli* (VTEC) can cause severe diseases in humans as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [16]. VTEC were isolated from humans, animals and from contaminated foodstuffs and were found to be heterogeneous in their O and H

Correspondence to: L. Beutin

serotypes [7, 16, 29]. Cattle, sheep and goats serve as important reservoirs for this group of toxigenic *E. coli* [6, 16, 29].

It is not clear if all types of VTEC are equally pathogenic for humans. Certain serotypes of VTEC called enterohemorrhagic *E. coli* (EHEC) are frequently implicated in human disease such as HC and HUS [16–18]. The mechanisms through which EHEC cause disease are not fully understood. The production of verotoxins and the ability of many EHEC to cause "attaching and effacing" lesions in the intestinal mucosa are regarded as important virulence factors [2, 16]. Other properties such as maintenance of "EHEC-specific" plasmids and production of enterohemolysins (E-Hly) are common in many EHEC strains [3, 18, 25]. However, it is not clear if these contribute to EHEC pathogenicity [26, 27].

Because of their phenotypical diversity, VTEC are difficult to discriminate from apathogenic *E. coli* in fecal samples. Most of EHEC O157:H7 and O157:H– strains were detected using specific antisera or selective media by screening for sorbitol-negative colonies [9, 12]. Moreover, the production of verotoxins is closely associated with E-Hly in some VTEC and EHEC [3, 6, 25, 28]. E-Hly synthesis was, therefore, suggested as a suitable epidemiological marker for detection of some serotypes of EHEC and VTEC [3, 6].

In the present study we have examined more than 2000 *E. coli* strains which were isolated from human patients in Germany between 1985 and 1992 for verotoxigenic activity. The association of different EHEC types with certain virulence markers and with human disease was investigated. By examination of epidemiological markers we could demonstrate that E-Hly is highly efficient for detection of most EHEC strains found in this study.

Material and methods

Bacteria

A total of 2165 *E. coli* strains were investigated for production of verotoxins. Most of the strains were from feces of human patients with enteric disease and were isolated in clinical laboratories in different parts of Germany between 1985 and 1992. Bacterial cultures were kept frozen at −70°C in a dilution of 1.5% Bacto Peptone (Difco Laboratories, Detroit, Michigan, USA) with an equal amount of 87% glycerol (Merck, Darmstadt). Toxicity of culture fluid of bacteria grown in tryptic soy broth (TSB, Difco) to Vero cells was tested as described [4]. *E. coli* strains H19 (O26:H11) and CB295 (O139:K12:H1) served as positive controls for VT1 and VT2, respectively [3]. Hemolytic activity of *E. coli* was observed on blood agar plates containing 5% sheep blood washed in phosphate-buffered saline (PBS) pH 7.2 [3]. α-Hemolysin was distinguished from E-Hly by the time at which lysis occurred and by the morphology of lysis zones [23]. The strains U4-41 (O4:K3:H5) and C4170 (O78:H–) served as positive controls for *E. coli* α-Hemolysin and the strains C3888 (O26:H–) and H19 as positive controls for enterohemolytic activity [3, 23]. Fermentation of sorbitol was detected on MacConkey Sorbitol agar (Difco) and on Flurocult *E. coli* O157:H7-Agar (Merck). The latter media were also used for detection of β-glucuronidase activity by observing fluorescence around grown colonies at 366 nm wavelength.

Biochemical and serological typing of E. coli

Isolates of *E. coli* were identified by typical biochemical reactions [8]. Serotyping of *E. coli* O-antigens (O1 to O171) and H-antigens (H1–H56) was performed according to standard methods [21].

Preparation of DNA probes

Gene probes specific for VT 1 [750-base-pair (bp) *Hin*cII fragment of plasmid NTP705], VT2 (850-bp *Ava*I-*Pst*I fragment of plasmid NTP707), α-hemolysin [approx. 6-kilobase (kb) size *Ava*I-A fragment of plasmid pSF4000] and EAF (1-kb *Bam*HI-*Sal*I fragment of plasmid pJPN16 were prepared as described [3–5]. Strains carrying recombinant plasmids containing *eae* (pCVD434) and EHEC-plasmid-specific (pCVD419) sequences were kindly supplied from A. E. Jerse, B. A. Kay and J. P. Nataro (Center for Vaccine Development, University of Maryland, Baltimore, Maryland, USA). The 1-kb size *Sal*I-*Kpn*I fragment of pCVD434 was used for detection of *eae* sequences (A. E. Jerse, personal communication) and the 3.4-kb size *Hin*dIII fragment of pCVD419 served as the EHEC-specific probe [18].

Colony blot DNA hybridizations

The preparation of DNA probes was performed as described [3, 20]. DNA fragments were labelled with digoxigenin-11-dUTP using the Boehringer DIG-DNA labeling and detection system (Boehringer Mannheim, Mannheim, Germany) according to the instructions of the supplier. Hybridization and washing of nitrocellulose membranes (Schleicher und Schüll, Dassel, Germany) were performed at conditions of high stringency. The hybridization reaction was performed for 20 h at 68 °C in 5 × SSC (1 × SSC = 0.3 M NaCl; 0.03 M sodium citrate; pH 7.0); 0.1% N-laurylsarcosine (Sigma, Deisenhofen, Germany); 0.02% SDS with 1% blocking reagent (Boehringer Mannheim). Washing of nitrocellulose membranes was performed twice for 5 min in 2 × SSC with 0.1% SDS at room temperature and then twice for 15 min in 0.1 × SSC/0.1% SDS at 68 °C. Labeled DNA was detected by an enzyme-linked color reaction (anti-digoxigenin ELISA) on the nitrocellulose filters.

Results

Detection of verotoxigenic E. coli *(VTEC) strains*

By examination of 2165 *E. coli* isolates from human patients, 54 strains with verotoxigenic activity were found. Single isolates of VTEC were typed for their O and H antigens and were examined for DNA hybridization with gene probes specific for VTEC, EHEC and for enteropathogenic *E. coli* (EPEC). The 54 VTEC belonged to 13 different serotypes (Table 1). Of these 46 (85.2%) were identified as EHEC belonging to serotypes O157:H7, O157:H−, O26:[H11], O111:[H8] and O145:H−.

Of the 54 strains 50 (92.6%) hybridized with one or both of the DNA probes specific for VT1 and VT2. The remaining 4 strains did not hybridize with the VT-DNA probes. One of these was an O22:H− strain which had lost its verotoxigenic activity during laboratory culture, one was a O4:H5 strain and two were O6:H− strains which exerted only a weak toxic activity to Vero cells. In contrast to the VT1 (H19)- and VT2 (CB295)-producing control strains this activity was not detectable in dilutions of bacterial culture fluid higher than 1:10.

Virulence markers of human VTEC

None of the 54 VTEC strains hybridized with the *eaf* (EPEC adherence factor)-specific DNA probe, indicating that these do not carry the genes for localized adherence. However, 46 (85.2%) strains were positive for *eae* (*E. coli* attaching and

Table 1. Virulence markers of verotoxin-producing *E. coli* (VTEC) strains isolated from human patients in Germany

Serotype	No. of isolates					
	Positive for verotoxin	Hybridizing with DNA probes specific for:				
		VT1	VT2	VT1 + VT2	EHEC-plasmid	*eae*
O111:H−	6	6	0	0	5	6
O111:H8	1	1	0	0	1	1
O145:H−	1	0	1	0	1	1
O157:H−	11	1	2	8	11	11
O157:H7	17	0	14	3	17	16
O22:H1	1	0	0	0	0	0
O23:H7	1	0	1	0	0	0
O26:H−	1	1	0	0	1	1
O26:H11	9	6	3	0	7	8
O4:H5	1	0	0	0	0	0
O6:H−	2	0	0	0	0	0
ONT:H−	2	1	1	0	1	1
O-Rough:H−	1	1	0	0	1	1
Total	54	17	22	11	45	46

effacing) sequences and 45 (83.3%) for the EHEC-plasmid-specific sequences present on pCVD419. Both, EHEC-plasmid and *eae* sequences were present in almost all EHEC strains but absent in those VTEC which were negative for VT1 and VT2 (Table 1). The distribution of VT1 and VT2 sequences was different between the serotypes. Strains carrying both VT1 and VT2 sequences were only found in O-group 157. All O111 and 7 of the 9 O26 strains were only positive for VT1.

Expression of phenotypes serving as epidemiological markers for detection of VTEC

Of the 54 human VTEC strains 49 (90.7%) produced hemolysins which were detected on blood-agar plates (Table 2). Only 5 strains of serotypes O22:H1, O23:H7, O4:H5 and O6:H− produced α-hemolysin and were positive for DNA hybridization with the hemolysin specific DNA probe. The other 44 strains showed the enterohemolytic phenotype (E-Hly$^+$) [23]. All EHEC except 3 O26:H11 strains were E-Hly$^+$ (Table 2).

The 54 VTEC were investigated for fermentation of sorbitol and for β-glucuronidase activity on MacConkey Sorbitol medium and on Flurocult *E. coli* O157:H7 agar. The results are shown in Table 2. All of the 17 O157:H7 (100%) and 9 of the 11 (81.8%) O157:H− strains were negative for fermentation of sorbitol and for β-glucuronidase activity. All other VTEC were positive for fermentation of sorbitol. β-glucuronidase activity was absent in 92.8% of the O157 strains, in 85.7% of the O111 and in 10.0% of the O26 strains. All other strains were β-glucuronidase positive (Table 2).

Table 2. Expression of phenotypes associated with O157:[H7] and other VTEC

Serotype	No. of isolates					
	Positive for				Negative for	
	VT	E-Hly	α-Hly	Hly−	Fermentation of Sorbitol	β-Glucuro-nidase
O111:H−	6	6	0	0	0	5
O111:H8	1	1	0	0	0	1
O145:H−	1	1	0	0	0	0
O157:H−	11	11	0	0	9	9
O157:H7	17	17	0	0	17	17
O22:H1	1	0	1	0	0	0
O23:H7	1	0	1	0	0	0
O26:H−	1	1	0	0	0	0
O26:H11	9	6	0	3	0	1
O4:H5	1	0	1	0	0	0
O6:H−	2	0	2	0	0	0
ONT:H−	2	1	0	1	0	0
O-Rough:H−	1	0	0	1	0	0
Total	54	44	5	5	26	33

Hly, Hemolysin; VT, verotoxin

Origin of VTEC strains and association with disease in humans

All VTEC were human fecal isolates from sporadic cases of disease. The strains originated from urban and rural areas in 10 of the 16 Federal States of Germany. Patient data were available for 42 of the 54 VTEC strains, representing a total of 53 individual cases (Table 3). In 1 case with HC 2 different types of VTEC O26:H11 strains (E-Hly$^+$ and Hly$^-$) were isolated. Of the patients 26 were males, 19 were females and sex and age of 8 patients were not known. Patients' age ranged between 3 weeks and 74 years. VTEC other than O157 all came from young infants up to 4 years of age. Adults were only present in the group of patients infected with *E. coli* O157. Six cases with HUS were reported, among these 5 infants and 1 adult. Of these 5 were infected with O157:[H7] and 1 infant with O22:H1. Three cases were reported as HC, these were 2 adults with O157:H7 and 1 infant with O26:H11. Four cases of bloody diarrhea were associated with the O157:[H7] group. The remaining 23 cases comprising 10 different VTEC serotypes presented less severe forms of disease reported as enteritis or uncomplicated diarrhea.

Discussion

In our study, production of verotoxins was found in 54 (2.5%) of 2165 human clinical isolates of *E. coli*. We do not know if this frequency corresponds to the incidence of these pathogens in human patients in Germany. Many of the collaborating laboratories forwarded only selected *E. coli* isolates; in particular

Table 3. VTEC associated disease in humans

Serotype	No. of strains and case reports					
	HUS	HC	Enteritis	Diarrhea	Other	Unknown
O111:H−	0	0	3	2	0	1
O111:H8	0	0	1	0	0	0
O145:H−	0	0	0	1	0	0
O157:H−	1	0	3	1[a]	2[b]	4
O157:H7	4	2	7	3[a]	0	1
O22:H1	1	0	0	0	0	0
O23:H7	0	0	1	0	0	0
O26:H−	0	0	0	0	0	1
O26:H11	0	2[c]	1	0	3[d]	3
O4:H5	0	0	0	0	0	1
O6:H−	0	0	2	0	0	0
ONT:H−	0	0	1	0	0	1
O-Rough:H−	0	0	1	0	0	0
Total	6	4	20	7	5	12

[a] Patients with bloody diarrhea
[b] A 21-year-old man with ulcerative colitis and a 74-year-old man with an abdominal abscess
[c] Two phenotypically different O26:H11 strains were isolated from a 2-year-old girl with HC
[d] Two boys and one girl with additional Salmonella infections

those that had agglutinated with antisera used for the detection of EPEC O-groups and *E. coli* O157.

The frequency of detection of VTEC in feces and foodstuffs depends largely on the laboratory methods which are employed. Most of our O157 strains were originally detected by agglutination reaction with O157 antiserum or by screening for sorbitol-non-fermenting *E. coli.* Similarly, many of the EHEC strains belonging to O-serogroups O26 and O111 were isolated by routine examination of fecal *E. coli* from infants with EPEC-specific antisera rather than by detection of verotoxins. Only some of the VTEC detected here were identified by direct examination of verotoxin production or VT-specific DNA sequences. The common use of O-antigen typing as the only method for detection of enteral pathogenic *E. coli* disfavors the detection of VTEC that differ in their O-antigens from the EPEC or O157 strains. This might explain why VTEC other than O26, O111 and O157 were detected at low frequency.

Only 89 of the 2111 verotoxin-negative strains from our study (4.2%) were positive for E-Hly production and 46 of these (51.7%) were *E. coli* O26:[H11] strains. In contrast, E-Hly was found to be closely associated with 81.5% of all 54 VTEC strains and with 88.0% of the 50 strains which carried VT1- or VT2-specific DNA sequences. These findings confirm previous results on the association of E-Hly and verotoxin production in human clincal isolates of *E. coli* [3, 25, 28].

By comparing sorbitol, β-glucuronidase and E-Hly as epidemiological markers for detection of VTEC, E-Hly proved to be most advantageous (Table 2, this study). For biotyping sorbitol and β-glucuronidase were only suitable as epidemiological markers in the detection of O157:H7 and most of the O157:H− strains. β-glucuronidase may, in addition, be useful for detection of O111 isolates;

this needs to be examined using larger numbers of O111 strains. Taken together, this and other studies demonstrate that E-Hly is a suitable epidemiological marker for the detection of many different VTEC types [3, 6, 25, 28].

Only 2 of the 11 O157:H− isolates from this study (18.2%) were positive for sorbitol and β-glucuronidase. It has been reported by others [1, 11, 15] that sorbitol-fermenting O157:H− strains represent a new clone of EHEC which is frequently isolated from human patients with HUS in Germany. The relatively low frequency of sorbitol-fermenting O157:H− EHEC found here might be explained by the fact that many of our *E. coli* O157 isolates were originally isolated on sorbitol indicator media, thus disfavoring the detection of sorbitol-positive O157 and other EHEC. In contrast, sorbitol-positive O157 EHEC were more frequently found when direct detection of VT (SLT)-specific DNA sequences was employed as the method for isolation of EHEC [11].

Five of the VTEC strains investigated produced α-hemolysin which is uncommon in VTEC of human origin but more frequent in animal VTEC [3, 6]. One of the α-hemolytic VTEC had lost its verotoxigenic activity and became negative for VT-specific DNA sequences; this might have occurred during subcultivation [14]. The other three strains exerted a low verotoxigenic activity and were negative for VT1- and VT2-specific DNA sequences. We do not know if these strains produce a verotoxin variant, similar to those described for the SLT-II (VT2) family, or a different type of cytotoxin [19, 13, 24].

All α-hemolysin-producing VTEC were characterized by the absence of EHEC-plasmid and *eae*-specific DNA sequences. Both of these markers are, however, highly associated with many EHEC strains and are putative virulence markers ([2, 18, 25, 28] and this study). Further investigations are necessary to characterize the "unconventional" VTEC which were detected in our study and their possible role in human pathogenicity.

Many infections with EHEC O157:H7 and O157:H− strains were reported as outbreaks of foodborne disease in humans [16]. The VTEC strains that we have described were all from sporadic cases of disease and the sources of individual infections are not known. The clinical presentation of individual cases with VTEC infections ranged from uncomplicated diarrhea to HC and HUS, similar to those previously reported [16, 19, 22, 29]. Bloody diarrhea, HC and HUS were more associated with O157:H7 and O157:H− than with other VTEC. This finding might be significant since similar results were reported from other epidemiological studies [1, 16, 22].

Interestingly, only O157:H7 and O157:H− strains were isolated from both children and adults, whereas all non-O157 VTEC, including O26 and O111 strains, were isolated exclusively from infants. We do not know if this is due to differences in the pathogenic potential between O157 and other EHEC. It might be also possible that this finding results from the different usage of serodiagnosis of enteric *E. coli* infections in infants and adult patients. EPEC-specific antisera including O-serogroup 26 and 111 are generally only employed for routine examination of fecal *E. coli* from young infants. Therefore, it might be possible that infections with EHEC O26 and O111 strains are particularly underdiagnosed in adults by many clinical laboratories.

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