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Subtyping Method for *Escherichia coli* Shiga Toxin (Verocytotoxin) 2 Variants and Correlations to Clinical Manifestations▽

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Shiga toxin 2 (Stx2) from Shiga toxin-producing *Escherichia coli* (STEC) was subtyped by a method involving partial sequencing of the $stxAB_2$ operon. Of 255 strains from the Danish STEC cohort, all 20 cases of hemolytic-uremic syndrome were associated with subtype Stx2 (11 cases), subtype Stx2c (1 case), or the two combined (8 cases).

Infections caused by Shiga toxin-producing *Escherichia coli* (STEC), alternatively known as verocytotoxin-producing *E. coli*, account for the most severe symptoms among those caused by diarrheagenic *E. coli*, sometimes leading to bloody diarrhea (BD) or hemolytic-uremic syndrome (HUS). STEC is defined by the ability to produce Shiga toxins (Stx or verocytotoxin) 1 and 2, which are two sequentially and antigenetically distinct toxin groups transcribed from the $stxAB_1$ and $stxAB_2$ operons, respectively. Besides the *stx* gene(s), STEC strains often carry the gene encoding the adherence factor intimin (*eae*), which is an outer membrane protein (14). It has previously been shown that there exists an increased risk for developing HUS when both stx_2 and *eae* are present in the infecting strain (4, 7, 11). Additionally, a number of studies have documented that subtypes stx_2 and stx_{2c} are more often associated with HUS than the other stx_2 subtypes (5, 6, 8, 15), but stx_{2d} (9, 13, 16)- and stx_{2e} (11)-containing strains have also been isolated from humans with HUS. PCR-restriction fragment length polymorphism has often been the preferred tool for subtyping stx_2 variants (2, 3, 5, 6, 8, 10, 17–19, 22). These methods are, however, vulnerable to single-nucleotide changes and are difficult to interpret if the strain contains more than one subtype or if the fragments generated are small or of similar sizes. For a new and improved subtyping method for the Stx2 toxin, we applied partial sequencing of the most variable part of the $stxAB_2$ operon. By applying this method to the stx_2-positive isolates from the Danish National STEC cohort from 1997 to 2003, we hoped to clarify the epidemiology of human stx_2 subtypes and to correlate specific subtypes with clinical manifestations of BD and HUS.

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All clinical STEC strains included in this study were characterized at the Unit of Gastrointestinal Infections, Statens Serum Institut (SSI), Denmark. About 90% of the strains were isolated at the SSI from individuals suffering from diarrhea, using DNA dot blot hybridization with broad-range polynucleotide DNA probes derived from plasmids *p*NTP705 for stx_1 (23), *p*DEP28 for stx_2 (21), and *p*CVD434 for *eae* (12). The remaining STEC strains originated either from patients with diarrhea who had stool cultures analyzed for the most common STEC O groups (at regional clinical laboratories) or from asymptomatic carriers analyzed because of close contact to STEC-infected individuals. The data set consists of 44 different O groups and is presumably not biased toward certain O groups; there were no general outbreaks within the study period. Retrieval of patient information concerning the illness was performed throughout the study period by patient interviews as described previously (7). STEC strains were grown on agar plates under standard conditions and prepared for PCR in the following way. One colony was transferred to 100 μl 10% Chelex 100 (Bio-Rad, Hercules) in 10 mM Tris-HCl, 1 mM EDTA, pH 8, boiled for 5 min, and centrifuged briefly. The supernatant was used directly for PCR. Each template was subjected to PCR with the following primers: F4, 5′-GGCACTGTC TGAAACTGCTCCTGT (matched all subtypes except stx_{2f}); R1, 5′-ATTAAACTGCACTTCAGCAAATCC (matched all subtypes except stx_{2e} and stx_{2f}); F4-f, 5′-CGCTGTCTGAGGCATC TCCGCT (matched stx_{2f}); and R1-e/f, 5′-TAAACTTCACCTG GGCAAAGCC (matched stx_{2e} and stx_{2f}). PCRs were carried out in a total reaction volume of 25 μl containing 1× PCR buffer [50 mM Tris-HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$, pH 8.3], 2.6 mM $MgCl_2$, 260 μM of each deoxynucleoside triphosphate, 1.25 U *Taq* polymerase (FastStart, Roche Diagnostics GmbH, Mannheim, Germany), 0.05 μM of each of the four primers, and 5 μl template. The thermocycler conditions were as follows: 95°C for 2 min, followed by 35 cycles of 94°C for 50 s, 58°C for 40 s, and 72°C for 50 s, and finally 72°C for 3 min. The completed PCR products were purified by a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced at MWG-BIOTECH AG, Ebersberg, Germany. If the first sequencing attempt using primer F4 or R1 failed, amplicons were sequenced with primer F4-f or R1-e/f. Se-

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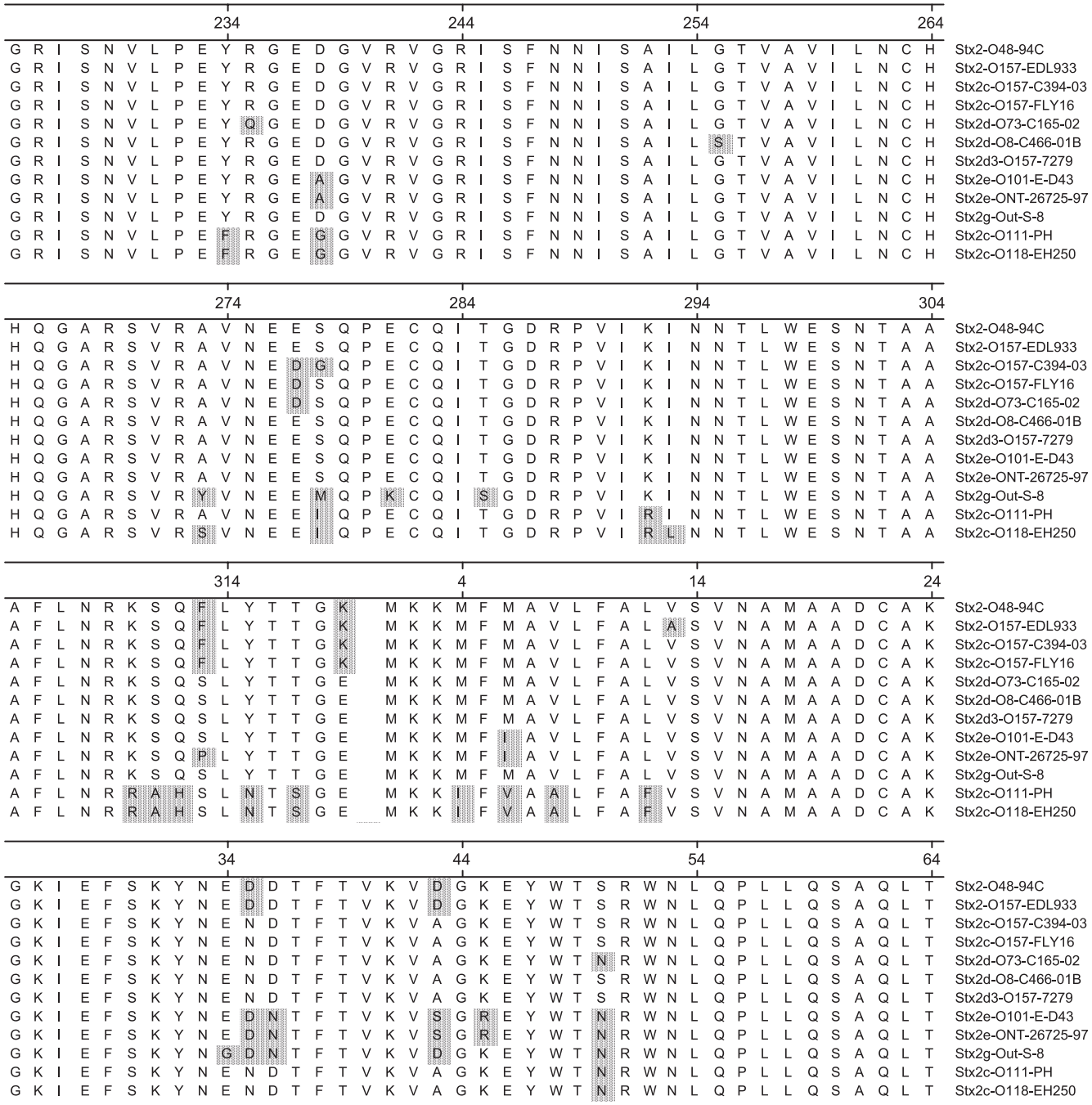

FIG. 1. A total of 255 STEC strains were subjected to partial sequencing of the *stxAB*$_2$ operon, and the coding regions were translated. The figure shows an alignment (ClustalW) of the resulting 159 amino acids (95 amino acids of the C-terminal part of subunit A [positions 225 to 319] and 64 amino acids of the N-terminal part of subunit B [positions 1 to 64]). The toxin variants are named by subtype, O group, and strain name. With respect to the 159 residues, the sequences for the following toxin variants are registered under the respective GenBank accession numbers: Stx2-O48-94C, Z37725; Stx2-O157-EDL933, X07865; Stx2c-O157-C394-03, DQ235774; Stx2c-O157-FLY16, AB015057; Stx2d-O73-C165-02, DQ059012; Stx2d-O8-C466-01B, DQ235775; Stx2d3-O157-7279, X61283; Stx2e-O101-E-D43, X81417; Stx2e-ONT-26725-97, AJ567998; Stx2g-Out-S-8, AB048227; Stx2c-O111-PH, L11078; Stx2c-O118-EH250, AF043627.

quencing with forward and reverse PCR primers generated a 491-bp internal high-quality sequence on the approximately 620-bp PCR product. The nucleotide sequence was translated into 159 amino acids, covering 95 residues of the C-terminal part of subunit A and 64 residues of the N-terminal part of subunit B (Fig. 1). By analyzing the positions of double peaks in the sequence chromatogram, different subtypes present in the same strain could be identified.

All observed chromatograms with two different toxins cor-

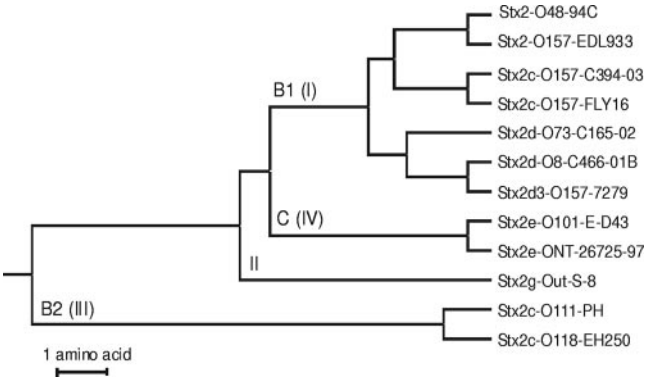

FIG. 2. Phylogenetic tree (unweighted-pair group method using average linkages) based on the alignment shown in Fig. 1 of the 159 amino acids partially covering the *stxAB*$_2$ operon of the 12 toxin variants from this study. Roman numbers (I, II, III, and IV) and capital letters (B1, B2, and C) refer to previous groupings introduced in references 1 and 2, respectively. The same clustering was observed when the entire sequence of 408 joined A- and B-subunit *stx*$_2$ holotoxin sequences in GenBank was analyzed.

responded to the superimposition of two known sequences; if previously unknown sequences are present in a strain harboring two different toxin variants, further confirmatory analyses may be required. Construction of alignments and phylogenetic trees was performed by DNAStar software (DNAStar Inc., Madison, WI).

This partial sequencing of the *stxAB*$_2$ operon offers an easy and specific subtyping method for the Stx2 toxins. Based on the 159 amino acids, 12 different variants were identified in the Danish cohort (Fig. 1 and 2), named by subtype (nomenclature according to reference 20), O group, and strain name. Nine of these were already present in GenBank, and among these, two were for the first time found in humans (Stx2c-O157-FLY16 and Stx2g-Out-S-8).

From 1 January 1997 to 28 October 2003, the Danish National STEC cohort contained 272 strains positive for the *stx*$_2$ gene. Of these, 17 could not be recovered from the archives or did not grow and the resulting 255 strains (of which 39 contained two different toxins) were subtyped by the present method (Table 1). Fifty-two patients (20%) reported being infected during foreign travel. Clinical data concerning HUS were available for 241 patients, and among these patients, 20 (8.3%) developed HUS. Clinical data concerning BD were available for 199 patients, of which 90 (45%) had BD. Eighteen of the 20 HUS cases, but only 40% of all cases, were from children 7 years of age or below. All 20 HUS cases were associated with subtype Stx2 and/or Stx2c, i.e., 19 HUS cases were associated with subtype Stx2 (Stx2-O157-EDL933 or Stx2-O48-94C) either alone (11 cases) or in combination with variant Stx2c-O157-FLY16 (8 cases). One strain associated with HUS was found to contain variant Stx2c-O157-FLY16 alone. Subtypes Stx2 (Stx2-O157-EDL933 and Stx2-O48-94C) and Stx2c (Stx2c-O157-FLY16) accounted for the majority of cases (83%) associated with BD. O157 was by far the most common O group, accounting for 109 (42.7%) of 255 Stx2-positive strains, and was associated with 12 HUS cases. All O157 strains from patients with HUS were *stx*$_1$ negative and *eae* positive. To calculate adjusted odds ratios (ORs), multivariate logistic regression analysis was performed using SAS version 9.1 (Cary, NC). The crude and adjusted ORs for the association between HUS and subtypes Stx2 and Stx2c and age are shown in Table 2. *eae* was strongly associated with HUS but

TABLE 1. Stx2 subtype distribution among 255 strains from the Danish STEC cohort, listed with the clinical outcomes for HUS and BD

Toxin variant(s)[a]	Stx2 subtype(s)	No. (%) of strains		No. (%) of patients with indicated disease	
		Total	Positive for *eae*	HUS[b]	BD[c]
Stx2-O157-EDL933	2	38 (14.9)	38 (23.8)	4 (20.0)	19 (21.1)
Stx2-O48-94C	2	32 (12.5)	27 (16.9)	7 (35)	10 (11.1)
Stx2c-O157-FLY16	2c	54 (21.2)	49 (30.6)	1 (5)	24 (26.7)
Stx2c-O157-C394-03	2c	16 (6.3)	16 (10)		6 (6.6)
Stx2d-O8-C466-01B	2d	2 (0.8)	1 (0.6)		1 (1.1)
Stx2d-O73-C165-02	2d	1 (0.4)			1 (1.1)
Stx2d3-O157-7279	2d	4 (1.6)			
Stx2c-O118-EH250	2c	29 (11.4)			2 (2.3)
Stx2c-O111-PH	2c	34 (13.3)			5 (5.5)
Stx2e-O101-E-D43	2e	1 (0.4)			
Stx2e-ONT-26725-97	2e	3 (1.2)	1 (0.6)		
Stx2g-Out-S-8	2g	2 (0.8)	1 (0.6)		
Stx2-O157-EDL933 + Stx2c-O157-FLY16	2 + 2c	26 (10.2)	26 (16.3)	7 (35)	20 (22.2)
Stx2-O48-94C + Stx2c-O157-FLY16	2 + 2c	1 (0.4)	1 (0.6)	1 (5)	
Stx2-O157-EDL933 + Stx2c-O118-EH250	2 + 2c	1 (0.4)			1 (1.1)
Stx2-O48-94C + Stx2d3-O157-7279	2 + 2d	1 (0.4)			
Stx2c-O111-PH + Stx2c-O118-EH250	2c + 2c	7 (2.7)			
Stx2-O48-94C + Stx2d-O8-466-01B	2 + 2d	2 (0.8)			
Stx2c-O157-FLY16 + Stx2d-O8-C466-01B	2c + 2d	1 (0.4)			1 (1.1)
Total		255	160	20	90

[a] The first 12 rows comprise the 216 strains that contained one toxin variant, and the remaining rows comprise the 39 strains that contained two different toxin variants. ONT, O nontypeable; Out, O untypeable.
[b] Information concerning HUS was available for 241 of the 255 patient strains; of these patients, 20 had HUS.
[c] Information concerning BD was available for 199 of the 255 patient strains; of these patients, 90 had BD.

TABLE 2. Crude and adjusted odds ratios for development of HUS

Determinant and value	No. of patients		OR (95% CI)[a] for indicated analysis	
	Total (n = 241)	With HUS (n = 20)	Crude	Adjusted
Subtype Stx2[b]				
No	145	1	1[c]	1
Yes	96	19	35.5 (4.67–270)	43.1 (4.9–380)
Subtype Stx2c[d]				
No	147	11	1	1
Yes	94	9	0.34[e] (0.15–1.0)	3.29 (1.04–10.5)
Age (yrs)				
>7	144	2	1	1
≤7	97	18	16.2 (3.66–71.5)	10.4 (2.71–50.0)
eae				
No	88	0	1	1
Yes	153	20	ND[f]	ND

[a] ND, not defined.
[b] Presence of Stx2-O157-EDL933 or Stx2-O48–94C.
[c] Reference value.
[d] Presence of Stx2c-O157-FLY16, Stx2c-O157-C394–93, Stx2c-O118-EH250, or Stx2c-O111-PH.
[e] The OR was 1.31 (95% CI, 0.52 to 3.29) when only subtypes Stx2c-O157-FLY16 and Stx2c-O157-C394–93 were included.
[f] $P < 0.001$ (Fisher exact test).

could not be included in the model, since this gene was identified in isolates from all 20 HUS cases. As expected (7), there was no association between HUS and individual O groups, including O group O157 (crude OR, 2.18; 95% confidence interval [CI], 0.86 to 5.56), in this data set of exclusively stx_2-positive isolates. Subtype Stx2 was strongly associated with HUS in both the crude and the adjusted analysis, while subtype Stx2c was associated only in the adjusted analysis. Comparable results were found when the analysis was restricted to the 63% *eae*-positive strains (not shown). Our results do not allow conclusions about a causal mechanism but strongly suggest that the stx_2 and the stx_{2c} genes, as opposed to other stx_2 genes, are associated with development of HUS. Because stx_{2c} (i.e., toxin type Stx2c-O157-FLY16) was present at the same time as subtype stx_2 in eight out of nine isolates from HUS patients, it is not clear from our data if stx_{2c} can contribute to the development of HUS on its own or merely assists subtype stx_2. It may also be speculated that the one strain associated with HUS containing stx_{2c} alone has lost a bacteriophage carrying an stx_2 gene.

Subtype Stx2c appeared to consist of two separate groups of toxins. The amino acid sequences of toxin variants Stx2c-O118-EH250 and Stx2c-O111-PH were divergent from those of the two other Stx2c variants. Furthermore, they were found only in *eae*-negative strains and never in strains from HUS cases. We therefore would suggest the introduction of a distinct nomenclature for these subtypes, which we propose to designate subtype stx_{2b}.

Nucleotide sequence accession numbers. Three sequences, Stx2d-O73-C165-02, Stx2c-O157-C394-03, and Stx2d-O8-C466-01B, were new. They were subjected to full gene sequencing and deposited in GenBank under accession numbers DQ059012, DQ235774, and DQ235775, respectively.

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