

## Non-O157:H7 Stx<sub>2</sub>-Producing *Escherichia coli* Strains Associated with Sporadic Cases of Hemolytic-Uremic Syndrome in Adults

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Received 2 December 1997/Returned for modification 17 February 1998/Accepted 17 March 1998

**From August 1996 to May 1997, six verotoxin-producing *Escherichia coli* (VTEC) strains were isolated from stool specimens of adults suffering from hemolytic-uremic syndrome (HUS). All the isolates were *stx*<sub>2</sub> positive and belonged to different serotypes: O6:H4, O91:H10, O91:H21, O rough:H16, OX3:H-, and O nontypeable: H-. The enterohemolysin (Ehly)-encoding genes were detected in two isolates, and none of the isolates harbors the intimin (Eae)-encoding gene. These findings suggest that *stx*<sub>2</sub>-positive non-O157:H7 VTEC is a major cause of HUS in adults and that several sources of pathogens are responsible for local endemic infections.**

Hemolytic-uremic syndrome (HUS) is characterized by acute hemolytic anemia, thrombocytopenia, and acute renal failure. In some cases, these three clinical features are associated with neurological manifestations and fever. The association between HUS and verotoxin-producing *Escherichia coli* (VTEC) infection is now well established, and usually prodromic gastroenteritis, frequently including bloody diarrhea, is observed (9). Cases of HUS caused by VTEC have been identified in all age groups but most frequently in infants and young children, and they are observed either during the course of outbreaks of VTEC infections or as sporadic cases. Contamination occurs via consumption of contaminated food, and most of the clinical signs observed are due to the absorption from the gastrointestinal tract of Shiga-like toxins (Stx) produced by the bacteria. Two types of Shiga-like toxins (also called verotoxins), Stx<sub>1</sub> and Stx<sub>2</sub>, which presumably cause microangiopathic hemolytic anemia as a result of endothelial-cell injury, have been isolated. Other bacterial virulence factors may play a role in the pathological process, including an outer membrane protein, intimin, the product of the chromosomal gene *eae*, which is involved in bacterial adhesion to intestinal cells (6), as well as a plasmid-encoded enterohemolysin (Ehly) which has a cytolytic effect (20).

*E. coli* O157:H7 is the worldwide serotype of VTEC most commonly isolated from HUS patients. Other serogroups have been implicated (O26, O55, O103, O111, and O128) (3, 14, 17, 23), but their occurrence is likely to be underestimated, because isolation of non-O157:H7 VTEC still remains a challenge. Unlike most of the O157:H7 isolates, the majority of non-O157:H7 VTEC strains ferment sorbitol and therefore cannot be isolated by using media such as sorbitol MacConkey agar. Molecular biological and immunological techniques based on the detection of verotoxin genes and toxins, respectively, are so far the most reliable methods for detecting these pathogens in clinical specimens.

**Patients and clinical features.** The average number of adults with HUS admitted to the medical intensive-care unit of the Clermont-Ferrand hospital used to be one every 18 months. (This hospital serves a large geographical area with approximately 1.3 million residents.) Between August 1996 and May 1997, this number increased considerably; 14 patients with clinical and biological evidence of HUS were admitted. In six cases, a VTEC strain was identified in the patients' stools by *stx*-specific PCR. The patients' mean age was 64 ± 19 years (range, 39 to 84 years). The male-to-female ratio was 1:5. All the patients developed HUS, defined as a Coombs-negative microangiopathic hemolytic anemia, thrombocytopenia without signs of disseminated intravascular coagulation, and acute renal failure (see Table 1). One of them (patient 1) had previously been admitted to the gastroenterology unit with severe abdominal pain and bloody diarrhea. Eleven days later, development of macroscopic hematuria and acute renal failure prompted her transfer to the intensive-care unit. Coombs-negative microangiopathic hemolytic anemia was defined as a hemoglobin level of <10 g/dl, intravascular hemolysis (serum haptoglobin, ≤0.1 g/liter), negative results of Coombs' test, and fragmented red cells and schistocytes on blood smear. Acute renal failure occurred in all the patients enrolled; four of them required renal replacement therapy. Fever (body temperature of >38°C) was present in four patients. Prodromal bloody diarrhea was observed in two patients, and nonbloody diarrhea was observed in four. All patients were treated with plasma exchanges, and none of them died. The mean number of plasma exchange treatments was 11 ± 2.

**Isolation of VTEC strains by *stx*-specific PCR.** Fecal samples were both cultured in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.) and streaked out on Drigalski plates (Biomerieux, La Balme les Grottes, France), and they were then incubated at 37°C for 18 h. Bacteria from 1 ml of the LB broth culture or from at least 10 single colonies grown on Drigalski agar and previously suspended in 1 ml of saline were harvested, resuspended in 200 µl of sterile water, and incubated at 100°C for 10 min. Following centrifugation of the lysate, 10 µl of the supernatant was used in PCR. Oligonucleotides specific for amplification were 5'-ACCCTGTAACGAA GTTTCGCG-3' and 5'-ATCTCATGCGACTACTTGAC-3' for

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TABLE 1. General, biological, and clinical data of patients during the acute phase and characteristics of the *E. coli* strains isolated from patients' stool specimens

Characteristic	Patient <sup>a</sup>					
	1	2	3	4	5	6
Sex <sup>b</sup>	F	M	F	F	F	F
Age (yr)	45	84	63	39	76	77
Prodromic diarrhea <sup>c</sup>	+ (B)	+ (NB)	+ (NB)	+ (NB)	+ (B)	+ (NB)
Body temperature (°C)	38.5	37	38.5	38	39	37.2
Biological parameter						
Hemoglobin level (g/dl)	6.6	7.4	8.6	5.9	7.1	9.8
Haptoglobin level (g/liter)	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Schistocytes	+	+	+	+	+	+
Platelets (count/ $\mu$ l)	94,000	25,000	22,000	22,000	32,000	25,000
White blood cells (count/ $\mu$ l)	16,000	7,530	4,260	10,810	13,960	9,200
Creatinine ( $\mu$ mol/liter)	647	454	240	1,127	370	542
<i>E. coli</i> characteristic						
Serotype <sup>d</sup>	O6:H4	O91:H10	O91:H21	O rough:H16	OX3:H-	Ont:H-
<i>stx</i> <sup>e</sup>	<i>stx</i> <sub>2</sub>	<i>stx</i> <sub>2</sub>	<i>stx</i> <sub>2</sub>	<i>stx</i> <sub>2</sub>	<i>stx</i> <sub>2</sub>	<i>stx</i> <sub>2</sub>
<i>ehly</i> <sup>e</sup>	-	-	+	+	-	-
<i>eae</i> <sup>f</sup>	-	-	-	-	-	-

<sup>a</sup> +, present; -, not detected.

<sup>b</sup> F, female; M, male.

<sup>c</sup> B, bloody; NB, nonbloody.

<sup>d</sup> Ont, not O serotypeable.

<sup>e</sup> *Stx*- and *Ehly*-encoding genes detected by PCR and specific hybridizations.

<sup>f</sup> *eae* detected by dot blot hybridization.

*stx*<sub>1</sub> and 5'-ATCCTATTCCCGGAGTTTACG-3' and 5'-GC GTCATCGTATACACAGGAGC-3' for *stx*<sub>2</sub> (4, 18). The PCR cycle included denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and extension for 1 min at 72°C (30 cycles) in a Perkin-Elmer Cetus DNA thermal cycler. Each of the primers was used at 0.125 mM, with 0.2 mM each deoxynucleoside triphosphate (Boehringer Mannheim, Meylan, France), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM MgCl<sub>2</sub>, and 1 U of *Taq* DNA polymerase (Appligène-Oncor, Illkirch, France). The reaction products were then analyzed by electrophoresis on 2% agarose gels after staining with ethidium bromide. DNA from the reference strain *E. coli* EDL 933 and a reagent blank, which contained all components except the template DNA, were included as positive and negative controls, respectively. The identities of the PCR products were then confirmed by Southern hybridization after transfer to Hybond N+ nylon membranes (Amersham International, Amersham, United Kingdom) and hybridization with a 1.1-kb *Bam*HI *stx*<sub>1</sub>-specific or a 0.8-kb *Pst*I *stx*<sub>2</sub>-specific DNA probe obtained from the recombinant plasmids pJPN37-19 and pNN111-19, respectively (16). DNA probes were labeled by random priming using the enhanced chemiluminescence system (ECL; Amersham International) according to the manufacturer's specifications, and hybridized filters were exposed to ECL-Amersham film. As shown in Fig. 1, PCR products of 584 bp were detected with the *stx*<sub>2</sub>-specific primers with all stool specimens, but none of them gave a positive reaction with the *stx*<sub>1</sub>-specific primers. Similar results were obtained by colony hybridization using *Stx*<sub>1</sub>- and *Stx*<sub>2</sub>-specific DNA probes (data not shown).

**Bacterial identification and characterization.** *stx*-positive isolates were identified biochemically by using an API 20E test (Biomérieux). All the isolates fermented sorbitol. Determination of their serotypes performed by the International *E. coli* and *Klebsiella* Reference Center in Copenhagen, Denmark, revealed that they belonged to different serotypes: O6:H4 (pa-

tient 1), O91:H10 (patient 2), O91:H21 (patient 3), O rough:H16 (patient 4), and OX3:H- (patient 5). The O-antigenic nature of the VTEC isolate from patient 6 could not be determined (O+:H-). *Ehly*-specific genes were detected by PCR using the primers 5'-CACACGGAGCTTATAATATTCTGT

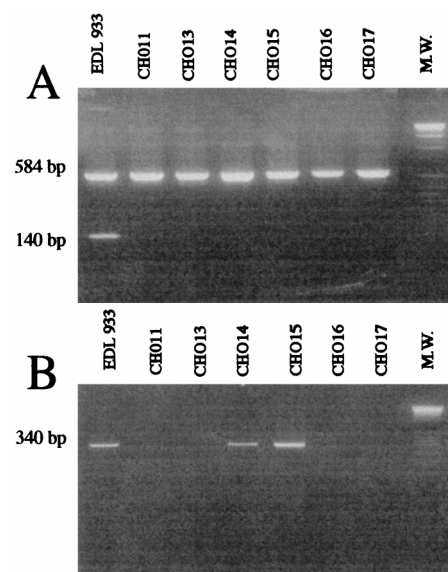


FIG. 1. Agarose gel electrophoresis of DNA fragments obtained by multiplex PCR with primers specific for *stx*<sub>1</sub> (140 bp) and *stx*<sub>2</sub> (584 bp) (A) and with primers specific for *ehly* (340 bp) (B) performed with genomic extracts from different *E. coli* strains: EDL 933, *stx*<sub>1</sub>-, *stx*<sub>2</sub>-, and *ehly*-positive O157:H7 reference strain; CH011, CH013, CH014, CH015, CH016, and CH017, isolates from patients 1 through 6, respectively. M.W., 1-kb ladder of molecular size markers (Boehringer Mannheim).

CA-3' and 5'-AATGTTATCCCATTGACATCATTGACT-3'. Conditions similar to those used for detection of *stx* genes were used, and the PCR products were identified by hybridization with a 3.4-kb *Hind*III fragment from pEO40 (20). Two strains, those isolated from patients 3 and 4, harbored Ehly-specific sequences as determined by PCR (Fig. 1) and hybridization; the same two isolates produced detectable hemolysis after 18 h of growth at 37°C on 5% washed sheep blood agar plates. The presence of *eae* was detected by dot blot hybridization; bacteria were grown in LB broth at 37°C overnight, and DNA was extracted by successive action of lysozyme, proteinase K, and Sarkosyl, followed by a purification step in a cesium chloride gradient. Hybridization was performed as described above by using a DNA probe specific for *eae*, i.e., a 1.4-kb fragment from an O157:H7 clinical isolate covering the entire *eae* open reading frame. DNAs from the reference strains *E. coli* EDL 933 and DH5 $\alpha$  were included as positive and negative controls, respectively. None of the VTEC isolates hybridized with this DNA probe when they were tested under high-stringency conditions.

All the VTEC strains isolated in this study harbored *Stx2*-encoding genes. A higher prevalence of infection with VTEC producing only *Stx2* among HUS patients has been reported in several investigations (10, 22). This may reflect the higher pathogenicity previously observed with *Stx2*- versus *Stx1*-producing strains both in *in vitro* assays with endothelial cells (13) and in murine models (24). All the bacterial strains were sorbitol fermenting, and none of them belonged to the O157:H7 serotype. However, although it is unlikely that we would have missed an O157:H7 isolate in the patients' stools, we cannot exclude the possibility of the occurrence of mixed infections with both a non-O157:H7 and an O157:H7 *E. coli* strain. Previous studies have described a few cases of mixed infections by detecting anti-O157 antibodies in patients' sera (2, 5). Unfortunately, we were not able to test patients' sera for anti-O157 antibody detection in this study. But if we had used routinely performed laboratory procedures with stool specimens, i.e., use of media such as sorbitol MacConkey agar or immunomagnetic separation techniques using anti-O157 antibody-coated beads, none of the present non-O157 isolates would have been detected. Analysis of their ribotype patterns (data not shown) did not reveal any homology, and they all belonged to different serotypes, indicating the sporadic nature of the cases. Three of them belonged to serogroups which have previously been associated with VTEC infections in humans (O91 and O6) (10, 12, 25) and isolated from meat and fecal samples of bovines in both the United States and Europe (15, 19). The O group OX3 is a provisional designation for a new O antigen, but a few isolates from this serogroup, differing from our isolate by the H antigen, have already been isolated from patients suffering from HUS in Europe. In Finland, an *Stx2*-positive *E. coli* OX3:H21 was detected in the stools of a 66-year-old woman, and in Denmark, *E. coli* OX3:H2 was detected in the urine of a patient (8, 11). Since strains belonging to this serogroup are detected in meat samples (19) and in domestic animals (1), they might represent another group of potentially life-threatening VTEC strains causing food infections.

**Virulence factors other than toxins are likely to be required during the pathological process, including adherence factors and/or cytolysins.** Among the six VTEC strains isolated in this study, none harbored the intimin-encoding gene (*eae*), which is involved in the attachment and effacing process, and Ehly sequences were detected in only two isolates. The presence of *eae* has mostly been described in O157:H7 isolates, but *eae*-negative non-O157 VTEC strains are also capable of causing disease indistinguishable from that caused by *eae*-positive

O157:H7 (7, 11). It is likely that *eae*-negative VTEC strains pathogenic for humans may possess adherence factors other than *Eae*; investigations are currently being performed with isolates from this study in order to identify their adherence factors.

The role of the plasmid-encoded Ehly in the pathologic process of VTEC strains is not yet known. Ehly's produced by VTEC strains belong to the RTX (defined as repeats in toxin) toxin family and are closely related to the *E. coli*  $\alpha$  hemolysin. They might act by lysing eucaryotic cells or by modulating the immune response, thus enhancing the virulence of VTEC. Previous studies demonstrated that patients infected with Ehly-positive VTEC were at a higher risk for developing HUS than patients infected with Ehly-negative strains (21). Only two bacterial isolates from this study harbored Ehly-encoding genes, indicating that synthesis of Ehly is not an absolute prerequisite for HUS development, although it might contribute.

From this study, we conclude that Shiga toxin-producing bacteria of serotypes other than O157:H7 can cause serious disease, as has been observed in several other instances. Cases of HUS due to non-O157:H7 *E. coli* are usually sporadic, unlike most of the infections due to serotype O157:H7. The reasons for this difference have not yet been addressed; it might be due to variations in the strains' virulence, but difficulties in identification of non-O157:H7 *E. coli* strains might also contribute to underestimation of their virulence potential. Although the cases of HUS observed in this study occurred in the same geographical area in a relatively short period (10 months), characterization of the VTEC isolates demonstrated that they were not related to each other. This might reflect an endemic situation, and since HUS represents the tip of an iceberg of clinical complications, it is likely that the number of mild infections is greatly underestimated. Development of diagnostic tools allowing detection of VTEC regardless of serotype is therefore urgently needed. Rapid and efficient detection of VTEC should be performed not only with patients suffering from HUS, but with anyone suffering from bloody diarrhea, in order to prevent both severe development of the disease and further spread of the pathogens.

We thank Kristin Swihart for critical review of this paper.

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