

Clinical *Escherichia coli* Strains Carrying *stx* Genes: *stx* Variants and *stx*-Positive Virulence Profiles

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Altogether, 173 Shiga toxin-producing *Escherichia coli* (STEC) serotype O157 ($n = 111$) and non-O157 ($n = 62$) isolates from 170 subjects were screened by PCR-restriction fragment length polymorphism for eight different *stx* genes. The results were compiled according to serotypes, phage types of O157, production of Stx toxin and enterohemolysin, and the presence of *eae*. The *stx* genes occurred in 11 combinations; the most common were *stx*₂ with *stx*_{2c} (42%), *stx*₂ alone (21%), and *stx*₁ alone (16%). Of the O157 strains, 64% carried *stx*₂ with *stx*_{2c} versus 2% of the non-O157 strains ($P < 0.001$). In the non-O157 strains, the prevailing gene was *stx*₁ (99% versus 1% in O157 strains; $P < 0.001$). In addition, one strain (O Rough:H4:*stx*_{2c}) which has not previously been described as associated with hemolytic-uremic syndrome (HUS) was found. Ten *stx*-positive virulence profiles were responsible for 71% of all STEC infections. Of these profiles, five accounted for 71% of the 21 strains isolated from 20 patients with HUS or thrombotic thrombocytopenic purpura (TTP). The strains having the virulence profile that caused mainly HUS or TTP or bloody diarrhea produced Stx with titers of $\geq 1:128$ (90%) more commonly than did other strains (51%; $P < 0.001$). These strains were also more commonly enterohemolytic (98% versus 68% for other strains; $P < 0.001$) and possessed the *eae* gene (100%) more commonly than did other strains (74%; $P < 0.001$). A particular virulence profile, O157:H7:PT2:*stx*₂:*stx*_{2c}:*eae*:Ehly, was significantly more frequently associated with HUS and bloody diarrhea than were other profiles ($P = 0.02$) and also caused the deaths of two children. In this study, the risk factors for severe symptoms were an age of <5 years and infection by the strain of O157:H7:PT2 mentioned above.

Shiga toxin-producing *Escherichia coli* (STEC) cells have emerged as new food-borne pathogens of clinical and public health concern (35). The most epidemic STEC serogroup has been O157, but about 200 other STEC serogroups have been identified (<http://www.microbionet.com.au/frames/feature/vtec/brief01.html>). Shiga toxins (Stx1 and/or Stx2) have a prominent role in the pathogenesis of STEC bacteria (15, 20, 26). The clinical picture of a STEC infection may vary from an asymptomatic state to bloody diarrhea and severe life-threatening complications such as hemolytic-uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP) (35). Children and elderly people have been more susceptible to STEC infections than healthy adults (32). It is estimated that the incidence of HUS varies from 2.0 to 3.0 cases per 100,000 children under 5 years of age (15) and from 0.9 to 1.2 cases per 100,000 in children under 18 years of age (5, 43).

The pathogenesis of STEC infection in humans is not fully understood. It is considered to be multifactorial and dependent on several bacterial virulence factors such as enterohemolysin (Ehly) and the *eae* gene, in addition to host factors (6, 35). A recent discovery of a quorum-sensing system, mediated by self-produced extracellular factors, may play an important role in the control of the colonization of STEC O157 on human cells (17, 18, 44). The main bacterial properties in the pathogenesis of HUS, however, are considered to be the Stx toxins encoded by the *stx* genes. It is known that STEC strains frequently carry more than one *stx* gene, and several variants of

these genes (e.g., *stx*_{1c}, *stx*_{1vO111}, *stx*_{2vha}, *stx*_{2vha}, *stx*_{2vha}, *stx*_{2vOX392}, *stx*_{2vOX393}, *stx*_{2c}, *stx*_{2d-Ount}, *stx*_{2d-OX3a}, *stx*_{2e}, *stx*_{2ev}, *stx*_{2f}) have been found (1, 16, 27, 30, 37, 41, 42, 46, 48). In the literature, different designations have been used for the same genes, causing confusion. However, recently, Scheutz et al. (40) compiled data on the designations of the *stx* genes. In the Stx2 toxin family, the amino acid homologies in the A subunit to the mature Stx2 are 93, 99, and 100% for Stx2e, Stx2d (also designated as Stx2vh [40]), and Stx2c, respectively (31). The corresponding percentages in the B subunit are 84, 97, and 97% (31).

Previously obtained data (13, 16, 37) have suggested that not only the main toxin type but also the toxin variant type could be important in determining the probability of developing HUS. For example, humans infected by strains producing Stx2 have developed HUS more frequently than those infected by strains producing Stx1 only (7, 34). Of the *stx*₂ variants, *stx*_{2c} has been associated with HUS but the risk of developing HUS after infection with STEC of the *stx*_{2c} genotype has been significantly lower than that after infection with STEC of the *stx*₂ genotype only (13, 16). The strains carrying the *stx*_{2d} genes encoding VT2d-Ount or VT2d-OX3a have been concluded to be less pathogenic for humans than other Stx2-class-toxin-producing strains (37). *stx*_{2d} has been associated with various forms of diarrhea but not with HUS (13). Of the other *stx* variants, *stx*_{2e} has been associated with edema disease in pigs (30, 46) though it has been detected rarely in human infections (13). *stx*_{2f} has been isolated in STEC isolates from pigeons, and only a single STEC human isolate harboring a similar *stx* gene has been found (41).

By combining PCR with restriction fragment length polymorphism (PCR-RFLP) (37), we investigated all the 173 hu-

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TABLE 1. *stx* genes expected to be detected by PCR or PCR-RFLP methods used in this study

Primer pairs for indicated <i>stx</i> genes ^a	Size (bp) of PCR product	<i>stx</i> gene	Sizes (bp) ^b of restricted PCR-RFLP products	Reference(s)
All (<i>HincII</i> and <i>AccI</i>) LIN (5' GAACGAAATAATTTATATGT) and LIN (3' TTTGATTGTTACAGTCAT)	ca. 900	<i>stx</i> ₁ <i>stx</i> _{1vO111} <i>stx</i> ₂ <i>stx</i> _{2c} <i>stx</i> _{2vha} <i>stx</i> _{2vOX393} <i>stx</i> _{2vbb} <i>stx</i> _{2vO111} <i>stx</i> _{2vOX392} <i>stx</i> _{2e} <i>stx</i> _{2ev}	705, 158, 32; 768, 127 705, 158, 32; 768, 127 555, 262, 62; 544, 351 555, 324, 16; 544, 351 555, 324, 16; 544, 351 555, 324, 16; 544, 351 555, 340; 544, 351 880, 15; 544, 351 880, 15; 544, 351 555, 340; 900 521, 374; 900	1, 27
<i>stx</i> _{2d} VT2-cm (AAGAAGATATTTGTAGCGG) and VT2-f (TAAACTGCACTTCAGCAAAT)	256	<i>stx</i> _{2d-Ount} <i>stx</i> _{2d-OX3a}	— ^c — ^c	37
<i>stx</i> _{2d} (<i>HaeIII</i> and <i>PvuII</i>) VT2-e (AATACATTATGGGAAAGTAATA) and VT2-f (TAAACTGCACTTCAGCAAAT)	348	<i>stx</i> _{2d-Ount} <i>stx</i> _{2d-OX3a}	216, 132; 200, 120 (28) ^d 167, 132 (49) ^d ; 200, 120 (28) ^d	37

^a Restriction enzymes are given in parentheses after gene designations.

^b Sizes are grouped according to the order in which the restriction enzymes are given in column 1.

^c —, detection of *stx*_{2d-Ount} (256 bp) and *stx*_{2d-OX3a} (256 bp) by PCR with no restriction enzymes.

^d This fragment did not resolve or was too small to be clearly visible under the electrophoretic conditions used.

man STEC strains isolated during the period from 1990 to 2000 from Finns with STEC infection to evaluate the prevalence of the *stx*_{2d} genes for VT2d-Ount (hereafter referred to as *stx*_{2d-Ount}) or VT2d-OX3a (hereafter *stx*_{2d-OX3a}) among these strains. We also subtyped all STEC isolates for the possession of the *stx*₁/*stx*_{1vO111} (hereafter *stx*₁), *stx*₂, *stx*_{2c}/*stx*_{2vha}/*stx*_{2vOX393} (hereafter *stx*_{2c}), *stx*_{2vbb}, *stx*_{2vO111}/*stx*_{2vOX392} (hereafter *stx*_{2vO111} and *stx*_{2vOX392}), *stx*_{2e}, and *stx*_{2ev} genes (1, 27). In addition, the association of these *stx* genes with the clinical pictures of the subjects and with several other bacterial characteristics (the serotype, the possession of the *eae* gene, the titers of the Stx toxins, the production of Ehly, and the phage types [PTs] of the serogroup O157 strains) was studied. We also searched for specific virulence profiles potentially associated with the clinical picture of STEC infection.

MATERIALS AND METHODS

Subjects. The age data and the clinical pictures (HUS or TTP, bloody diarrhea, nonbloody diarrhea, or asymptomatic carriage) of the 170 subjects were indicated on a special form that always accompanied the isolate that was received from the clinical microbiology laboratory, or the diagnosis was inquired from the hospital over the telephone. Also, the relationship between asymptomatic carriers and STEC-infected patients was indicated. The subjects were divided into seven age groups: <1, 1 to 5, 6 to 15, 16 to 25, 26 to 55, 56 to 65, and >65 years old (38).

STEC strains. One hundred seventy-three STEC strains (O157 isolates, *n* = 111; non-O157 isolates, *n* = 62) were isolated from Finns (*n* = 170) during an 11-year period (1990 to 2000). The pure cultures of the isolates or fecal cultures of the STEC-infected patients were referred to the Finnish routine microbiological laboratories for microbiological verification and detection of the *stx*₁, *stx*₂, and *eae* genes by PCR. All STEC isolates were also serotyped and their enterohemolytic activity was investigated, and the STEC O157 isolates were phage typed as previously described (12, 25, 39). Certain single characteristics of 161 strains have been reported previously (12, 22, 23, 24, 25, 39). Of the 173 strains, 15 were derived from one major outbreak (36) caused by strains of serotype O157:H7:PT2:*stx*₂:*stx*_{2c} (39). In addition, 65 strains were associated with small family clusters in 24 families.

Shiga toxin production. The production of Stx1 and Stx2 by the isolates was determined by using a reversed-passive latex agglutination kit (VTEC-RPLA; Denka Seiken Co., Ltd., Tokyo, Japan) after having been grown and shaken in 5 ml of Casamino Acids-yeast extract broth overnight at 37°C. Of this suspension, 1 ml was shaken with 1 ml of polymyxin B (5,000 U) solution (2) for 1 h at 37°C and was then centrifuged for 30 min at 3,000 rpm (BiofugeA instrument Heraeus, Sepatech, West Germany). The titer of the supernatant was determined in the VTEC-RPLA test according to the manufacturer's instructions up to 1:128. All strains were tested for the production of Stx1 and Stx2. Titers lower than 1:4 were interpreted as negative.

Detection of *stx* genes by PCR-RFLP. PCRs for the *stx*₁, *stx*₂, and *stx*₂ variants (1, 27, 37) (Table 1) were executed with minor modifications: boiled bacterial supernatant (1.0 μl) was used as a template, and a final elongation step (10 min at 72°C) was added to each PCR run (39). Amplified DNA fragments of specific sizes were located in electrophoresis gels by UV fluorescence after staining with ethidium bromide. Strain ATCC 43895 (RH 4270) was used as a positive control in the run according to Lin et al. (27) and Bastian et al. (1). Strain LMG 18459 (RH 4872) (the Belgian Coordinated Collections of Microorganisms-Laboratorium voor Microbiologie Universiteit Gent bacterium collection, Universiteit Gent, Ghent, Belgium) was used as a positive control in the run according to Piérard et al. (37). In all runs, strain ATCC 25922 (RH 1484) was used as a negative control. The *stx*-positive PCR products were restricted with 20 U of four restriction enzymes (New England Biolabs Inc., Beverly, Mass.): a ca. 900-bp fragment with *HincII* and *AccI* (1, 27) and a 348-bp fragment with *HaeIII* and *PvuII* (37).

Statistical methods. Epi-Info 2000 version 1.1.2 was used for Fisher's exact two-tailed test. A *P* of <0.05 indicated statistical significance.

RESULTS

***stx* genotypes.** Of the 173 STEC strains studied, 168 (97%) gave *stx*-positive results in PCR for the *HincII* and *AccI* restriction enzymes. Three strains were repeatedly negative in PCR, and two showed very weak positive reactions. The 168 STEC strains that were clearly positive in PCR were divided into 11 groups after *HincII* and *AccI* endonuclease restriction (Table 2). Seventy-two (42%) of all 173 strains possessed *stx*₂ in combination with *stx*_{2c}. The *stx*₂ gene alone was found in 36 strains (21%); the *stx*₁ gene was found alone in 28 strains

TABLE 2. *stx* genes in 173 STEC isolates and toxin production of the strains

Method and related <i>stx</i> genes	No. (%) of strains [n = 173]	No. of samples with indicated titers of Stx toxin			
		≤1:2	1:4-1:16	1:32-1:64	≥1:128
PCR for <i>stx</i> according to Bastian et al. (1) and Lin et al. (27)					
Positive strains	168 (97)	3	11	47	117
Weak positive strains ^a	3 (2)	0	0	1	2
Negative strains ^a	2 (1)	1	0	1	0
<i>stx</i> genes detected after restriction (<i>HincII</i> and <i>AccI</i>) ^b					
<i>stx</i> ₁	28 (16)	0	1	18	9
<i>stx</i> ₁ and <i>stx</i> ₂ or <i>stx</i> _{2c} variant	10 ^b (6)				
Stx1 titers		0	1	9	0
Stx2 titers ^c		0	2	2	6
<i>stx</i> ₂ and <i>stx</i> _{2c}	72 (42)	0	0	0	72
<i>stx</i> ₂	36 (21)	0	1	13	22
<i>stx</i> _{2c}	9 (5)	0	4	3	2
<i>stx</i> _{2vhhb}	5 (3)	1	0	0	4
<i>stx</i> ₂ and <i>stx</i> _{2vhhb}	3 (2)	0	0	1	2
Undigestible	5 ^d (3)	2	2	1	0
PCR for <i>stx</i> _{2d} according to Piérard et al. (37)					
Positive strains	2 ^d (1)	1	1	0	0
Negative strains	171 (99)	3	11	49	119
<i>stx</i> genes detected after restriction (<i>HaeIII</i> and <i>PvuII</i>) ^e					
<i>stx</i> _{2d-Ount}	2 ^d (1)	1	1	0	0

^a This result was obtained repeatedly.

^b No *stx*_{2e}, *stx*_{2ev}, *stx*_{2vO111}, or *stx*_{2vOX392} genes were detected.

^c The corresponding *stx*₂ genes were *stx*_{2c} (titers of 1:4 to 1:16 [two strains], 1:32 to 1:64 [two strains], and ≥1:128 [two strains]), *stx*₂ (titer of ≥1:128 [one strain]), *stx*_{2vhhb} (titers of ≥1:128 [two strains]), and *stx*₂ and *stx*_{2vhhb} (titer of ≥1:128 [one strain]).

^d Two undigestible strains were positive for *stx*_{2d-Ount}.

^e No *stx*_{2d-OX3a} genes were detected.

(16%) and in combination with either *stx*₂ or some *stx*₂ variants (*stx*_{2c} and *stx*_{2vhhb}) in 10 strains (6%). Of the latter strains, one possessed three *stx* genes: *stx*₁, *stx*₂, and *stx*_{2vhhb}. Nine strains (5%) were positive for *stx*_{2c} only, five strains were positive for *stx*_{2vhhb}, and three strains were positive for both *stx*₂ and *stx*_{2vhhb}. Five strains were positive in PCR but were undigestible by the *HincII* or *AccI* restriction enzymes (Table 2).

In PCR for screening *stx*_{2d}, only two strains of the 173 were positive (1%). Both of them were confirmed as the *stx*_{2d-Ount} variant type after *HaeIII* and *PvuII* restriction (Table 2). These strains were undigestible by the *HincII* or *AccI* restriction enzymes in the PCR according to Lin et al. (27) and Bastian et al. (1).

Stx production. Of the 173 strains, 169 (98%) produced Stx1 or Stx2 or both (Table 2). The majority of the strains (119 of 173 [69%]) produced Stx with high titers (≥1:128) not depending on their *stx* type. However, of the 168 PCR-positive strains, 98 (87%) of the 113 strains possessing the *stx*₂ gene with or without any other genes produced Stx with titers of ≥1:128 whereas the strains without the *stx*₂ gene did not (24 of 55 strains [44%]; $P < 0.001$). Lower titers (<1:128) were observed especially in the strains possessing *stx*_{2c} (7 of 9 [78%]) and in the strains possessing *stx*₁ with or without other genes (29 of 38 strains [76%]). Of the strains producing Stx1 only, 32% (9 of 28) produced the Stx toxin with high titers (≥1:128) compared with 78% (102 of 130) of the strains producing Stx2 only ($P < 0.001$). Four strains (2%), three positive and one negative for *stx* in PCR, did not produce Stx. Of the two strains carrying *stx*_{2d}, one did not produce detectable levels of Stx2 and the Stx

titer of the other was low (1:4). However, both strains produced Stx1 with titers of 1:16 although *stx*₁ was not detected by the method used.

Serotypes, presence of *eae*, and Ehly production. Of all the strains, 111 (64%) belonged to the O157 serogroup. Within this O group, the most common *stx* gene combination was *stx*₂ with *stx*_{2c} (71 strains [64%]) whereas among the 62 non-O157 strains, this gene combination was found in only one strain (2%; $P < 0.001$) (Table 3). In contrast, among non-O157 strains, the most common *stx* gene was *stx*₁ (27 of 62 strains [44%]), which, among the 111 O157 strains, was found in one strain only (1%; $P < 0.001$). The *stx*₂ gene alone was equally common among the O157 (21 of 111 [19%]) and non-O157 (15 of 62 [24%]) strains. Almost all the strains carrying these *stx* genes also had the *eae* gene, and they produced Ehly (Table 3). On the other hand, of the nine strains carrying the *stx*_{2c} gene only, six (67%) strains of the non-O157 group were negative for both *eae* and Ehly. The two strains carrying *stx*_{2d-Ount} were both of serogroup non-O157 (O Rough:H⁻ and O76:H19); neither of them carried *eae*, but both produced Ehly.

PTs. Distribution of the *stx* genes by PTs among the strains of the O157 serogroup showed that 55 of 59 (93%) of the PT2 strains and all 10 PT49 strains had the *stx*₂ and *stx*_{2c} genes (Table 4). The PT4 strains had *stx*₂ only (7 of 13 [54%]) almost as often as *stx*₂ with *stx*_{2c} (5 of 13 [38%]), whereas the PT8 strains had most commonly *stx*₁ (8 of 9 [89%]) with the *stx*₂ genes (*stx*_{2c} [five strains], *stx*_{2vhhb} [two strains], and *stx*₂ with *stx*_{2vhhb} [one strain]). The nine strains belonging to the reacts-

TABLE 3. *stx* genes in 173 STEC isolates and other characteristics of the strains

<i>stx</i> gene(s)	Total no. of strains (<i>n</i> = 173)	No. of strains possessing the indicated characteristic (%):					
		O157 (<i>n</i> = 111)	Non-O157 (<i>n</i> = 62)	<i>eae</i> positive (<i>n</i> = 151)	<i>eae</i> negative (<i>n</i> = 22)	Ehly positive (<i>n</i> = 144)	Ehly negative (<i>n</i> = 29)
<i>stx</i> ₁	28 (16)	1 ^a (1)	27 (44)	26 (17)	2 (9)	27 (19)	1 (3)
<i>stx</i> ₁ and <i>stx</i> _{2c}	6 (3)	5 (5)	1 (2)	6 (4)	0 (0)	5 (3)	1 (3)
<i>stx</i> ₁ and <i>stx</i> ₂	1 (1)	1 (1)	0 (0)	1 (1)	0 (0)	1 (1)	0 (0)
<i>stx</i> ₁ and <i>stx</i> _{2vhhb}	2 (1)	2 (2)	0 (0)	2 (1)	0 (0)	1 (1)	1 (3)
<i>stx</i> ₁ , <i>stx</i> ₂ , and <i>stx</i> _{2vhhb}	1 (1)	1 (1)	0 (0)	1 (1)	0 (0)	0 (0)	1 (3)
<i>stx</i> ₂ and <i>stx</i> _{2c}	72 (42)	71 ^a (64)	1 (2)	71 (47)	1 (5)	72 (50)	0 (0)
<i>stx</i> ₂	36 (21)	21 (19)	15 (24)	35 (23)	1 (5)	28 (19)	8 (28)
<i>stx</i> _{2c}	9 (5)	3 (3)	6 (9)	3 (2)	6 (27)	3 (2)	6 (21)
<i>stx</i> _{2vhhb}	5 (3)	3 (3)	2 (3)	3 (2)	2 (9)	3 (2)	2 (7)
<i>stx</i> ₂ and <i>stx</i> _{2vhhb}	3 (2)	1 (1)	2 (3)	1 (1)	2 (9)	2 (1)	1 (3)
<i>stx</i> _{2d-Ount}	2 (1)	0 (0)	2 (3)	0 (0)	2 (9)	2 (1)	0 (0)
Undigestible ^b	3 (2)	0 (0)	3 (5)	0 (0)	3 (14)	0 (0)	3 (10)
Weak positive strains ^c in PCR for <i>stx</i> ^b	3 (2)	1 (1)	2 (3)	1 (1)	2 (9)	0 (0)	3 (10)
Negative strains ^d in PCR for <i>stx</i> ^b	2 (1)	1 (1)	1 (2)	1 (1)	1 (5)	0 (0)	2 (7)

^a *P* < 0.001 compared with that for non-O157.

^b PCR-RFLP for *stx* according to Bastian et al. (1) and Lin et al. (27).

^c Repeatedly weak results.

^d Repeatedly negative results.

but-does-not-conform phenotype group had a variable set of the *stx* genes.

Association of *stx* with clinical symptoms. The clinical diagnosis was available for 160 (94%) of the 170 patients (Fig. 1). Of them, three patients were simultaneously infected by two different STEC strains (O157 and non-O157 strains). HUS or TTP was diagnosed in 20 (12%) patients, bloody diarrhea occurred in 77 (45%) patients, nonbloody diarrhea occurred in 37 (22%) patients, and asymptomatic carriage was determined in 26 (16%) subjects. Twenty-four subjects were family members of the children with HUS (11 subjects), bloody diarrhea (6 subjects), nonbloody diarrhea (6 subjects), or an unknown clinical picture (1 subject). However, of the 11 patients with HUS, a STEC strain could be isolated from four of the subjects. Among the asymptomatic subjects, two findings were connected to enhanced screening of travelers (22). Of the 20 patients with HUS, most were 1- to 5-year-old children (13 of 20 subjects [65%] versus 47 of 150 [31%] for other clinical groups; *P* = 0.007), and of the asymptomatic carriers, most were 26- to 55-year-old subjects (15 of 26 subjects [58%] versus 20 of 144 [14%] for other clinical groups; *P* < 0.001).

In the strains found in subjects with HUS or TTP, bloody diarrhea, nonbloody diarrhea, or asymptomatic carriage, the most common *stx* genes were *stx*₂ with *stx*_{2c} (detected in 50, 53, 30, and 35% of patients, respectively) and *stx*₂ only (detected in 25, 14, 22, and 31% of patients, respectively) (Table 5). Of the nine asymptomatic persons infected by STEC harboring *stx*₂ and *stx*_{2c}, all were associated with a patient with HUS, bloody diarrhea, or nonbloody diarrhea (two, three, and four subjects, respectively). Five were 16- to 55-year-old family members of the STEC-infected patients and four were <1- to 15 year-old siblings. Of the eight asymptomatic carriers from whom a STEC strain possessing *stx*₂ was isolated, six were connected to patients with HUS or to a patient with nonbloody diarrhea (one subject). One infection was sporadic. Of these eight subjects, five were 16- to 55-year-old adults and three were siblings under 10 years old.

In addition, among the strains associated with HUS or TTP,

there were three (15%) strains possessing *stx*_{2c} alone, an undigestible strain, and a PCR-negative strain. None of the 38 *stx*₁-positive strains (28 *stx*₁ only, 10 with *stx*₂ or the *stx*₂ variant) were associated with HUS or TTP, although they were the third most common genes of all the STEC strains and were equally common in all other clinical groups. The only strain possessing three distinct *stx* genes (*stx*₁, *stx*₂, and *stx*_{2vhhb}) was isolated from a patient with bloody diarrhea.

The strains of 10 different *stx*-positive virulence profiles (P1 through P10) were responsible for the majority (120 of 170

TABLE 4. *stx* genes among sorbitol-negative and sorbitol-positive STEC O157 strains belonging to different PTs

PT (<i>n</i>)	<i>stx</i> gene(s)	Sorbitol fermentation of strains (<i>n</i> = 111)		Total no. of strains (%)
		Negative (<i>n</i> = 103)	Positive (<i>n</i> = 8)	
PT2 (59)	<i>stx</i> ₂ and <i>stx</i> _{2c}	55	0	55 (93)
	<i>stx</i> ₂	4	0	4 (7)
PT4 (13)	<i>stx</i> ₂ and <i>stx</i> _{2c}	5	0	5 (38)
	<i>stx</i> ₂	7	0	7 (54)
PT8 (9)	<i>stx</i> ₂ and <i>stx</i> _{2vhhb}	1	0	1 (8)
	<i>stx</i> ₁ and <i>stx</i> ₂ variant ^a	8	0	8 (89)
	<i>stx</i> _{2c}	1	0	1 (11)
PT14 (1)	<i>stx</i> ₂	1	0	1 (100)
PT21/28 (1)	<i>stx</i> ₁ and <i>stx</i> ₂	1	0	1 (100)
PT34 (1)	<i>stx</i> ₁	1	0	1 (100)
PT49 (10)	<i>stx</i> ₂ and <i>stx</i> _{2c}	10	0	10 (100)
PT50 (2)	<i>stx</i> ₂	2	0	2 (100)
PT88 (6)	<i>stx</i> ₂	0	4	4 (67)
	Weak positive ^b	0	1	1 (17)
	Negative ^b	0	1	1 (17)
RDNC ^c (9)	<i>stx</i> ₂	1	2	3 (33)
	<i>stx</i> ₂ and <i>stx</i> _{2c}	1	0	1 (11)
	<i>stx</i> _{2vhhb}	3	0	3 (33)
	<i>stx</i> _{2c}	2	0	2 (22)

^a *stx*₂ variants were *stx*_{2c} (five strains), *stx*_{2vhhb} (two strains), and *stx*₂ and *stx*_{2vhhb} (one strain).

^b Repeatedly obtained result in PCR-RFLP for *stx* according to Bastian et al. (1) and Lin et al. (27).

^c RDNC, reacts but does not conform phenotype.

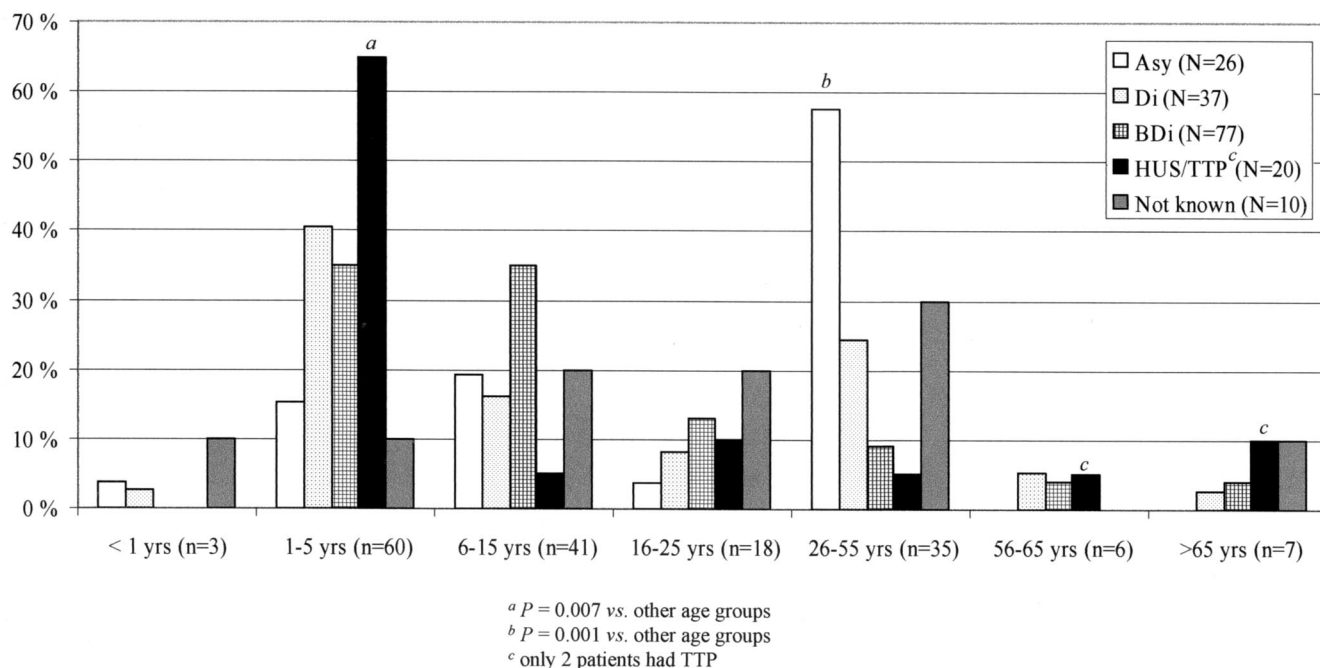


FIG. 1. Percentage of STEC-infected subjects in different age groups according to the clinical picture of the infection (asymptomatic carriage [Asy], nonbloody diarrhea [Di], bloody diarrhea [BDi], HUS or TTP, or clinical picture not known).

[71%] of all STEC infections (Table 6). The strains belonging to five virulence profiles (P1 through P5) accounted for 71% (15 of 21) of all the strains found in patients with HUS or TTP but only 4% (6 of 152) of all remaining strains associated with HUS or TTP (P = 0.03). Of the strains associated with HUS or TTP, nine (43%) were of the non-O157 serogroup: three strains of O145:H28/H⁻:stx₂ and one strain each of OX174:H21:stx_{2c}, O Rough:H4:stx_{2c}, O101:H⁻:stx₂, O Rough:H49:stx_{2c}, O107:H27 lacking stx, and O2:H29:undigestible. P1 was the most prevalent virulence profile, with 55 strains, and

these strains more often caused HUS and bloody diarrhea (39 of 55 [71%]) than other clinical symptoms (16 of 55 [29%]). This association with HUS and bloody diarrhea (71%) was statistically significant compared with that for all other strains (60 of 118 strains [51%]; P = 0.02). The P1 virulence profile also caused the deaths of two children under 5 years of age. The strains belonging to profiles P1 through P10 (95 of 120 strains [79%]) produced Stx toxin with titers of ≥1:128 more commonly than did other strains (24 of 53 strains [45%]; P < 0.001). These strains were also enterohemolytic (112 of 120

TABLE 5. Distribution of different stx genes in STEC strains according to the clinical pictures of the 160 patients for whom clinical data were available

stx gene(s) (n)	No. of patients with symptom or diagnosis (%):				
	HUS or TTP ^f (n = 20) ^a	Bloody diarrhea (n = 77) ^b	Nonbloody diarrhea (n = 37)	Asymptomatic (n = 26) ^c	Not known (n = 10)
stx ₂ only (34)	5 ^a (25)	11 ^b (14)	8 (22)	8 (31)	2 (20)
stx ₂ and stx _{2c} (72)	10 (50)	41 (53)	11 (30)	9 (35)	1 (10)
stx _{2c} (9)	3 (15)	2 (3)	1 (3)	2 (8)	1 (10)
Undigestible (2)	1 ^f (5)	0 (0)	0 (0)	1 ^c (4)	0 (0)
PCR negative ^e (2)	1 ^f (5)	1 ^b (1)	0 (0)	0 (0)	0 (0)
stx ₂ and stx _{2v} hb (3)	0 (0)	1 (1)	2 (5)	0 (0)	0 (0)
stx ₂ or stx ₂ variant and stx ₁ (10)	0 (0)	5 (6)	3 (8)	1 (4)	1 (10)
stx _{2v} hb (5)	0 (0)	3 (4)	2 (5)	0 (0)	0 (0)
stx _{2d-Ount} (2)	0 (0)	1 (1)	1 (3)	0 (0)	0 (0)
stx ₁ (28)	0 (0)	12 (16)	7 (19)	4 (15)	5 (50)
Weak positive ^d (3)	0 (0)	0 (0)	2 (5)	1 (4)	0 (0)

^a One patient had a simultaneous infection with O157:H⁻:PT88:stx₂ and O145:H28:stx₂ strains (the number of isolates is 21 when including the O145:H28:stx₂ strain).
^b One patient had a simultaneous infection with O157:H⁻:PT88 lacking stx and O145:H28:stx₂ strains (the number of isolates is 78 when including the O145:H28/H⁻:stx₂ strain).

^c One patient had a consecutive infection with O102:H7:undigestible and O Rough:H18:undigestible (the number of isolates is 27 when including the O Rough:H18:undigestible strain).

^d Repeatedly obtained result in PCR for stx according to Bastian et al. (1) and Lin et al. (27).

^e Negative results in PCR for stx according to Bastian et al. (1) and Lin et al. (27).

^f Two patients had TTP.

enzymes or the strains may have changed during storage and cultivation.

A considerable share (21 or 42%) of the strains carried *stx*₂ only or *stx*₂ with *stx*_{2c}. Also, in Belgium and Germany, *stx*₂ alone and *stx*₂ with either *stx*_{2vha} or *stx*_{2c} have been prevalent (7, 13, 37). In our strains, the third most common gene was *stx*₁, which was present alone in 28 strains and in combination with *stx*₂, *stx*_{2c}, or *stx*_{2vha} in 10 strains. In Germany, of the 212 human isolates harboring *stx*₁, almost half also carried *stx*₂, *stx*_{2c}, or *stx*_{2d} (48). Of our strains, only two (1%) were of the *stx*_{2d-Ount} variant type. In Belgium and Germany, the *stx*_{2d} gene has been more prevalent and has been found in 7% of 359 human or animal isolates (37) and in 4% of 626 human isolates (13). These data indicate similar distributions of the *stx* genes in different European countries.

By the VTEC-RPLA assay, the strains carrying the *stx*₂ gene with or without any other genes produced Stx significantly more than did the strains without the *stx*₂ gene. Lower titers were observed especially within the strains possessing *stx*₁, *stx*_{2c}, or *stx*_{2d}. The VTEC-RPLA assay has previously been found to be a reliable method for the detection of Stx1, Stx2, and Stx2c but not for the Stx2e porcine or Stx2d-Ount variant toxins (2, 3, 21). Our results might indicate a weak capability of the gene to express the Stx1 toxin or a putative carriage of the *stx*_{1c} gene and thus a possible weak ability to detect the Stx1c toxin by the VTEC-RPLA, as suggested by Zhang et al. (48). Of our two strains carrying *stx*_{2d-Ount}, both produced Stx1. However, these strains were negative for *stx*₁ although they were positive in PCR (12) with primers described by Olsvik and Strockbine (33). Similarly, four strains (O107:H27 lacking *stx*, O157:H- lacking *stx*, O157:H7:*stx*₁, and O43:H2:*stx*₁) negative for any *stx*₂ gene on the basis of the method of Lin et al. (27) and Bastian et al. (1) had previously been positive for *stx*₂ either as a sole gene (O107:H27 lacking *stx* and O157:H- lacking *stx*) or in combination with *stx*₁ (O157:H7:*stx*₁ and O43:H2:*stx*₁) (12, 25, 39). In addition, one strain (O91:H40) possessed *stx*₁ with *stx*_{2c} in this study, although previously it has been shown to possess only *stx*₂ and to produce Stx2 toxin only (12, 25). These results clearly demonstrate the differences among different protocols in the detection of the *stx* genes.

The comparison of the distribution of the *stx* genes in strains of the O157 and non-O157 serogroups showed that *stx*₂ with *stx*_{2c} was statistically significantly associated with strains of the O157 serogroup. Also in Belgium, *stx*₂ with *stx*_{2vha} was prevalent among the human STEC O157 isolates, unlike *stx*₁, which was more prevalent in other O serogroups (37). Also among our non-O157 strains, the significant association with *stx*₁ was seen. This gene was most common in strains of serotypes O26:H11/H- and O103:H2/H-. In France, the serotype O103:H2 has commonly possessed *stx*₁ (29).

*stx*₂ with the *stx*_{2c} gene was found in strains associated with all symptoms but mostly in strains associated with bloody diarrhea or HUS. In contrast, none of the strains carrying *stx*₁ alone or in combination with another gene were associated with HUS or TTP. However, in France, strains of the serotype O103:H2 carrying *stx*₁ have been found in HUS patients (29). Also, in Germany, a child infected by STEC O118:H- positive for *stx*₁ suffered from HUS (47). Neither of our *stx*_{2d-Ount}-infected patients had HUS or TTP; this supports other studies

describing the presence of the *stx*_{2d} gene in isolates from patients with symptoms milder than HUS or TTP (13, 35, 47, 48).

One of our strains isolated from a patient with bloody diarrhea carried three distinct genes simultaneously. This finding is an example that the infection caused by STEC bacteria possessing several *stx* genes does not always cause a severe clinical picture, such as HUS, as observed previously also by others (14).

Most of the patients with HUS were 1- to 5-year-old children, and most of the asymptomatic carriers were 26- to 55-year-old adults. Thus, our data are in accordance with other studies concerning the age as a risk factor for STEC infection (8, 11, 13, 32).

Three of the 170 subjects, one with HUS, one with bloody diarrhea, and one an asymptomatic carrier, had infections caused by two STEC strains of different serotypes: correspondingly, O157:H-:PT88:*stx*₂ and O145:H28:*stx*₂, O157:H-:PT88 lacking *stx* and O145:H28:*stx*₂, or O102:H7:undigestible and O Rough:H18:undigestible. Similarly, in the Czech Republic, some HUS patients were infected with two different STEC serotypes: O157:H7 and O5:H-, O157:H7 and O55H?, O157:H7 and O111:H-, O26:H11 and O5:H-, or O111:H- and O1:H- (4). These findings support the possibility of people being infected by several STEC strains simultaneously.

The strains belonging to five virulence profiles (from P1 to P5) accounted for 71% of all strains found in patients with HUS. In addition, of all the strains studied, those carrying *stx*₂ with *stx*_{2c} mainly belonged to the virulence profile P1 (O157:H7:PT2:*stx*₂:*stx*_{2c}) and, among them, the majority (71%) were associated with HUS or bloody diarrhea. All these strains possessed *eae*, were enterohemolytic, and produced Stx2 with high titers. Strains of the virulence profile P1 also caused the deaths of two children under 5 years of age. Our results are also supported by other studies concerning the carriage of *stx*₂ with *stx*_{2c} (7, 13). Friedrich et al. (13) found that 68 (25%) of 268 STEC isolates from HUS patients harbored *stx*₂ with *stx*_{2c}, and among all the 626 STEC isolates investigated, these 68 HUS-associated strains accounted for 11% of the cases of HUS infection. Also, in a study by Cornu et al. (7), nine STEC isolates of serogroup O157 and four STEC non-O157 isolates carrying *stx*₂ only or *stx*₂ with *stx*_{2vha} were identified in 13 cases of HUS.

In our study, seven virulence profiles of STEC non-O157 were associated with HUS or TTP (O145:H28/H-:*stx*₂, OX174:H21:*stx*_{2c}, O Rough:H4:*stx*_{2c}, O101:H-:*stx*₂, O Rough:H49:*stx*_{2c}, O107:H27 lacking *stx*, and O2:H29:undigestible). In the literature (10; <http://www.microbionet.com.au/frames/feature/vtec/brief01.html>; <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>), serotype O Rough:H4 has never been published as being associated with patients suffering from HUS.

In this study, the risk factor for HUS in STEC-infected subjects was young age (1 to 5 years) and an infection especially by STEC O157:H7:PT2:*stx*₂:*stx*_{2c}:*eae*:Ehly but also by O145:H28/H-:*stx*₂:*eae*:Ehly. Of the 21 strains causing HUS or TTP, almost half (43%) belonged to the non-O157 serogroup. This high prevalence of the non-O157 STEC strains indicates the importance of the detection of all STEC serogroups in clinical diagnostics. PCR and Stx toxin detection assays have proven to be essential methods worldwide (10), and they should be used routinely in all hospital laboratories. However,

the development of molecular methods of high capacity for detecting all *stx* genes should be considered in future studies. In addition, the further characterization of the *stx* genes can be exploited for predicting the clinical outcome of STEC infection, especially in young children.

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